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EVALUATION OF PEPTIDE-MEDIATED DENDRITIC CELL TARGETING AND PARTICULATE ADJUVANTS FOR IMMUNOTHERAPY

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Table of contents

Summary

Introduction. In the last decades we observed a steep increase in the worldwide prevalence of allergic diseases. Although rarely fatal, allergies have a significant impact on the quality of life, as well as on the general economy. The most common treatments, mostly corticosteroids and antihistamines, aim at alleviating symptoms but are not curative. An attractive alternative to symptomatic control is the so called allergen-specific immunotherapy, which significantly and persistently reduces symptoms, or even cures allergic diseases. Allergen-specific immunotherapy is known since more than a century and consists of treating the patient with the very same substance that causes the disease (allergen). Repeated injections lead to a re-programming of the immune system and the establishment of tolerogenic and T_H1/T_{reg} dominated immune responses. To avoid adverse reactions the allergen needs to be administered slowly, starting from a very low amount and gradually increasing the dose over several years. The long duration of treatment, as well as therapy-induced side-effects, strongly reduce the patients' compliance, which is why immunotherapy is chosen only for severe cases. For this reason, there is a strong need for the development of novel therapies. They need to be shorter, exempt from significant side-effects, and require less personal and medical efforts.

Aim. In this dissertation we aimed to evaluate two strategies to increase efficacy, and thus patient compliance, of immunotherapies: the use of particulate adjuvants and the targeting of dendritic cells. The first approach aimed to increase the persistence of the antigen in the tissues by means of Strontium-doped hydroxyapatite microspheres (SHAS). The second approach exploited the dendritic cell targeting peptide DCpep to increase the contact of antigens with antigen presenting cells. The ultimate goal of both strategies was to enhance the uptake by antigen presenting cells, thereby accelerating the induction of a T_H1/T_{reg} dominated immune response. This should result in reduced side-effects and/or the need of fewer injections to obtain a therapeutic effect.

Results. Our first approach, which makes use of particulate adjuvants to increase the efficacy of immunotherapies, was successful. We demonstrated the suitability of SHAS for the creation of an allergen depot *in vivo*, and the resulting sustained stimulation of $CD8^+$ and $CD4^+$ T-cells. We further identified $CD11b^+$ migratory dendritic cells to be the cell subset that mostly contributed to the prolonged allergen persistence in lymph nodes (LNs). Additionally, using SHAS as an adjuvant showed a low inflammatory profile and reduced side-effects in allergic mice.

In contrast, the second approach, which consisted of targeting dendritic cells with DCpep, showed no efficacy *in vivo*. Through *in vitro* investigations of both, mouse and human cells, we identified a lack of specificity as the main factor responsible for the unexpected outcome. DCpep not only bound the analyzed dendritic cell subsets indiscriminately, but also enhanced construct uptake by monocytes and macrophages.

Conclusion. Our data showed which subsets of antigen-presenting cells play a role in the antigen transport upon SHAS-assisted vaccination. Based on our findings of reduced side effects and low inflammatory profile, we suggest SHAS as a low-side effect adjuvant for immunotherapy.

Additionally, we identified lack of specificity as a major issue for antigen-presenting cell targeted therapies. Despite the numerous subsets of antigen presenting cells that have already been identified, no targeting approach has been discovered yet, which is able to discriminate among them. Our data strongly suggested that the accuracy of the targeting has to be carefully determined *in vivo* before drawing conclusions about a potential therapeutic efficacy.

The technical efforts associated with this work led to the development of a method, which allows the measurement of antibody affinity on cells with the Quartz Crystal Microbalance device Attana Cell™ 200. Besides enabling the measurement of affinity in more physiological settings, this novel technology can potentially be used to track cell-antigen interactions during immunotherapy.

Sommario

Introduzione. La prevalenza delle malattie allergiche a livello mondiale è aumentata molto negli ultimi 30 anni. Nonostante un decorso generalmente non-letale, queste malattie hanno un notevole impatto sia sulla qualità di vita del paziente, che a livello economico. La terapia più commune consiste nel combatterne i sintomi con la somministrazione di corticosteroidi e antistaminici. L'unica cura vera e propria è rappresentata dall'immunoterapia allergene-specifica, capace di ridurre sensibilmente i sintomi e, in alcuni casi, di curare le malattie allergiche. L'immunoterapia allergene-specifica consiste nel trattare il paziente con la stessa sostanza che causa la malattia (l'allergene). Iniezioni ripetute portano ad una riprogrammazione del sistema immunitario che è così portato verso una risposta tollerogenica di tipo T_H1. Per evitare reazioni allergiche, che possono sfociare un uno shock anafilattico, la dose iniziale di allergene per il trattamento deve essere molto bassa. Durante un periodo di anni, questa verrà gradualmente aumentata finchè la riprogrammazione sarà completa. Per questo, e a causa degli inevitabili effetti collaterali, la terapia allergene-specifica non gode di una buona reputazione. Solamente pazienti con allergie gravi intraprendono di norma il percorso terapeutico, e sono in molti ad interromperlo. C'è quindi il bisogno di terapie nuove, veloci e con effetti collaterali ridotti e, nel migliore dei casi, anche di una riduzione cospicua del numero delle visite da parte del personale sanitario.

Obiettivi. In questa tesi di dottorato si sono valutate due nuove strategie per aumentare l'efficacia degli interventi immunoterapeutici e quindi la loro reputazione. Il primo approcio consiste nell'assorbire l'allergene sulla superficie di microsfere di idrossiapatite contenenti stronzio (SHAS). In questo modo se ne aumenta la persistenza nel tessuto sottocutaneo, riducendo il numero di iniezioni necessarie per un effetto terapeutico. Somministrare l'allergene in forma particellare potrebbe inoltre risultrare in una maggior assimilazione da parte di cellule presentatrici di antigene e quindi in una modulazione benefica della risposta immunitaria. Il secondo approcio ambisce ad aumentare la presentazione dell'allergene direzionandolo verso le cellule dendritiche, con la conseguente genesi di risposte immunitarie dominate da linfociti del tipo T_H1/T_{reg}. Per direzionare l'allergene abbiamo sfruttato le proprietà di DCpep, un peptide che lega selettivamente le cellule dendritiche. Con questo approcio ci auguravamo di ridurre il tempo di riprogrammazione del sistema immunitario, riducendo quindi il numero di iniezioni necessarie per l'immunoterapia.

Risultati. In questo lavoro abbiamo potuto dimostrare quanto le SHAS siano adatte per la creazione di un deposito di antigene. L'uso delle SHAS ha portato ad una stimolazione prolungata di cellule T $CD4^+$ e $CD8^+$. Questo effetto è principalmente dovuto all'azione di cellule dendritiche migratorie $CD11b^+$, che hanno trasportato l'antigene al sistema linfatico per una settimana dall'iniezione. I risultati mostrano quanto le SHAS siano un adiuvante promettente per le immunoterapie. Nel topo

hanno infatti mostrato effetti collaterali ridotti e un ridotto potenziale infiammatorio.

In contrasto, DCpep non ha dato i risultati attesi, soprattutto *in vivo*. Le nostre analisi hanno mostrato come DCpep non sia sufficientemente specifico. DCpep ha potenziato il legame non solo con tutti i tipi di cellula dendritica analizzati, ma anche con monociti e macrofagi. Questo comportamento è stato osservato *in vitro*, sia per cellule del topo, sia per cellule umane. La sottrazione di antigeni da parte di cellule con una ridotta capacità di presentazione (monociti e macrofagi) potrebbe rappresentare un importante svantaggio non solo per questa, ma per tutte le terapie che mirano ad intervenire sulle cellule dendritiche.

Conclusioni. Questo lavoro analizza la questione della specificità nelle terapie che mirano alle cellule presentatrici di antigene. Numerosi sottotipi sono stati identificati *in vivo*, con svariate funzioni e capacità di presentazione. A tutt'oggi però nessuna strategia è in grado di mirare specificatamente all'uno o all'altro sottotipo. I nostri dati mostrano come l'accuratezza in questi interventi mirati sia però di grande importanza e debba essere valutata con cura, prima di poter trarre conclusioni sull'efficacia terapeutica. Sull'altro fronte diamo delle prime indicazioni su quail siano i sottotipi di cellule presentatrici di antigene che sono coinvolte nel trasporto di antigene tra la periferia e il sistema linfatico quando questo è applicato associato a microparticelle. Suggeriamo inoltre l'uso delle SHAS come adiuvante per l'immunoterapia, vista la consistente riduzione degli effetti collaterali. Inoltre, la necessità di sviluppare nuovi metodi ha portato allo sviluppo di un nuovo medodo per la misurazione dell'affinità degli anticorpi direttamente sulla superficie di cellule usando l'Attana Cell™ 200, una microbilancia a cristallo di quarzo, che potrebbe essere usato, fra altro, per analizzare come i diversi sottotipi di cellule reagiscono a un trattamento immunoterapeutico.

Aim

The aim of this dissertation was to evaluate innovative strategies for the delivery of antigens to Dendritic Cells (DCs) in the skin and to investigate which subsets are responsible for antigen uptake, transport and presentation to T-cells.

First, we aimed to investigate hydroxyapatite microparticles as a delivery system for native antigens. The experimental work involved the analysis of *in vitro* and *in vivo* release dynamics, as well as particle-antigen uptake and presentation by different DC subsets. Additionally, we wanted to establish a mouse model for specific immunotherapies to evaluate the immunogenicity and the immunotherapeutic potential of adsorbed allergens (Chapter 1).

In a second approach we aimed to identify the cell subsets bound by the DCs targeting peptide DCpep both in the mice skin and in *in vitro* differentiated human dendritic cell subsets. We also wanted to understand if this targeting leads to enhanced antigen presentation and polarization of T-cells responses, an effect that could be exploited for the development of safer and more efficient vaccines (Chapter 2).

A third project emerged by the need of determining the affinity of DCpep for different DC subsets, without knowing the ligands involved. For this purpose, we aimed to develop a proof of concept method to measure the affinity of antibodies and proteins for membrane-bound epitopes on whole cells. For this approach we decided to use of the Quartz Crystal Microbalance device Attana Cell™ 200, designed for cell-ligand binding assays and offering superior sensitivity (Chapter 3).

Recurring Abbreviations

APC	Antigen presenting cell
Bet v 1	*Betula verrucosa* allergen 1
CD	Cluster of Differentiation
cDC	Conventional Dendritic Cell
DC	Dendritic Cell
DCpep	Dendritic Cell targeting peptide 3 (FYPSYHSTPQRP)
Dec1	Dectin-1
Der p 1/2	*Dermatophagoides pteronyssinus* allergens 1 and 2, respectively
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EPIT	Epicutaneous Immunotherapy
Fel d 1	*Felis domestica* allergen 1
FDA	Food and Drug Administration
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HA	Hydroxyapatite
HAD	Hypoallergenic Allergen Derivatives
HIV	Human Immunodeficiency Virus
HIV-TAT	HIV derived Trans-activator of Transcription protein
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILIT	Intralymphatic Immunotherapy
kDa	Kilo Dalton
LN	Lymphnode
LT	Lymphoid Tissue
MAT	Modular Antigen Translocation
M-CSF	Macrophage Colony-Stimulating Factor
MF	Macrophage
MFI	Mean Fluorescence Intensity
MHC-1/2	Major histocompatibility complex class 1/2
mdDC	Monocyte-derived Dendritic Cell (*in vitro* derived)
mdMF	Monocyte-derived Macrophage (*in vitro* derived)
moDC	Monocyte-derived Dendritic Cell (natural population)
mRNA	Messenger Ribonucleic Acid
OVA	Ovalbumin from *Gallus gallus domesticus*
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid Dendritic Cell
PE	Phycoerythrin
PFA	Paraformaldehyde
P.L.E.A.S.E	Precise Laser Epidermal System
PLGA	Poly lactic-co-glycolic acid
QCM	Quartz Crystal Microbalance
SCIT	Subcutaneous Immunotherapy
SHAS	Strontium-doped Hydroxyapatite porous Spheres
(A)SIT	(Allergen)-Specific Immunotherapy
SLIT	Sublingual Immunotherapy
SPR	Surface Plasmon Resonance
TGF-β	Tumor Growth Factor beta
TNF-α	Tumor Necrosis Factor alpha
TLR	Toll-like receptor
T_H1	T-helper cell type 1
T_H2	T-helper cell type 2
T_{reg}	T-regulatory cell

Introduction

1. Allergy and allergic diseases

Over millions of years the immune system evolved a variety of mechanisms to fight against environmental threats. The result is a fine tuned network that allows a very efficient elimination of most pathogens ranging from viruses, to bacteria, fungi, helminthes, and protozoa. In this picture allergic diseases are like a bull in a china shop: hard to control, hard to explain, and potentially devastating [1].

Allergies are abnormal adaptive immune responses directed against non-infectious normally innocuous environmental substances called allergens. Allergic disorders include allergic rhinitis (hay fever), atopic dermatitis (eczema), allergic asthma, and food allergies. The most serious life threatening allergic reaction is called anaphylaxis, which can induce severe life-threatening systemic reactions within minutes after exposure to an allergen [2]. Allergic diseases have also a considerable economical impact resulting from absences from work and reduced working capacity of allergic individuals. The costs associated with allergic diseases were recently estimated by the European Union to 55-151 billion euro per year for Europe only [3].

1.1. Epidemiology and environmental risk factors

The prevalence of allergic diseases shows great differences among countries. Studies by the International Study of Asthma and Allergy in Children (ISAAC) on 13-14 years old children indicate worldwide prevalences of 3.4 - 31.2 % for asthma, 4.5 - 45.1 % for rhinoconjunctivitis and 1.4 - 21.8 % for atopic eczema [4]. Compared with results from five years earlier a tendency for higher prevalence was observed, especially in India and Asia-Pacific countries. The evident correlation between the prevalence of allergic diseases and the stage of development of a country has been extensively analyzed and explanations have been proposed and controversially discussed (reviewed in [5]). Studies in non-industrialized countries undergoing the industrialization process strongly suggest an increased risk for allergic diseases for i) people living in urban areas, ii) cigarette smokers (asthma), iii) obese children, iv) contact with certain types of air pollution (e.g. kerosene smoke), and v) workplace exposure. Also vi) parasitic infections may play an important role for the development of allergic diseases. Several studies have indeed shown a reduction of the prevalence of allergic symptoms in patients that were seropositive for a variety of parasites (e.g. the giant roundworm *Ascaris lumbricoides*, the dog roundworm *Toxocara canis*, or *Toxoplasma gondii*) [5]. An interesting theory in this context is the so called hygiene hypothesis, which explains the higher prevalence of allergic diseases in developed countries with the associated increase in cleanliness and the reduced family size. The hygiene hypothesis is supported by the numerous negative

correlations between the prevalence of atopic diseases and the contact with parasitic, infectious and noninfectious organisms as well as microbial components [6]. In the last decade new findings have been challenging and reshaping the hygiene hypothesis [7, 8]. Even if the correlation with "dirtiness" still convinces, the picture appears much more complicated. For example it became evident that protection is triggered by contact with specific organisms but only at particular times of life. It also became evident that the protection of farmers may not only be driven by microorganisms and their components, but also by the close contact with animals and the increased consumption of (raw) animal products [9]. The molecular mechanism of this animal-driven protection is largely unexplained. Recent findings suggest a role of N-Glycolylneuraminic acid (Neu5Gc), a sialic acid molecule found in most mammals, which cannot be synthesized by humans (Frei R., manuscript in preparation).

1.2. Genetic risk factors

It is nowadays clear that significant genetic and hereditary components influence the onset and the severity of allergic diseases. Genetics of complex diseases (such as allergy, asthma, diabetes, obesity and inflammatory bowel disease) is challenging, and several issues are affecting the reputation of this field of study. First, the inconsistency and the poor reproducibility of published genetic associations. Second, the large phenotypic variations and post-translational modifications that are not directly visible at genomic level. Because of their complexity these diseases are hard to address genetically: they show a variety of symptoms and causes, they are characterized by a disturbance of huge and largely unexplained cellular and cell biological networks, they can arise at any age, and are strongly influenced by environmental and epigenetic factors. Conclusions like "*the CD14-159TT genotype protected against atopic dermatitis, but only in children with a dog at home*" [10] are, indeed, not uncommon [11].

Fact is that several candidate genes have been proposed to play a role in allergic diseases. These genes can be arranged in four main groups. i) innate immunity and immunoregulation: this group contains innate immunity genes involved in the initiation of immune responses such as pattern recognition receptors (TLR2, TLR4), extracellular (CD14) and intracellular receptors (NOD1, NOD2), and cytokines (IL-10, TGF-β). ii) T_H2 cell differentiation and effector function: including transcription factors (GATA3, STAT6), IL-4, and its receptor IL-4R. iii) Epithelial cells: including genes for chemoattractants secreted by epithelial cells (RANTES, eotaxins) and for the protection and the maintenance of epithelial barriers (SPINK5, FLG). iv) Lung function: including a variety of genes involved in airway remodeling and lung functions (LTC4s, TNF) [11].

It is evident that the gene groups mentioned contain almost every gene product known to play a role in allergic diseases. The immune system is so redundant and

diversified, that the combinations of alleles potentially generating allergic disorders could be almost unlimited.

1.3. Immunological mechanism

The first phase of an allergic disease, the sensitization phase, is defined by the first interaction of an allergen with the immune system. Numerous factors such as genetic predisposition of the host, biochemical properties and concentration of the allergen, presence of enhancers or adjuvants and failure of tolerance mechanisms can trigger a T_H2 dominated immune response. The associated production of IL-4 and IL-13 leads to class-switch recombination in B-cells, which results in the production of allergen-specific IgE that bind to the high-affinity IgE receptor (FcεRI) on mast cells and basophils [2].

Upon a second encounter the allergen cross-links membrane-bound IgE on effector cells, starting a series of downstream signaling cascades that initiate the early phase of the allergic response characterized by an immediate release of preformed mediators such as histamine, proteases or heparin from the granules of mast cells and basophils, accompanied with the release of *de novo* synthesized mediators like prostaglandine D2 and leukotrienes. This very rapid reaction results in increased vascular permeability, mucus production, smooth-muscle contraction, and the release of cytokines, chemokines and growth factors (e.g. IL-8, IL-5, TNF-α) that recruit and activate cellular mediators at the inflammation site, which in turn drive the late phase response [2, 12, 13].

The late phase response takes place hours after allergen encounter as a consequence of the influx and activation of T-cells, macrophages, neutrophils, eosinophils and basophils in the tissues. It seems to be regulated by early phase mediators together with antigen-stimulated T-cells, and typically resolve within 1-2 days [2, 13].

The variety of symptoms of allergic diseases is as large as the different ways allergens can encounter the immune system: air-borne allergens cause asthma, rhinoconjunctivitis and airway inflammation; food allergies mostly result in oral and gastrointestinal symptoms; and contact allergies, after exposure to environmental allergens or small chemical compounds, in atopic dermatitis. If untreated, some allergic reactions can become systemic, leading to life-threatening anaphylactic reactions, the most well known type being hymenoptera (bee and wasp) venom hyper reactivity. Other allergic inflammations become chronic (e.g. because of persistent allergen challenge), leading to structural changes and loss of functionality in the affected organs [2].

Anaphylaxis is associated with a number of symptoms and can involve several organs. Most afflicted are: the skin (80-90%, urticaria), the respiratory tract (70%) and, in the case of food allergies, the gastrointestinal tract (30-45%). Anaphylactic

reactions can affect also the nervous and the cardiovascular system. Untreated, severe cases of anaphylaxis can have fatal outcome [14].

1.4. Specific tolerance in healthy individuals

The environment is burdened with a plethora of antigens that do not represent a threat. While allergic patients develop deregulated responses to some of these antigens, healthy individuals either ignore them or develop a state of immune tolerance where the antigens are cleared without inflammatory responses [15].

Immune tolerance can be divided in two main branches: central and peripheral tolerance. Establishment of central tolerance occurs during T-cell maturation and consists in the deletion of self-reactive T-cells, and the development of T regulatory cells (T_{regs}) from $CD4^+$ T-cells that have escaped negative selection in the thymus [16]. For the prevention and the treatment of allergy major efforts are aiming at inducing peripheral tolerance. Peripheral tolerance is characterized by multiple mechanisms including apoptosis of immune effector cells during inflammation by death-inducing ligands [17], and mitigation of tissue inflammation by the secretion of suppressive cytokines from T_{regs}, dendritic (DCs), and tissue cells.

The reasons why some allergens induce inflammatory responses in atopic patients remains elusive: breaking of immune tolerance is a complex process that has been shown to involve genetic susceptibility, antigen dose, time and route of exposure, structural characteristic of the allergen, and simultaneous exposure to infections or commensal bacteria that act as immunostimulants [16]. Also the cellular players involved in tolerance are many and involve all branches of the immune system. Current data suggest a vast number of molecules and cell types to be involved in maintaining a tolerogenic state. However, the data reported in the literature are fragmentary and often contradictory. It is therefore not yet possible to draw a complete, reliable and generally valid picture.

There is strong evidence supporting an important role of breast-feeding in the development of tolerance in the infant [18]. Protection against allergy appears to be conferred by the milk-mediated transfer of antigen to the child [19]. From a cellular point of view, studies comparing atopic and non-atopic individuals as well as allergic patients before and after immunotherapy revealed a predominant role of T_{regs} in the maintenance and restoration of allergen tolerance [16, 20, 21]. In this context the balance between T_{regs} and T_H2 appears to play a fundamental role [22]. Importantly, allergen-specific T_{regs} are present and functional in both atopic and non-atopic individuals [23] and have been shown to recognize the same epitopes [24].

Regulatory T-cells

T_{regs} are the key modulators of immune tolerance, they steer T_H1, T_H2 and T_H17 immune responses in order to avoid excessive inflammatory or allergic damage.

Various subsets of T_{regs} have been suggested. The most robust classification divide these cells in two major subsets: naturally occurring T_{regs} (nT_{reg}, $CD4^+$ $CD25^+$ $FoxP3^+$), which arise during the normal process of T-cell maturation in the thymus, and inducible T_{regs} (iT_{regs}) which differentiate from $CD4^+$ T-cells under specific conditions [15]. The induction of the transcription factor FoxP3 (e.g. by TGF-β) is sufficient for the conversion of naïve T-cells into T_{reg} cells in the periphery [25].

T_{regs} act on different levels to prevent inflammation. They exert their inhibitory potential by contact mechanisms or by secretion of specific cytokines. Among others, T_{regs} have been shown to: i) inhibit the maturation of DCs and down-regulate their expression of the T-cell priming ligand CD80/86 [26], ii) to suppress FcεRI-dependent mast cell degranulation [27] and iii) to secrete or induce the secretion of the immunosuppressive cytokines IL-10 and TGF-β, which in turn act on innate and adaptive immune cells to reduce inflammation [16, 28].

2. Immunotherapy

After many years of research, the best prophylaxis to avoid symptoms in established allergic diseases remains allergen avoidance. Escaping an allergen exposure is, however, not always possible. Subjects with food allergies know well how hard it is to be absolutely certain of the absence of a contaminating allergen in industrially processed foods. Additionally, big efforts have been devoted to the reduction of the house dust mite burdens (microporous barriers, air filtration, acaricidal sprays, etc.) with, however, very limited reduction in patients' exposure [29].

When avoidance is not possible the symptoms of allergic diseases are treated with immunosuppressive or anti-inflammatory drugs such as corticosteroids, antihistamines, antileukotrienes or β2 adrenergic receptor agonists. More recently the first biological targeting allergic diseases reached the market. Omalizumab is a monoclonal humanized mouse IgG1 antibody that recognizes the Cε3 region of human IgE, thus blocking IgE binding to its high affinity receptor (FcεRI) on effector cells. Omalizumab has been proven efficient in reducing exacerbation of allergic asthma and the consequent use of corticosteroids [30]. For these reasons the therapy, although not curative, has a positive impact on the quality of life in numerous atopic diseases and contributes to the baseline control of asthma. To be noted, Novartis has a new anti-IgE antibody called QGE031 in the pipeline which should be 12 times more potent than Omalizumab [31].

All the drugs mentioned have to be administered regularly, because they alleviate the symptoms but not the disease. The only long term treatment is allergen-specific immunotherapy, which modulates the immune system towards the correct immune response.

2.1. Antigen-specific immunotherapy

The first report of immunotherapeutic interventions for allergic diseases dates back to the beginning of the 20th century, when Leonard Noon was able to prevent the symptoms of allergic rhinitis during the pollen season by subcutaneous injections of pollen extracts [32]. Since then the repeated subcutaneous administration of increasing doses of allergens has been the method of choice for allergy immunotherapy [33]. Antigen-specific immunotherapy (SIT) has been effective in reducing symptoms for patients with asthma, allergic rhinitis and bee venom allergy [34]. The remission of symptoms achieved by SIT is long-lasting, and persist for many years after discontinuation of the therapy as demonstrated, for example, by studies on grass-pollen SIT [35].

Our knowledge about the immunological mechanisms underlying SIT is still incomplete and is continuously being elucidated. At an early stage (days) of SIT a decrease in mast cell and basophil degranulation can be observed, correlating with reduced risk of systemic anaphylaxis [34]. A possible, partially supported explanation is that, during SIT, the release of inflammatory mediators occurs in small bursts, unable to trigger severe symptoms but sufficient for a long-term decrease in the amount of inflammatory mediators contained in the granules [36, 37].

Mast cells and basophils desensitization is followed by a marked reduction of these cell subsets in blood and the induction of a tolerant state in peripheral T-cells, possibly favored by the decrease in IL-4 and IL-13 secretions [38]. Peripheral T-cell tolerance is orchestrated by T_{regs}. The beneficial effects of T_{regs} in the treatment of allergic diseases has been demonstrated in many ways and includes at least five different mechanisms: i) suppression of antigen presenting cells that would otherwise stimulate the generation of effector T_H2 and T_H1 cells, ii) direct suppression of T_H2 and T_H1 cells through the secretion of IL-10 and TGF-β, iii) shift of the balance between IgE and IgG4/IgA on B cells through the effect of IL-10, iv) tissue remodeling (mainly through the effects of TGF-β) and v) further suppression of mast cells, eosinophils and basophils [39]. The tolerogenic state triggered by T_{regs} is accompanied by enhanced T_H1 responses, resulting in the production of IgG that interfere with the pathological IgE response [40]. The increased production of IgG4 antibodies is considered an indicator for successful immunotherapy. In fact, IgG4 antibodies can become bivalent by exchanging IgG half molecules (one H- plus L-chain). Bivalency is thought to avoid the formation of large IgG4 cross-linked aggregates, supposed to lead to the degranulation of mast cells and basophils [41]. The amount of IgG1 antibodies is also increased upon various SIT approaches. Its protective role in allergic diseases, if any, is poorly investigated. Epitope-blocking and the generation of large phagocytable complexes represent the most plausible benefits of IgG1 in SIT [40].

More recently, numerous new application routes for SIT were suggested, such as mucosal (including sublingual, nasal, bronchial and rectal), intralymphatic [42], and epicutaneous allergen delivery. All of these routes target areas that are densely populated by DCs, and aim for a maximization of antigen presentation and the reduction of therapy-related side-effects. Interestingly, the immunological mechanisms involved in the success of the different immunotherapies vary depending on the application route. Therefore, different routes may be chosen depending on the affected organs [43].

2.2. Sublingual immunotherapy

Sublingual immunotherapy (SLIT) was extensively reviewed by G. W. Canonica in a World Allergy Organization position paper in 2009 [44]. SLIT consists in the application of the immunotherapeutic substance (mostly an allergen extract) under the tongue. SLIT is supposed to exploit the natural tolerogenic environment of the mouth's mucosa, which is maintained free of inflammation by the presence of a network of langerhans cells, epithelial cells and monocytes secreting IL-10 and TGF-β.

After the first proofs of concept much research has focused on the establishment of safety profiles and in the robust demonstration of mid- and long-term clinical efficacy. Unfortunately many results are still controversial and some studies were not adequately powered to provide conclusive answers. Meta-analyses generally suggested benefits for grass SLIT ranging from 10% to 45% in comparison to placebo [45]. Phase III trials showed grass SLIT being capable of long-lasting disease modification in terms of symptom reduction and antibody responses [46]. Even though SLIT is increasingly popular and recently got approved for the first time in the United States [47] further investigation is necessary. Reliable data are still missing about the efficacy of SLIT for allergens other than the usual grass extracts. Moreover, optimal dosages and schedules aimed to increase compliance and reduce side-effects need to be further developed [44].

Mechanistically SLIT appears to act in a different fashion in comparison with the classical subcutaneous immunotherapy (SCIT). In fact, while by SCIT the reduction of symptoms correlates with the production of allergen-specific IgG competing with IgE for allergen binding [48], the sublingual route induces a tolerogenic response dominated by T_{regs} and the cytokines IL-10 and TGF-β but does not alter the IgG response. On the contrary, it boosted allergen-specific IgE responses [49, 50]. Both strategies induce immune deviation, i.e. inhibit T_H2 in favor of T_H1 responses [33].

2.3. Intralymphatic immunotherapy

The injection of an antigen directly in LN allows rapid antigen presentation to a large number of T-cells with different antigen specificity. The greatly enhanced

probability that the presented antigen encounters its specific T- or B-cell results in a faster response [51]. Intralymphatic immunotherapy (ILIT) exploits this mechanism to achieve allergen desensitization with only a few injections and with reduced side-effects. Intralymphatic injections are given in the inguinal LN under ultrasound guidance; common sense would suggest such a treatment being associated with pain. However, no more discomfort than subcutaneous injections was recorded during therapy [52].

A first evaluation in mice showed that intralymphatic injections enhanced immunogenicity and T-cell responses in comparison with the subcutaneous route. Interestingly, only the intralymphatic route resulted in the production of T_H1-dependent IgG2a antibodies. As predictable consequence, in an immunotherapy model ILIT was proven to be more protective than SCIT against anaphylaxis [53].

In a randomized clinical trial involving 165 rhinoconjunctivitis patients sensitized to pollen, 3 ILIT injections resulted in ameliorated hay fever symptoms, reduced allergen-specific IgE in serum and skin-prick test reactivity. The tolerance induced was comparable with the one obtained with 54 SCIT injections administrated over a period of three years and less adverse reaction were recorded [52].

2.4. Epicutaneous immunotherapy

The skin represents one of the most interesting organs for immunotherapy for three reasons. First, the application of compounds to the skin in form of cream or needle-patches, or by using laser drilling is easy, mostly painless, and well accepted by the patients. The absence of syringes allows large-scale treatments without the need of highly trained staff and minimizes the risk of infections. Second, the skin is populated by a variety of antigen presenting cells, which bring applied antigen to the draining LN [51]. The amount of DCs in the apical dermis (0-30 μm) is extraordinarily high and is estimated in around 10-fold the number of DCs that circulate in the blood [54]. Third, the epidermis is deprived of mast cells, which reside in perivascular sheets in the upper side of the dermis (60-150 μm) [54, 55]. An antigen applied on the apical dermis may therefore be processed and presented without triggering mast cell degranulation and the resulting side-effects.

The efficacy of Epicutaneous Immunotherapy (EPIT) for the treatment of allergic diseases was verified by a double-blind, placebo controlled clinical trial involving 132 patients with grass-pollen induced rhinoconjunctivitis. Treatment with allergen-patches resulted in a 30% reduction of hay fever symptoms but was accompanied by a significant dropout rate of 8.3% due to adverse events [56]. In order to have a better controlled EPIT recent trials involved the use of laser-generated micropores, allowing precise control of depth and area of allergen application, which may result in reduced side-effects in comparison to other application strategies [57].

3. Vaccines for allergy

Originally, SIT was performed using natural allergen extracts. Within the last decades the scientific community realized how the effects of these drugs were too unpredictable in terms of efficacy and especially side-effects for a broad clinical use [58]. Later, the development of DNA cloning and protein purification techniques allowed the production of improved and more defined drugs. More importantly, it opened the door for the design of sophisticated vaccines that promise to be safer and more effective [59].

3.1. T-cell epitopes

The use of short synthetic peptides for the delivery of T-cell epitopes was proposed in 1993 [60, 61]. The approach is based on the observation that the presentation of T-cell epitopes by nonprofessional antigen-presenting cells results in anergy or tolerance [62]. Additionally, it exploits the impossibility for a short peptide to crosslink adjacent immunoglobulins, thereby avoiding major side-effects.

The efficacy of this type of treatment is supported by numerous studies in mice covering allergen sensitization models (e.g. Bet v 1, Fel d 1, Der p 2) and autoimmunity models (multiple sclerosis, arthritis, diabetes) [63]. Clinical trials performed with Fel d 1 derived peptides showed contradicting outcomes. Haselden et al. observed that the vaccine used was effective but poorly tolerated in many subjects, mostly resulting in IgE-independent asthma manifestations [64]. Another study used a peptide cocktail with a safer profile, which resulted in improved clinical indicators such as reduced late-phase reactions in the skin, and reduced T_H1 and T_H2 cell proliferation and cytokine production. Unfortunately, follow up investigations reported no significant improvement in the quality of life following peptide-based immunotherapy [65]. Recent still ongoing studies with a novel peptide preparation (Cat-PAD) showed more promising results in terms of safety and tolerance, but also efficacy, recorded as reduction of symptoms after challenge [66]. The few studies with bee venom peptides showed similar results as obtained with Fel d 1 peptides [63].

3.2. Hypoallergenic allergen derivatives

Hypoallergenic Allergen Derivatives (HAD) are recombinantly produced versions of the naturally occurring allergens. These proteins maintain the T-cell epitopes of the original allergen, but are engineered for a reduced IgE reactivity, potentially resulting in reduced side-effects [67]. Clinical trials showed that the main mechanism of action for HAD is the production of allergen-specific blocking IgG antibodies. HAD have been engineered in many different ways including rational sequence reassembly, covalent coupling to vitamin D3, hypoallergenic trimers, protein folding variants, and *in vitro* evolution [59].

3.3. Modular MAT Vaccines

The mechanism exploited by Modular Antigen Translocation Vaccines (MAT-Vaccines) origins from the observation that antibody subclass switch in allergic individuals depends on the dose of allergen used for priming whereas low doses favor IgE production, while high doses favor IgG production [68] and the induction of IL-10 secreting T_{regs} [69]. Since, during SIT, a treatment with high doses of the allergen is not possible due to the hypersensitivity itself, a modular recombinant protein construct was developed aimed to increase MHC class II presentation without increasing the amount of allergen applied. The construct, produced in *E. coli*, consists of a his-tagged version of the allergen of interest linked to a HIV-TAT derived translocation peptide that brings the vaccine into the cell [70] and to the first 110 amino-acids of the human Invariant Chain (Ii) that drives the construct to the lysosomal / endosomal compartment, where antigen loading to MHC class II molecules takes place. MAT Vaccines have been shown to trigger PBMCs proliferation *in vitro* at 10-100 lower doses compared to the control allergen. Moreover, they switched the secreted cytokine pattern from T_H2 (IL-4, IL-5) to T_H1 (IFN-γ) and the tolerance-inducing cytokine IL-10 [71].

The major cat allergen Fel d 1 version of the MAT-Vaccine has been tested in mice and humans in combination with ILIT. In mice, both applied s.c. or i.l. MAT constructs resulted in more T_H1 skewed immune responses, as shown by immunoglobulins and cytokine profiles. In an immunotherapy model MAT-Vaccine ILIT was proven to be more protective against anaphylaxis compared to treatments with cat fur extract or recombinant Fel d 1 allergen [53]. In untreated cat allergic individuals, the MAT vaccine showed reduced skin and basophil reaction. Following immunotherapy a T_H1 skewing of the immune response was observed in terms of increased IgG4 production, together with an increased nasal and dermal tolerance [72]. Unfortunately, the study didn't include a control group treated with the recombinant version of the allergen (Fel d 1 alone). It is therefore hard to evaluate at what extent the benefits result from the modules included in the MAT-Vaccine design.

3.4. Carrier-bound non allergenic peptides

In this approach allergen-derived peptides are coupled to a carrier protein, e.g. keyhole limpet hemocyanin [73], or viral proteins [74]. The resulting constructs are first selected for low IgE and T-cell reactivity, then for the ability to induce blocking IgG responses. This strategy allows providing T-cell help without activation of allergen-specific T-cells, which are responsible for late-phase reactions and may, therefore, result in the reduction of both IgE and T-cells mediated side-effects during immunotherapy [73].

3.5. Nucleic acid vaccines

The principles behind nucleic acid vaccinations are similar but not identical to the ones behind other allergy vaccines. Nucleic acid vaccines appear to achieve allergy protection mainly by recruiting IFN-γ producing $CD4^+$ and $CD8^+$ T-cells, thereby inducing a shift from a T_H2 dominated immune response towards a rather T_H1/T_H2 balanced one [75]. The induction of a T_H1 shift is an intrinsic property of DNA, known since at least 20 years [76]: where the presence of unmethylated DNA from bacterial origin plays a determinant role. Importantly, the T_H1 response induction is antigen specific: T_H2 responses against other antigens remain unaffected.

DNA vaccination faces similar problems as protein vaccines (anaphylaxis, duration of therapy, etc.) and uses similar strategies to overcome them. Some of the published modifications are: i) use of mutant or truncated variants of the allergen; ii) cytokine co-expression or fusion; iii) carrier mediated administration (e.g. using poly lactic-co-glycolic acid (PLGA) particles [77]); iv) modular construction to enhance the presentation of the gene product [78]. DNA vaccinations offer numerous advantages in comparison with protein vaccination: i) less stability issues; ii) easy to purify as LPS free preparation and with good reproducibility; iii) absence of therapy induced IgE; iv) no direct cross-linking of IgE and subsequent mast-cell and basophil activation [79].

Three are the main disadvantages of DNA vaccines. First, the reduced efficacy (the weekly dose for animals is 0.2-1 mg/kg [80]). Second, the need for cell penetration: it is relatively easy to transfect rapidly-dividing mammalian cells in culture. However, strategies employing synthetic delivery systems (cationic lipids, polymers or peptides) have been proven inefficient *in vivo*, mainly because of a drastic drop in transfection efficiency compared with the *in vitro* results or the poor stability of the constructs in serum [80]. Third, the long term safety issues, including concerns about integration into the genome followed by uncontrollable prolonged expression of the encoded antigen. These worries are amplified upon the use of viral vectors for DNA delivery. The mentioned issues severely impact the patient's acceptance of DNA vaccines as well as their commercial development [81].

Some of the security issues concerning DNA vaccines would be mitigated by using mRNA as allergen-delivery vector. In fact, by vaccinating with mRNA there is no need of introducing foreign DNA sequences (such as plasmid backbones or nuclear localization sequences) and there is limited risk of genome integration. Moreover, the expression of the foreign transgene is short-lived [82]. To date, only one publication dealt with mRNA vaccination and showed a clear preventive effect for the development of allergy in mice. It was shown that the T_H1 response induced by the vaccination prevented the consecutive onset of a T_H2 biased allergic response. The introduction of self-replicating viral elements improved the efficacy of the vaccination, but also raised safety issues [82]. The high production costs and the limited stability of mRNA hold back the development of mRNA vaccines. Future

studies will determine if this approach will be valuable and applicable for therapeutic interventions.

3.6. The role of adjuvants in allergy vaccines

The importance of adjuvants and vector systems in allergy vaccination has been optimally summarized in a review [83].

Until today the use of adjuvants for immunotherapy was rather pointless, because allergen extracts are already highly immunogenic. However, new molecular approaches are emerging, which make use of highly purified components that require immunopotentiators [83].

An adjuvant for allergy immunotherapy should have the following properties. i) Like all adjuvants it should be safe and cheap. ii) It should lower the local reactions to the absorbed allergen, e.g. by rendering it less accessible to mast cells, eosinophils and basophils, or by reducing the allergen dose that is needed to achieve a benefit. iii) It should trigger a T_H1/T_{reg} dominated immune response able to contrast the T_H2 response typical for allergic diseases [83].

In the context of allergy vaccination, following adjuvants display interesting properties and/or have been used for immunotherapeutic approaches.

Mineral adjuvants (e.g. aluminum hydroxide, calcium phosphate) are common components of subcutaneous allergy vaccines in Europe. They have been shown to elicit inflammatory responses with induction of the inflammasome, followed by a reduction of T_H2 responses in mice and humans [84].

TLR ligands (e.g. monophosphoryl Lipid A (MPL), CpG) reduced airway inflammation and T_H2 responses in murine models of asthma and are now under investigation for human use in various preclinical and clinical studies. MPL-adjuvated vaccines induced the production of IgG1 and IgG4 antibodies [83].

Small synthetic molecules (combination of oral steroids and Vitamin D3) have been shown to enhance the efficacy of OVA immunotherapy in mice involving IL-10 and TGF-β and are therefore considered as potential inducers of T_{reg} responses [85].

Heat-killed bacteria (*Mycobacterium vaccae*) induced T_{regs} secreting IL-10 and TGF-β [86].

Notably, different immunotherapeutic approaches require different adjuvants. For SLIT, for instance, an exclusive triggering of T_H1 responses was insufficient and T_{reg} stimulating properties were needed for it to be effective [87].

3.7. The use of micro- and nanoparticles as adjuvant

In the last decades progresses in nanotechnology lead to a number of new approaches for the manipulation and improvement of immune responses: a growing field of research that is far from being exploited (outstandingly reviewed by Moon [88] and Smith [89]). Among others, synthetic particles can be designed to act as artificial antigen presenting cells and/or provide co-stimulatory signals. The particulate administration of antigen has been shown to boost and modulate phagocytic processes, and careful design allowed a significant enhancement of MHC-I antigen presentation. Coupling small- and macromolecules to micro- and nanoparticles results in higher stability and hence a longer persistence in the tissues: for this reason particles are often used for mucosal applications. Additionally, the limited diffusion of particles-bound cytokines can be exploited to localize the application of immunostimulatory cytokines, thereby limiting the associated toxic effects [88, 89].

Adjuvation using nano- (1-1000 nm) or microparticles (1-1000 μm) offers numerous advantages. i) The uptake by APCs is facilitated in comparison to soluble antigens. ii) The permanence of the antigen in the injection site is prolonged (depot effect) and results in better immune responses. iii) The antigens are protected against degradation (this is particularly true when antigens are encapsulated into the particle material). iv) The antigen delivered in particulate form can be cross-presented, leading to the generation of $CD8^+$ immune responses. v) The immune response can be modulated by changing material, size and/or particulate carrier, as well as by combination with immunostimulatory compounds [90].

The most widely used materials that have been used for the preparation of adjuvant particles are synthetic polymers, such as polystyrene, poly-lactic acid, or PLGA. Common is also the use of natural polymers (e.g. gelatin, collagen and chitosan) [90] and salts (calcium phosphate, hydroxyapatite) [91]. Although the impact of particle size on the adjuvant properties of micro- and nanoparticles has been extensively studied (reviewed in [90]) it was not possible to determine which particle size is best suitable for the induction of strong, long lasting, or polarized immune responses. The reason is the vast experimental diversity in terms of materials, loaded antigens, routes of administration, size, and homogeneity. In general, the published studies observed an optimal range for particle size for immunization [92]. There is, however, no accordance between the results from the different groups.

Particle size also determines the fate of the particle itself after injection. Small particles (20-200 nm) injected intradermally will freely migrate to the draining LN allowing uptake by LN-resident cells, while bigger particles (0.5-2 μm) require transport by dermal APCs to reach the LN [93]. By increasing the particle size the extent of uptake by APCs gradually decreases and no uptake was observed for particle sizes above 32 μm [94].

Depending on the particle type the antigen can be loaded in three different ways. i) Trapping the antigen inside the particle provides protection and allows slow release avoiding exposure of the immune system to high antigen concentrations upon injection. Trapping could, however, damage the epitopes and sustained release is difficult to control. ii) Chemical conjugation is technically challenging and potentially detrimental for the antigen. iii) Physical absorption, which is easily achievable and mostly maintains the integrity of the antigen and is hence preferred from a pharmaceutical point of view. This method offers, however, little antigen protection and potentially exposes the immune system to high doses of antigen upon injection. The loading method has to be carefully evaluated depending on the application route, the particle type, and the nature of the loaded antigen [90].

3.8. Micro- and nanoparticles as adjuvants for allergy-immunotherapy

For an extensive, but unfortunately slightly outdated, review of the particulate formulations used in allergy immunotherapy please refer to the work of Schöll *et al.* [95].

Numerous are the particle formulations that have been tested in allergy immunotherapy. Many of them represent commercial and patented products, whose safety has already been demonstrated. The huge particle diversity has led to many, rather shallow studies that only marginally address their benefits in immunotherapeutic settings [95]. In animal immunotherapy models it appears clear that most particulate formulations are superior to the soluble antigen *in vitro* [96] in terms of DCs activation, induction of T_H1- or T_{reg}-dominated immune responses and protection from hypersensitivity reactions [97].

For SIT it is important that the particulate adjuvant induces a T_H1 dominated immune response. In this context Kanchan and Panda observed how smaller PLA nanoparticles (200-600 nm) induce more T_H1 biased immune responses in comparison with 2-6 μm microparticles [98]. On the contrary, Mann *et al.* observed that smaller lipid vesicles (10-100 nm) were not as potent as bigger particles (400-2000 nm) in the induction of T_H1 biased immune responses [99]. The same contrasts are observed in the induction of humoral vs. cellular responses [90].

To the best of our knowledge, the only report of the use of hydroxyapatite in the context of allergy immunotherapy evaluates the potential of aquasomes, which consis of a hydroxyapatite core coated with trehalose and loaded with an antigen by absorption. The study showed how immunotherapy with OVA-aquasomes was more protective against anaphylaxis than immunotherapy with OVA-Alum [100]. The robustness of the data is however doubtful, and because of the trehalose coating, these particles cannot be compared directly with pure Hydroxyapatite particles.

4. Dendritic cells, macrophages and antigen presentation

4.1. Introductory remarks

For simplicity, with the abbreviation DCs, I will refer to all subsets of Dendritic Cells, including Langerhans Cells (LCs). Any deeper classification will be clearly disclosed.

Mankind's knowledge on Dendritic Cells (DCs) experienced a boom during the past two decades, where not only their function and cellular biology has been deeply elucidated, but also several potential subsets with different surface markers, but unclear function, have been discovered. For obvious technical reasons most of these advances have been achieved in mice. Therefore it has to be kept in mind that the relevance of many studies for humans remains to be demonstrated.

4.2. History and introduction

In 1973, R.M. Seinmann, by looking at cells from the mouse spleen that adhere to glass and plastic surfaces observed a new population, characterized by a stellate morphology, with a variety of branching forms. He proposed these cells to be named Dendritic Cells (DCs), from the greek word for tree (déndron) [101].

DCs are the sentinels of the immune system and reside in exposed tissues and organs. DCs recognize and classify dangers through pattern recognition receptors such as Toll Like Receptors (TLRs) and consequently instruct the body about how to deal with threats. Upon encounter with a pathogen, DCs capture foreign antigens and display them on MHC-II molecules (and in some cases MHC-I) and migrate to the local draining LN where they prime and activate naïve T-cells. Likely, transcription factors such as Ikaros, PU.1, Gfi1 and Id2 are involved in DCs maturation [102].

4.3. DC subsets in the mouse skin

The most investigated organs for the study of DCs are lung, gut and skin. Depending on the location different DCs subsets, characterized by different markers, have been identified. Here, I will focus on the mouse skin, which is the relevant organ for our targeting and immunotherapeutic approaches. An overview of the involved DC subsets, their function and cellular markers can be found in section 4.5. and Table 1.

Table 1 Dendritic cell subsets in mouse and human skin

The table compares murine and human DC subsets and shows the most relevant surface markers. Markers that are currently used for subset identification are underlined. The subsets shown in grey represent DC subsets having phenotypes very close to monocytes. It is still controversial if these cell subsets can be considered DCs or not.

Mice	Human
XCr1$^+$ cDC CD11c$^+$, CD8a$^+$, CD64-, CD103$^+$, Xcr1$^+$, CD172a$^-$ Clec9a$^+$, MERTK$^-$	**CD141$^+$ cDC** CD11c^{lo}, CD141$^+$, Xcr1$^+$, CLEC9A$^+$, CD14$^-$, langerin$^-$, CD11b^{lo},
CD11b$^+$ cDC CD11c$^+$, CD11b$^+$, CD64$^-$, CD172a$^+$, Ly6C$^-$, MerTK$^-$, XCr1$^-$, ESAM$^+$	**CD1c$^+$ cDC** CD11c$^+$, CD1c$^+$, CD11b$^+$, CD13$^+$, CD33$^+$, CD45RO$^+$, CD172$^+$, CLEC7A$^+$, CLEC6A$^+$
DoubleNegative cDC CD11c$^+$, CD11b$^-$, XCr1$^-$	
Plasmacytoid DC (pDC) CD11c^{int}, CD11b$^-$, Bst2^{hi}, SiglecH$^+$, CD45R$^+$, PDCA-1$^+$	**Plasmacytoid DC (pDC)** CD11c$^-$, CD123$^+$, CD303$^+$, CD304$^+$, CD4$^+$, CD45RA$^+$,
moDC CD11c$^{-\text{ to }lo}$, CD11b$^+$, CCR2$^+$, CD64$^{lo\text{ to }+}$,MerTK$^{-\text{ to }lo}$, CD24$^-$,	**moDC** CD11c$^+$, CD11b$^+$, CD1a$^+$, CD1c$^+$, CD206$^+$, CD209$^+$, HLA-DR$^+$, BDCA1$^+$, CD16$^-$
Langerhans Cells (LCs) MHC-II$^+$, langerin$^+$, CD24$^+$	**Langerhans Cells (LCs)** CD11c^{lo}, MHC-2$^+$, EpCam$^+$, CD1a$^+$, CD83$^+$, langerin$^+$
Tissue DC CD11c$^+$, CD11b$^+$, CD64$^+$, CD14$^+$	**CD14+ cDCs** CD14$^+$, CD11c^{hi}, MHC-II^{hi}, CD163$^+$, CD11b$^+$, CD209$^+$, CCR7$^+$, CXCR3$^+$, FXIIIa$^+$
Gr-1/Ly6C low monocyte Ly6C^{lo}, CX3CR1^{hi}, CCR2$^-$	**SLAN DC** CD16$^+$, LacNAc$^+$, MHCII^{hi}

Conventional DCs (cDCs)

cDCs (previously known as myeloid DCs or mDCs) are the classical DCs found in both lymphoid and peripheral tissues. They are characterized by high MHC-II expression and the typical morphology with long dendrite protrusions. Due to the short half-life of 3-5 days they are constantly replaced from blood-borne precursors originating from the bone marrow. cDCs are currently subdivided in two subpopulations: Xcr^+ cDCs and $CD11b^+$ cDCs.

$Xcr1^+$ cDCs

The expression of Xcr1 and Clec9a newly defines $Xcr1^+$ cDCs (also called CD8a-type cDC), because their expression is more specific than the formerly used markers CD103 and CD8a, which are also expressed on pDC and some DC progenitors [103]. $Xcr1^+$ DCs play a major role in the induction of $CD8^+$ T-cell responses against viruses and cancer cells. Additionally they are considered the most efficient cell subset for cross-presentation, i.e. the presentation of phagocytized exogenous antigens on MHC-I molecules [104]. $Xcr1^+$ cDCs can either be $CD103^+$ or $CD103^-$; positivity for the surface marker CD103 correlates with the ability to cross-present keratinocyte-derived self antigens.

$Xcr1^+$ cDCs can further be classified according to their anatomical localization. Thus, Lymphoid Tissue cDCs (LT-cDCs, $CD11c^{hi}$, MHC-II^{lo}) reside their whole life in secondary lymphoid tissues and migratory cDCs (mig-cDCs, $CD11c^{lo}$, MHC-II^{hi}) reside in non-lymphoid tissues and migrate to the draining LN after encounter with an antigen [103].

$CD11b^+$ cDCs

$CD11b^+$ cDCs are the most abundant DCs. It is not trivial to distinguish this subset from monocyte-derived DCs (moDCs) or macrophages (MF). For this reason beside CD11b expression, lack of Ly6C, CD64 (FcγRI) and MerTK (a protein-tyrosine kinase receptor for the uptake of apoptotic cells) expression has to be ascertained [105]. Little is known about $CD11b^+$ cDCs: they appear to be involved in the presentation of soluble antigens and in the production of proinflammatory cytokines [106]. In the LN, they have been identified as the principal subset inducing T_H2-mediated immunity [107]. A portion of mouse $CD11b^+$ cDCs that has been shown to produce retinoic acid is able to induce T_{regs} and exert therefore a sort of negative feedback control on the continuous antigen challenge from the skin [108].

Double Negative cDCs

The mouse dermis is also populated by a rare DC subset expressing neither Xcr1 nor the marker langerin, defining LCs (see below). This population has not yet been characterized functionally and no human homologue could yet be identified [109].

Plasmacytoid DCs

Mouse pDCs play a major role in antiviral defense. Accordingly, they express high levels of the virus-specific pattern-recognition receptors TLR7 (detects ssRNA) and TLR9 (detects CpG DNA) and are characterized functionally by their ability to produce high amounts of type 1 interferons (e.g. IFN-γ) upon contact with viruses [110]. Morphologically they resemble plasma cells prior activation, hence the name "plasmacytoid". Dendrites become visible only in activated cells. pDCs possess a less efficient MHC-II presentation machinery and express less costimulatory molecules than cDCs. For this reason they are poor stimulators of $CD4^+$ T-cells.

Some groups propose a subdivision of mouse pDCs in different subsets based on the expression of surface markers like CCR9, Siglec-H, CD8α and CD8β. This subdivision is still controversial, and more data is needed to determine if these are real subsets with defined function or different activation states of pDCs [111].

moDC

As the name says monocyte-derived Dendritic Cells (moDCs) develop from extravasated $Ly6C^{hi}$ blood monocytes recruited at the site of inflammation. Their maturation in the mouse dermis goes through three different stages named P1 to P3 characterized by the gradual up-regulation of MHC-II and CD64 expression and the down-regulation of Ly6C [105].

Upon antigen contact moDCs can represent the major DC subset within the affected tissue. Only few of these cells can be found in the draining LN, indicating poor migratory properties compared to cDCs. moDCs are capable of antigen presentation to CD8 and CD4 T-cells, but their capacity is reduced in comparison to $CD11b^+$ DCs [105]. moDCs have been shown to transfer antigens to LN-resident DCs [112].

Under particular inflammatory conditions moDCs differentiate into Tip-DCs, which possess potent antimicrobial properties [113]. However, it has to be pointed out that the affiliation of Tip-DCs to the DCs is controversial, because of their close similarity to MFs [114].

Langerhans cells

LCs populate the supra basal layer of the epidermis. They are easily found in the epidermis of the ear, where they reach a concentration of 1000 LCs/mm^2. Notably,

LCs have also been detected in mucosal tissues. In addition to the expression of langerin LCs can be recognized by the presence of the Birbeck granule, a tennis racket shaped intracellular organelle possibly involved in endosomal recycling [115].

Despite extensive research the function of LCs is far from being completely elucidated. They appear to exert a variety of function depending on the stimuli and the mouse model used. *In vitro*, murine LCs can have two distinct states: a "processing state", which efficiently processes antigens for the presentation on MHC-II molecules and a "presenting state", characterized by high MHC-II expression and high T-cell stimulatory capacity, which appears after 3 days of cell culture. These two states suggest an *in vivo* situation where LCs first take up and efficiently process the antigen in the periphery, and then change properties while migrating to the draining LN where they efficiently present the antigen to T-cells [116]. The relatively modest *in vivo* data only partially confirm this view. In fact, in a hypersensitivity model, the immunostimulatory functions of LCs appeared to be important only when the antigen couldn't reach any other APC [117]. Moreover, in a *Leishmania* infection model LCs were merely involved in the transport of the antigen to the LN, while the actual presentation was carried on by LN-resident DCs [118].

A role of LCs in the establishment of tolerance has been suggested and is supported by the observation of T_{reg} induction and of the tolerogenic properties of epidermally expressed OVA antigen. All together these observations indicate a still uncharacterized degree of plasticity that allows LCs to act differently according to the nature of the challenge [115].

In 2007 a novel rare population of dermal langerin$^+$ DCs has been identified [119]. These cells are present in the mouse dermis at concentrations 10-20 times reduced with respect to epidermal LCs.

4.4. Human DC subsets

Conventional DCs (cDCs)

CD141$^+$ cDCs

The CD141 (BDCA-3)$^+$ DCs subset in humans is believed to be the homologue of mouse Xcr1$^+$ DCs. This subset is indeed positive for Xcr1 and CLEC9A [103]. Like the mouse counterpart, CD141$^+$ DCs can be further classified according to the anatomical location and by using the same surface markers. The LT-resident population is CD11c^{hi}, MHC-II^{lo}, while the migratory population shows a CD11c^{lo}, MHC-II^{hi} phenotype [120]. CD141$^+$ DCs are considered the human cross-presenting DCs: they present epitopes from virus-infected cells to CD8$^+$ T-cells after sensing viral nucleic acids through TLR3 and TLR8 and possess the ability to phagocyte

necrotic cells via CLEC9A. In accordance with the cross-presenting potential, $CD141^+$ DCs have been shown to secrete TNF-α, IP-10, IFN-γ, IL-12p70.

CD1c+ cDCs

CD1c DCs are the homologue of mouse $CD11b^+$ DCs [121]. These cells are found in blood (1% of all mononuclear cells) but also in lymphoid and non-lymphoid tissues. *In vitro* experiments showed that, comparably to their mouse counterpart, $CD1c^+$ DCs are superior stimulators of $CD4^+$ T-cells responses but possess reduced ability for the stimulation of $CD8^+$ T-cells [122].

Plasmacytoid DCs

Human pDCs have been identified in blood (1% of all mononuclear cells), tonsils, and in the T-cell zones, close to the high endothelial venule, in the LN. Like their mouse counterpart human pDCs are involved in antiviral defense, express high levels of TLR7 and TLR9, and secrete high levels of type 1 interferons upon viral infections [123]. They have also been shown to present exogenous viral antigen to $CD8^+$ T-cells via MHC-I after internalization and processing within endocytic organelles [124].

pDCs are supposed to play a role in the onset and therapy of allergic diseases because of their ability for T-cell polarization. pDCs have been reported to induce T_H1, T_H2 or T_{reg} cell responses depending on additional stimuli [125, 126]. They also play a role in the maintenance and breakage of tolerance and in the induction of autoimmune diseases by influencing and inducing T_{reg} cells [111].

$CD14^+$ DC

$CD14^+$ DCs are a clear example of the thin line that separates DCs from macrophages and monocytes. This cell population, which can be found in both lymphoid and non-lymphoid tissues, induces B-cell differentiation and play a role in the formation of follicular helper T-cells. These functional aspects and the marked expression of the surface markers CD11c and MHC-II classify them as DCs. However, their functional distinction from macrophages is difficult: they do not efficiently stimulate T-cells and up to date no clear migration to the LN could be observed. Moreover, they express numerous surface markers that are typical for macrophages. $CD14^+$ DC can be generated *in vitro* by cultivating $CD14^+$ monocytes together with IL-10 and vitamin D3.

moDC

A population of inflammatory DCs expressing surface markers distinct from LCs has been identified in the psoriatic skin [127]. It is plausible that this was the first

report of human monocyte-derived DCs (moDCs), but by that time no convincing evidence could be provided. More recently Segura and co-workers also characterized a population of human DCs in inflamed tissues displaying a unique phenotype. No direct derivation from $CD14^+$ monocytes could be demonstrated; nevertheless transcriptome analysis sugeested monocyte-origin. moDCs have been observed to induce IL-17 production and appear to be involved in the differentiation of T_H17 cells from naïve $CD4^+$ T-cells [128].

Langerhans cells

Most of the information on LCs in relies on mouse data, mainly because of the difficult access to human tissues due to ethical issues. Human LCs are supposed to share most of the properties with their mouse counterpart, as shown by numerous recent studies (reviewed in [115]). Human LCs are localized in the epidermis, interposed between the keratinocytes. In the LN they reside in close proximity to T-cells in paracortical areas. In contrast to their murine counterpart, human LN-resident LCs localize together with $langerin^-$ DCs [115].

SLAN DCs

SLAN DCs are a subpopulation of $CD16^+$ monocytes that express 6-sulfo-LacNAc on their surface. Their existence as a separate subset is, however, challenged by data where they appear indistinguishable from $CD16^+$ monocytes [129].

Other DC subsets have been suggested to populate inflamed tissues (psoriatic skin). However, the available data do not allow yet a clear functional characterization of these subgroups and it is still unclear if they are defined subpopulation or activated versions of existing populations of DCs, monocytes or macrophages. For this reason they will not be further discussed.

4.5. Homology between human and mouse DC populations

Numerous subsets of DCs have been identified in the mouse skin up to now and for most of them the human counterpart has been identified. Table 1 summarizes the mouse subsets described so far together with the human counterpart. The literature on DC subsets is very heterogeneous: the different groups use different names and markers to identify DC subpopulations. This summary table is mostly based on the works of B. Malissen and M. Collin [102, 109].

4.6. Macrophages

MFs differ from DCs by their increased phagocytic and lytic activity, and the reduced antigen presenting capabilities. At a first glance they could be considered as rough relatives of DCs, greedier for materials to phagocyte and digest, but

without assiduously practicing antigen presentation. MFs relatives can be found throughout the whole body: beside skin, lung, spleen and peritoneal MFs, foam cells can be found in the blood vessel plaques, Kuppfer cells in the liver and microglial cells in the brain. All these variants share the same mission: to sample the environment and decide for fighting or fixing, depending on additional stimuli, and are therefore crucial for the maintenance of tissue homeostasis [130, 131].

Fight or fix behavior by MFs can be identified by looking at specific biochemical markers. Fighting MFs produce nitric oxide (NO) for the inhibition of proliferation and are called M1-macrophages (M1-MFs). Fixing MFs produce ornithine, an arginine derivative that promotes proliferation and repair. Fixing MFs are called M2-macrophages (M2-MFs). The nomenclature, dividing MFs in the two functional families M1 and M2 results from the observation that they stimulate T_H1 and T_H2 cells, respectively, but do not require T- or B-cells to be generated. Hence, and importantly, the appearance of M1 and M2 precedes the contact with T_H1 or T_H2 cells [132]. Observations *in vitro* have pointed out that T-cell cytokines (IL-4, IL-13) have an influence on the behavior of MFs [133]. This lead to a further subdivision of M2-MFs in M2a, M2b and M2c depending on the stimulus [134], which, however, has been practically abandoned nowadays, after the observation of extensive plasticity within M1 and M2 phenotypes. In fact, some cells display a pure phenotype (e.g. NO or ornithine production only), while other cells can produce both compounds in variable amounts.

Functional differences such as M1 and M2 appear to correlate with different anatomical localizations. Depending on the organ and the field of research a variety of additional MF subsets have been observed. The brief summary on macrophages that follows will deal with skin and LN MFs only, which have been relevant for the study.

M1 macrophages

M1-MFs are activated by IFN-γ, LPS, and other Toll-Like Receptor ligands. In addition to the NO production M1-MFs are characterized by the secretion of citrulline and the pro-inflammatory cytokines IL-1, IL-8 and IL-12, as well as by the up-regulation of MHC-II molecules. This response provides protection against microorganisms, but also damages neighboring tissues. For this reason M1-MFs are believed to participate in various autoimmune and chronic inflammatory diseases [130, 131].

M2 macrophages

M2 is considered to be the default activation state for MFs. Ornithine production is accompanied by the secretion of TGF-β, PDGF, IL-10 and type 1 interferons, as well as chitinases, metalloproteinases and scavenger receptors [130]. The stimulatory activity of these compounds for epithelial cells and fibroblasts reflects the important role played by M2-MFs in wound healing and fibrosis. Additionally, M2-

MFs display potent anti-inflammatory activities able to counter-act the detrimental effects triggered by monocytes and M1-MFs [131].

Lymph Node macrophages

According to their localization two major MFs populations in the mouse LN have been identified.

Subcapsular Sinus macrophages (SSMs) CD169^{hi}, CD11b^{+}, F4/80^{-}

SSMs assume a para-cellular position, with a head located into the sinus of the LN and a stable tail that extends in the underlying follicle. Up to now the major function that could be attributed to SSMs is the capture of various lymph-borne antigens (viruses, nanoparticles, immune-complexes) by the head and their transfer to follicular B-cells by the tail [135, 136].

Medullar macrophages

Medullar MFs can be located either in the medullar sinus or the medullar chord and have been proposed to play a role in the uptake and sensing of lipids [137]. Depending on their location, medullar MFs have distinct functions and may even represent two distinct subpopulations. Medullary sinus MFs are highly phagocytic and are located on and sometimes in the wall of the medullary sinus. Medullary chord MFs' main functions appear to be the trophic support of plasma cells and their clearance by phagocytosis [135].

Skin macrophages

In mice most dermal MFs arise from blood LY6C^{hi} monocytes in a CCR2-dependent manner. However, some MFs (e.g. Kupffer cells) derive from yolk sac progenitors and are maintained in the dermis throughout life by proliferation, without hematopoietic input [105, 109].

Recently, extensive analysis of dermal DCs and MFs assessed the surface markers expression pattern of dermal MFs (CD11b^{+}, CD24^{-}, CD64^{hi}, MerTK^{+}, CCR2^{lo}, Ly6C^{lo}). According to the expression of MHC-II these cells segregate into two distinct populations called P4 (MHC-II^{-}, CD11c^{-}) and P5 (MHC-II^{+}, CD11c$^{-\text{ to }lo}$). Up to now no functional distinction between P4 and P5 could be identified, nevertheless these cells share a number of properties characteristic for MFs. P4 and P5 dermal MFs are autofluorescent cells with a foamy cytoplasm. In mice they do not migrate to the LN and have anti-inflammatory potential by the secretion of IL-10 [105]. In addition to immunoregulation, dermal MFs are thought to have a sentinel role, exerted by the secretion of chemotactic cytokines that trigger neutrophil extravasation [105].

From a morphological point of view P4 and P5 MFs resemble the $CD1a^-$ $CD14^+$ $FXIIIa^+$ MFs found in human dermis [138]. The identification of P4/P5 and their distinction from $CD11b^+$ DCs is not possible using the traditional MF markers F4/80, CD68, CX3CR1 and Lysozyme M [105].

The role of macrophages in allergic diseases

The increase in allergic diseases in the last decades is considered to be at least partially influenced by the important advances in public health and sanitation, which are thought to have made us more prone to the development of T_H2 dominated immune responses [139]. A role for MFs is suggested by the observation that chronic stress, improved hygiene and standardized foods have led not only to unbalanced T-cell responses, but also to unbalanced MFs responses. These responses are predominantly characterized by M2 phenotypes, which could exacerbate allergic diseases, but also promote the development of cancer and other autoimmune diseases such as Systemic Lupus Erythematosus [130].

The importance of MFs in airway inflammation is suggested by the higher number of activated MF observed in murine models of allergy [140]. Moreover, MFs have been reported to modulate T-cell responses in the lung, contributing to homeostasis [141]. In fact, the depletion of MFs in sensitized mice resulted in more elevated allergic symptoms [140]. In food allergy and oral tolerance the picture is more fragmented, with a role for MFs merely suggested by the increased numbers observed in the appendix of sensitized mice [142].

Human macrophages

Our knowledge on MFs mostly relies on mouse models. For obvious reasons human studies focused on the generation of MFs *in vitro*. Human MFs are generally polarized from blood monocytes ($CD14^+$ cells). The most widely used protocol for the generation of M1-like MFs *in vitro* makes use of M-CSF (or GM-CSF), followed by polarization with IFN-γ, alone or in combination with LPS or TNF-α. M2-like MFs are generated by stimulation with M-CSF or GM-CSF followed by IL-4 or IL-13 treatment. It has to be pointed out that a huge variety of differentiation protocols have been suggested, resulting in only partially overlapping phenotypes [143].

Human MFs generated *in vitro* can be identified by the enhanced expression of CD68. Depending on the differentiation protocol used, M1-MF can be identified by the expression of the surface markers CD40,CD80, CD86, CD64, CD32, while M2-MF express higher levels of CD163 [144-146].

4.7. Distinction between dendritic cells and macrophages

The traditional distinction between DCs and MFs is based on the enhanced ability of DCs to process and present antigens on their surface and to migrate from the tissues to the secondary lymphoid organs where antigen presentation to T- and B-cells takes place. More recently large efforts have been undertaken to characterize biomarkers able not only to distinguish between DCs and MFs, but also allowing their allocation in distinct subpopulations. Initially the molecules F4/80, CD68 and LYZ2 were suggested as discriminatory markers in mice. Later they have, however, shown to lack the desired specificity. Nowadays the most supported MFs markers distinguishing them from DCs are CD64 and MERTK [109].

David A. Hume proposes an interesting alternative vision on the differences between DCs and MFs. In his review "Macrophages as APC and the Dendritic Cell Myth" [114] Hume discusses with a critical view the literature supporting a clear distinction between DCs and MFs and tries to convince the scientific community that the functional and molecular markers used to define DCs do not necessarily correlate with their ability to act as APC and that other cells (such as some MF subsets) possess similar properties without being considered DCs.

Among others, Hume supports his idea with the following arguments: First, he criticizes the *in vivo* models that use a particular stimulus for the differentiation of DCs or MFs. As a matter of fact, in *in vivo* settings both DCs and MFs are influenced by the typical growth factors GM-CSF and M-CSF, it is therefore misleading to consider M-CSF as the MF growth factor and GM-CSF as the DC growth factor [147]. Second, there are studies showing that both DCs and MFs can present antigens to T-cells to a similar extent but with different target cells and outcomes [148]. Third, the fact that DCs express CD11c does not correlate with their ability to act "as DCs". Many more endocytic receptors exist and the use of CD11c for the distinction of DCs is arbitrary. Moreover some MF subsets are $CD11c^+$ [149]. With his visions Hume definitively stands out of the crowd, not supported by the scientific community. For this reason I will maintain here the traditional, clear distinction between DCs and MFs. Hopefully the next decade of research will be able tell us if he was right or not.

4.8. Maturation of dendritic cells and macrophages

Mouse cDCs, monocytes and MFs derive from a common progenitor that resides in the bone marrow (MDP, macrophage and DC precursor). This precursor gives rise to the monocyte precursor and to the common DC precursor (CDP), which in turn gives rise to the pre-DC precursor, which leaves the bone marrow and migrates through the blood to lymphoid and non-lymphoid organs where it differentiates into the various cDC subsets [150]. In humans it is known that DCs can arise from BM-resident precursors, however, no homologues have been yet identified for the mouse MDP, CDP and pre-DC [151].

MFs arise from circulating inflammatory monocytes that are recruited into the tissues and quickly differentiate into MFs or DCs. The fast differentiation process is thought to be necessary for the protection of the tissues against the excessive inflammation triggered by extravasated monocytes. This classical view has been recently complemented by the observation of macrophage self-renewal in the tissue, triggered by IL-4 for both M1-MFs and M2-MFs during helminth infection [152]. Self-renewal could therefore be an additional source of macrophages in the tissues in inflammatory settings.

Some MFs, including Kuppfer and microglia cells, as well as radiation resistant LCs develop from primitive macrophages that are recruited in the epidermis during embryonic life and maintain themselves without further input from the bone marrow in adulthood [153, 154]. The epidermis of bone marrow chimeras will therefore be populated by the recipient's LCs. This unique pathway, clearly distinct from DC-subsets appears to be shared between mice and humans [155].

The origin of mouse pDCs is still under debate. Both common myeloid and lymphoid progenitors have been shown to give rise to pDCs upon transfer into irradiated mice [156]. More recently a study identified a $CD115^-$ pDCs-specific subset within the CDP population, which commits to pDCs by up-regulating the E2-2 transcription factor [157].

4.9. Dendritic cell migration to and inside the lymph node

For obvious technical and ethical reasons DCs migration has been intensively investigated in mouse models only. Their behavior in human may or may not be similar.

To reach the LN, DCs have often to travel for several millimeters and across collagenous connective tissue. For this reason proteinases (especially matrix metalloproteinases such as MMP-2 and MMP-9) play a pivotal role, as shown in studies where broad spectrum matrix metalloproteinases inhibitors or antibodies were able to prevent DCs and LCs migration [158]. The picture describing the migration mechanisms to the LN is far from being complete and new reports are showing the importance of many chemokines and receptors, upregulated under inflammatory conditions [159]. Well supported are among others the importance of CCL1, CCL19 and CCL21, whom receptors (CCR8 and CCR7) are abundantly expressed on the surface of mature DCs [160, 161]. The adhesion molecules ICAM-1 and JAM-1, and their interaction with the skin lymphatic endothelium also play an important role by facilitating cell motility [162, 163].

Once reached the LN, DCs originating from the skin accumulate in the vicinity of high endothelial venules (HEVs). In this way they are supposed to selectively trap antigen specific T-cells that are passing by [164]. Later, DCs occupy distinct areas

within the T-cell zone where they show continuous micromotility behaviors to probe this environment, more marked in the first 2-3 days [165].

The majority of antigen-bearing DCs will reach the LN within the first two days after exposure. Interestingly the langerin$^+$ LCs reach their maximum only after 3-4 days, as the immune responses are already initiated. LCs are therefore supposed to predominantly have an immunoregulatory role. A different role for LCs is corroborated by the observation that they assume distinct positions inside the LN: in fact, LCs are localized in the deep paracortex of the T-cell zone, while the other dermal DCs localize in proximity of B-cell follicles [159].

4.10. Antigen processing and presentation

Depending on its localization the antigen is presented on class 1 or class 2 major histocompatibility complex molecules (MHC-I, MHC-II) on the cell surface.

Peptides generated in the cytoplasm by proteasome degradation are presented through the MHC-I complex. Every cell presents self and non-self peptides on MHC-I molecules. The presented peptides interact with the T-cell receptor (TCR) on CD8$^+$ T-cells. If the peptide is recognized as non-self (e.g. because of a viral or bacterial infection) and in the presence of defined co-stimulatory stimuli, the CD8$^+$ T-cell will react by triggering apoptosis of the target infected cell, thereby minimizing the spread of the infection [166].

MHC-II presentation is a matter for professional APCs. In fact, MHC-II expression can only be detected on DCs, MFs and B-cells. These cells phagocyte exogenous proteins and present them to the TCR of CD4$^+$ T-cells after endosomal processing and loading on MHC-II. At the same time they provide signals in form of cytokines, which additionally define the threat, and in form of membrane bound co-stimulatory receptors such as CD40 (binds to CD40L on T-cells) or CD80/CD86 (cooperatively bind to CTLA-4 and CD28 on T-cells), which have an activatory and survival-promoting function [166]. With this information CD4$^+$ T-cells are able to drive the immune response towards a resolution of the infection.

The MHC class II presentation pathway

The two chains of the MHC-II molecule (α and β) are assembled in the ER and coupled with the Invariant chain (Ii).

Ii is thought to play a dual role. First, its cytoplasmatic tail contains di-leucine sorting motifs that direct the Ii-MHC-II complex to endocytic compartments [167]. In mature DCs this transport appears to occur directly from the Golgi compartment; while in HeLa cells and immature DCs the complex travels to the plasma membrane and gets endocytosed [168]. Second, it prevents the binding of unwanted endoplasmatic reticulum peptides to the MHC-II groove. This is accomplished by the

class II-associated Ii peptide (CLIP) region at the end of the cytoplasmic domain, which occupies the peptide binding groove.

Through the Golgi apparatus, the complex is transported to a late endosomal compartment called MHC-II compartment (MIIC) where Ii is almost completely degraded, leaving only the residual CLIP bound to the MHC-II binding groove. Early endosomes containing phagocytized proteins fuse with the MIIC, where their content is further processed by a variety of proteases. The resulting peptides take over the CLIP position on the MHC-II molecule with the help of HLA-DM (H2-DM in mice): the new complex is subsequently presented on the plasma membrane [169].

Cytoplasmic peptides can enter the MHC-II pathway when a peptide-MHC-I complex enters an endosome. The acidic conditions release the peptide from MHC-I making it available for MHC-II presentation. This pathway is rare under normal conditions, but may be more prevalent in the context of cross-presentation [170].

Even if the picture is far from being complete, many regulators of MHC-II presentation, expression and stability have been identified. Fist, the activation state of the cell: the half-life of a MHC-molecule is greatly increased in mature DCs [171]. Second, IL-10, which down-regulates the surface expression of MHC-II [172]. Third, the effect of co-stimulatory molecules, for instance LPS has been shown to trigger a re-distribution of MHC-II molecules on the plasma membrane [173].

Role of DCs in allergy

By the onset of an allergic disease naïve T-cells are induced to differentiate into T_H2 cells, which in turn orchestrate the immune response that later leads to an allergic reaction. Two of the big unanswered questions in allergology are “What makes a protein an allergen?” and “What is triggering the B-cell immunoglobulin class switch to IgE?”. In this context, and being the sentinels of the immune system, DCs and APCs in general are thought to play a pivotal role.

One possible motive for the allergenicity of a protein may be its ability to act as an adjuvant and drive the DC to induce T_H2 differentiation [174]. This explanation is supported by several publications demonstrating the T_H2-skewing properties of allergens. More specifically, T_H2 adjuvation appears to derive from the glycan structures on the allergens and the consequent binding to C-type lectin receptors. This mechanism is common to some food (e.g. the major peanut allergen Ara h 1 [175]) and inhaled allergens (e.g. the house dust mite allergens Der p 1 and Der p 2 [176]). Still, the picture remains controversial. Various studies support this view and suggest an auto-adjuvant potential for some allergens. Others seem to be in contrast with this explanation, like the observation that generalized binding to C-type lectin receptors is mostly tolerogenic [177].

Several studies have linked the tolerogenic properties of pDCs to the control of allergic diseases both in human and mice. A clinical study showed that the number

of pDCs in infancy inversely correlate with the development of asthma during the first 5 years of life [178]. Palomares and co-workers showed that FoxP3$^+$ T-cells in human palatine tonsils inhibit antigen-induced T-cells proliferation and that FoxP3$^+$ T_{regs} are generated by pDCs from naïve T-cells. Moreover, they observed a co-localization of pDCs and FoxP3$^+$ T_{regs} [179]. In mice pDCs play a role in the prevention of food allergy and their depletion causes sensitization and increased lung inflammation in OVA-Alum sensitized mice [111, 180]. The tolerogenic potential of pDCs is supported by the presence of probiotic microorganisms such as *Bifidobacterium infantis* and is abrogated by the presence of danger signals deriving e.g. from viral or bacterial infections [111, 181].

5. Dendritic cell targeting

DCs are key regulators of the immune system and, more than any other cell type, possess the ability to process antigens and present them to responder cells. For this reason, being able to specifically target these cells with selected antigens, has a great immunological potential and may significantly enhance current vaccinations and immunotherapies.

The increase in T-cell mediated immune responses following DC-targeting was initially observed by D.P. Snider and D.M. Segal in 1987, who exploited the discovery of the benefits of opsonization [182] to target antigens specifically to FcγRs or MHC molecules, thereby reducing by a factor 100-1000 the amount of antigen required for antigen presentation (at that time measured by lymphokine release) [183].

From then on many other potential targets have been identified that are primarily expressed on DCs. However, it has to be kept in mind that no exclusive DC receptor has yet been identified. Therefore targeting of unwanted cell populations can never be excluded [184].

It is likely that targeting antigens to selected DC subsets will result in different immune responses, and first evidences for this assumption have been published in the last few years. For example, in mice, targeting CD8$^-$ DCs resulted in more prominent antigen-specific CD4$^+$ T-cell responses, while targeting Xcr1$^+$ DCs resulted in enhanced CD8$^+$ T-cell responses [185]. Deeper knowledge of target epitopes on the various DC subsets may therefore allow disease-specific targeted therapies [186].

5.1. DC targeting in allergen immunotherapy

The interest for DC targeting in allergic diseases stems from the observation that increasing the antigen concentration in cell cultures of PBMCs from allergic individuals, favors IFN-γ over IL-4 production [68]. Experiments *in vitro* and clinical observations during allergen-specific immunotherapy clearly showed that mostly IL-4 is responsible for the switch of B-cells towards the production of the allergen-

specific IgE antibodies associated with the atopic condition, while IFN-γ rather results in the production of protective IgG4 antibodies [68]. In this context, being able to load DCs with large amounts of allergens may induce an IFN-γ dominated (T_H1) immune response, beneficial in an allergic background.

It is broadly recognized that targeting steady-state DCs without danger signals or adjuvants results in the induction of tolerance. Most of the work involves targeting of the C-type lectin DEC-205 (discussed below). The induction of tolerance involves the deletion or unresponsiveness of antigen-specific CD4 and CD8 T-cells and the induction of T_{regs} [187]. This property of DCs may be exploited in immunotherapeutic strategies, but more studies aiming at identifying potent tolerance inducing target molecules and at the optimization of *in vivo* approaches are needed [186].

5.2. DC targeting strategies

C-Type lectin receptors

C-Type lectin receptors (CLR) are a large family of carbohydrate-binding membrane proteins that are primarily involved in microbial recognition and in the modulation of innate immune responses. The restricted expression on few cell subsets including APCs, together with cell-activating properties, made CLR the most widely used target molecule for immunization approaches. The following CLRs can be found on the surface of DCs or LCs: DEC-205 (CD205), Mannose Receptor (CD206), Langerin (CD207), DC-SIGN (CD209), Dectin-1 (Clec7A), Dectin-2 (Clec9A), BDCA-2, DCIR, DLEC, and CLEC-1.

CLR targeting can be achieved both by using an antibody or a specific ligand. Experiments performed with the C-type lectin DC-SIGN have shown that antibody targeting has to be preferred, because, with some exceptions, it resulted in higher binding, uptake and presentation of antigens [188].

DEC-205 (CD205)

DEC-205 has been suggested to be a promiscuous receptor that binds various ligands in addition to the carbohydrate structures interacting with its 10 C-type lectin like domains. A recent study has actually identified non-methylated cytosine-guanosine (CpG) motifs as additional binders for DEC-205, which may therefore possess undisclosed immunoregulatory potential [189]. DEC-205 is a suitable candidate for the targeted delivery of epitopes to be recognized by helper T-cells, because it recirculates through lysosomes and late endosomes, mediating antigen presentation through the MHC-II pathway [190]. Targeted constructs delivered without additional stimulus lead to the deletion of antigen-specific T-cells and the induction of T_{reg} cells [191]. This induction of tolerance has been shown to prevent

the onset and the progression of type 1 diabetes in mice and hence represents a promising approach for the treatment of autoimmune diseases [187]. On the other hand, co-administration of maturation stimuli (CpG or anti-CD40 antibodies) resulted in antigen-specific T-cell responses and tumor remission in mice [192]. The usefulness of DEC-205 for human use *in vivo* is limited by its presence on cells other than DCs that may act as a sink for the applied biological [193].

Mannose Receptor (CD206)

Antibody-mediated targeting of CD206 enhances uptake and MHC-I/II presentation [194]. Targeting CD206 using the natural ligand would most probably result in unspecific binding, since mannose and mannan are recognized by other lectins as well. Cancer clinical trials performed up to date and using different targeting strategies showed variable outcomes [195]; the most promising being an immunotherapy of breast cancer with mannan conjugated tumor-derived mucin-1, where the targeted vaccine resulted in significantly increased survival and reduced recurrence [196].

DC-Sign (CD209)

DC-SIGN is the most DC-specific receptor in humans [197] and has, therefore, a great clinical potential. The downside of DC-SIGN is the lack of a functionally homologous receptor in mice [198], which result in obvious technical limitations. *In vitro* DC-SIGN-targeted antigens are rapidly internalized and localize inside lysosomes and late endosomes. Targeting antigens to DC-SIGN resulted in both MHC-I and MHC-II presentation and enhanced naïve and memory $CD4^+$ and $CD8^+$ T-cell responses [197, 199, 200]. Experiments performed with cross-reactive mouse anti-human DC-SIGN in monkeys showed efficient DCs targeting *in vivo* [200]. Up to date no clinical trials involving DC-SIGN targeting have been published.

Dectin-1 (Clec7A)

Dectin-1 is preferentially expressed on $Xcr1^+$ cDCs in the mouse and on their human homologues $CD141^+$ cDCs. Targeting of an antigen to Dectin-1 promotes T-cell responses, DCs activation and antibody production. It is believed that Dectin-1 is involved in the recognition of dead cells and that the targeting benefit may exploit the natural mechanisms of antigen presentation associated with their elimination [201]. Dectin-1 is discussed in more detail in Chapter 3.

Peptides

Targeting peptides (usually 3-12 aa) are usually identified by phage display or, more recently, by chemical generation of highly complex libraries, which have the advantage of including D- or non-natural amino acids. Most targeting peptides have

been directed against tumors for imaging or for the delivery of cytotoxic compounds. The biggest advantages of targeting peptides in comparison with antibody targeting are the small size, which may result in increased tissue penetration, and the reduced costs (short peptides are easily synthesized chemically with good yields and antigen-peptide fusions can be cloned and produced in *E. coli*) [202].

To the best of our knowledge, up to date only two publications deal with peptides targeting DCs. Both publications demonstrated the benefits of their targeting peptides extensively, but only *in vitro*. Significant experiments measured T-cell proliferation and the induction of specific T-cells, or combined DCs and targeted antigens *in vitro* before injecting them into the mouse [203, 204]. For this reason the *in vivo* efficacy of peptide targeting is still far from being proven.

The DC targeting peptide DCpep

In 2004, Curiel and coworkers identified by phage display three DC-binding 12-mer peptides: pep3 (FYPSYHSTPQRP), pep12 (AYYKTASLAPAE) and pep18 (SLSLLTMPGNAS). Out of these pep3 (later renamed DCpep), had the interesting property to bind both human mdDCs and mouse $CD11c^+$ $I\text{-}A^+$ DCs and was therefore investigated further [203]. More recent studies showed that DCpep binds DCs from a broad range of species, including feline, avian, canine and equine DCs ([205] and A. Ziegler personal communication). DCpep bound to both mature and immature DCs in a saturable way through a yet unidentified epitope, which is distinct from the one bound by pep12 or pep18. *In vitro*, DCs pulsed with DCpep fused to NS3 (hepatitis virus nonstructural protein 3) were superior in the activation of autologous $CD4^+$ and $CD8^+$ T-cells from hepatitis C virus (HCV) infected patients in comparison with NS3 alone. *In vivo* DCpep was shown to improve the efficacy of *Lactobacillus acidophilus* mediated vaccination against *Bacillus anthracis*. Oral vaccination of mice with *L. acidophilus* expressing *B. anthracis* protective antigen (PA) fused with DCpep resulted in higher and more protective antibody titers and a different cytokine pattern involving IL-6, IL-10, TNF-α and MCP-1 when compared with control peptide fusions [206].

Recently DCpep was adopted for the creation of nested MHC-II epitopes for vaccination. In this approach a MHC-II peptide was flanked with DCpep on the C-terminus and the invariant chain-derived Ii-Key peptide (LRMK) on the N-terminus with the aim of combining DC-targeting and the exchange of peptides in the MHC-II molecule at the site of action of HLA-DM promoted by Ii-key. DCpep and Ii-key nesting resulted in higher levels of antigen presentation from DCs and the activation of high-avidity antigen-specific CD4 T-cells *in vitro* [207]. Nesting with a control peptide instead of DCpep was not investigated. It is therefore hard to evaluate at what extent the observed benefits derived from the DC-targeting properties of DCpep.

The data showing increased targeting and presentation *in vitro* and the increased functionality of DCpep-antigen pulsed DCs *in vitro* and *in vivo* are convincing. However, up to now, no studies dealt with a direct DCpep-mediated targeting *in vivo* with soluble constructs.

Peptide multimers and cyclation

Once a binding peptide has been identified, the affinity of the targeted construct can be enhanced by multimerization. Increased affinities through multimerization are mainly achieved in two ways: First, binding to two adjacent receptors (usually made possible only by the addition of more or less flexible spacers between the targeting moieties); second, the increase in local concentration that facilitates the reattachment of the targeted construct to its epitope through the presence of additional binding sequences. Studies with the αVβ3 integrin receptor and one of its ligands, the anti-cancer peptide RGD, suggested the increase in local concentration to be the dominating mechanism for the increased affinity [208]. The number of repetitions of the targeting peptide has also been matter of study using the RGD peptide: the results are not completely consistent but in general suggested 4 moieties to be sufficient for an increase in binding over the monomer without creating too many solubility, size or complexity problems [202, 209].

Another strategy to improve the affinity of a peptide is its cyclation. A linear peptide in solution assumes a number of conformations, but only some of them have the desired affinity and specificity. Cyclation, by introducing fixed geometries that limit the number of possible conformations often results in increased affinity and specificity [210]. The tumor binding RGD-motif for example binds integrin subtypes with little specificity in its linear form. In contrast, cyclic derivatives of RGD peptides, showed high specificity for particular integrin receptors [211]. The versatility of cyclation chemistry also represents a tool for the creation of unusual constructions with novel properties such as the incorporation of metal ions (metal coordination) [212]. Moreover, cyclation also usually increases the protease stability of the otherwise rather unstable short peptides [210]; a property that may be crucial for the production of biologicals with longer shelf-life.

Lectins

Until present, no reports of DC-binding lectins were found. Binding approaches focused on antibody- or C-type lectin receptor-mediated targeting. Using lectins as targeting domains may be of interest due to the high binding-avidity. However, concerns may arise because of the immunogenic potential of lectins [213], which may lead to unwanted immune reactions such as autoimmunity or allergic diseases. Moreover, their potential as specific targeting molecules is only theoretical. In fact, no DC-specific sugar structures have yet been identified.

Other Targeting Proteins

Other proteins have been proposed for DC targeting. For instance, the dual ability of Heat-shock proteins (hsp) to bind polypeptides and to modulate immune responses by binding to cellular receptors has been investigated for the creation of targeted vaccines. The receptors involved in hsp binding to DCs are poorly known and have not been assessed *in vivo*. However, hsp have been able to deliver pathogen-specific peptides as cargo to DCs, resulting in MHC-I presentation and $CD8^+$ T-cell responses. The clinical efficacy of hsp as adjuvants or targeting modules in cancer vaccines is currently under evaluation, mostly in phase I and II clinical trials [214].

Fc Receptor Targeting

Fc Receptors are expressed on most innate immune cells and are classified according to the immunoglobulin subtype that they bind in FcαR (IgA), Fcα/γR (IgA and IgM), FcεR (IgE), and FcγR (IgG). They can be subdivided in high-affinity receptors that bind monomeric antibodies, and low-affinity receptors that bind antibody complexes. Furthermore, they can be functionally divided into activating and inhibitory FcγR depending on the motif initiating the signal transduction process. Activating FcγR possess a cytoplasmatic immunoreceptor tyrosine-based activation motif (ITAM) (e.g. FcγRI, FcγRIIA), while inhibitory FcγR possess an immunoreceptor tyrosine-based inhibitory motif (ITIM) (FcγRIIB).

FcγR have been shown to favor the internalization of opsonized material [215]. According to the type of FcγR the internalized material is addressed to different compartments. Activating receptors lead to degradative pathways and subsequent antigen presentation to T-cells, while the inhibitory receptor FcγRIIB preserves the antigen for subsequent transfer to B-cells [216].

FcγRs are selectively expressed on the different subsets of DCs, monocytes and macrophages. Inhibitory FcγR are mainly expressed by cells that populate the peripheral tissue during homeostasis, while the cells that are recruited upon inflammation express virtually all types of FcγR.

FcγR targeting was one of the first targeting approaches attempted to promote activation of moDC. FcγR targeting enhanced antigen uptake and its localization in MHC class II-rich late endosomes, leading to efficient processing and presentation to $CD4^+$ T-cells and T_H1 polarization *in vitro*. The T_H cell polarizing effect upon FcγR binding is, however, still unclear. In fact, using different models other groups observed the development of T_H2 responses after FcγR targeting [217]. In addition, the usefulness of FcγR targeting *in vivo* is questioned by the observation that migratory cDCs, the main DC subset responsible for T-cell polarization, express very low levels of FcγR. At the moment there is therefore no consistent theoretical support for the benefits of this targeting strategy [215].

Particles

One of the main mechanisms by which Alum exerts its adjuvation potential is the presentation of antigens in form of particles that are easily taken up by APCs (discussed in sections 3.6 and 3.7). Hence, another simple way to target phagocytic cells is to provide the antigen in particulate form. In this context a major role is played by the particle size [94], which can determine the success of the targeting approach. Nowadays much effort is put in the development of particulate materials carrying antibodies or natural ligands for the targeting of selected cell populations [88].

Anatomical targeting of skin APCs

As already discussed in section 2.4 (Epicutaneous immunotherapy) a simple way to target specific cell populations is to exploit their anatomical localization. During this thesis work we employed the Precise Laser Epidermal System (P.L.E.A.S.E) from Pantec Biosolutions (Ruggell, Liechtenstein) to target APCs in the dermis of the mouse ear. P.L.E.A.S.E is a fractional laser ablation system that creates pores by triggering the explosive evaporation of water molecules in the skin. It allows the control of pore density, ablated surface and, more importantly, pore depth. Depending on the purposes it is therefore possible to perform intra-epidermal, intra-dermal or trans-dermal applications. The presence of a restricted number of DCs subsets and LCs in relatively high number [54, 115] and their non-homogeneous distribution throughout the various layers of the skin allows the preferential targeting of some cell subsets to specifically modulate the immune response [218]. Laser microporation is not the only way to target skin cells: other common methods are the epidermal powder immunization [219] or the use of micro-needle patches (reviewed in [220]).

5.3. Achieving cell penetration

It is still unclear if the addition of cell penetrating peptides (CPP) to targeting constructs is beneficial. Older studies showed that conjugation with CPP resulted in enhanced MHC-I presentation, and speculated that the *tat* peptide used was able to trigger endosomal escape, resulting in MHC-I presentation [221]. More recent studies questioned these ideas. It appears that the endosomal escape triggered by CPP is minimal and hardly detectable by fluorescence microscopy. However, if carefully designed and dosed, CPP-derived peptides acquire membrane disruptive abilities that result in cytoplasmic escape [222]. This fact may explain why targeting DC-SIGN with a specific antibody or with a poly-Arginine CPP resulted in similar binding and presentation, but the combination of the two targeting strategies showed no additional benefits [223].

Antigen presentation through the MHC-II pathway does not require endosomal escape. The fact that internalization via *tat* peptide results in vesicle-trapped antigens is exploited by modular antigen translocation (MAT) vaccines. After internalization MAT vaccine are thought to intercept the MHC class II molecules bearing vesicles leading to the presentation of the transported antigen [71].

The combination of targeting and cell-penetrating moieties has been attempted before. In the case of antibodies, the presence of the *tat* cell penetration peptide resulted in a dramatic decrease in specificity [202], probably because of interactions with the cell membrane. For this reason such an approach would be possible only by designing the delivery system in a way that masks the penetration peptide until successful targeting.

5.4. Combination with adjuvants

The general rule states that targeting of immature DCs will lead to tolerance, while aiming for activated DCs will immunize. The elicitation of an immune response while targeting immature DCs will therefore require adjuvation (e.g. LPS, TLR ligands, anti-CD40). This statement appears to be broadly valid for the generation of cellular responses involving cytotoxic lymphocytes [186], with the apparent exception of CD36 [224]. In contrast, numerous studies reported the adjuvant-free generation of antibody responses [225]. These differences appear to be determined by the nature of the targeted receptor. Notably, the broadly studied DEC-205 does not induce any antibody response upon targeting [191].

Care has to be taken in the interpretation of the results reported for DEC-205 targeting. Not all the studies carefully ruled out all the possible contaminations of their reagents and the presence of low amounts of microbial products with adjuvant properties can never be completely excluded.

CHAPTER 1

Novel microparticles create a slow releasing depot for sustained immunostimulation

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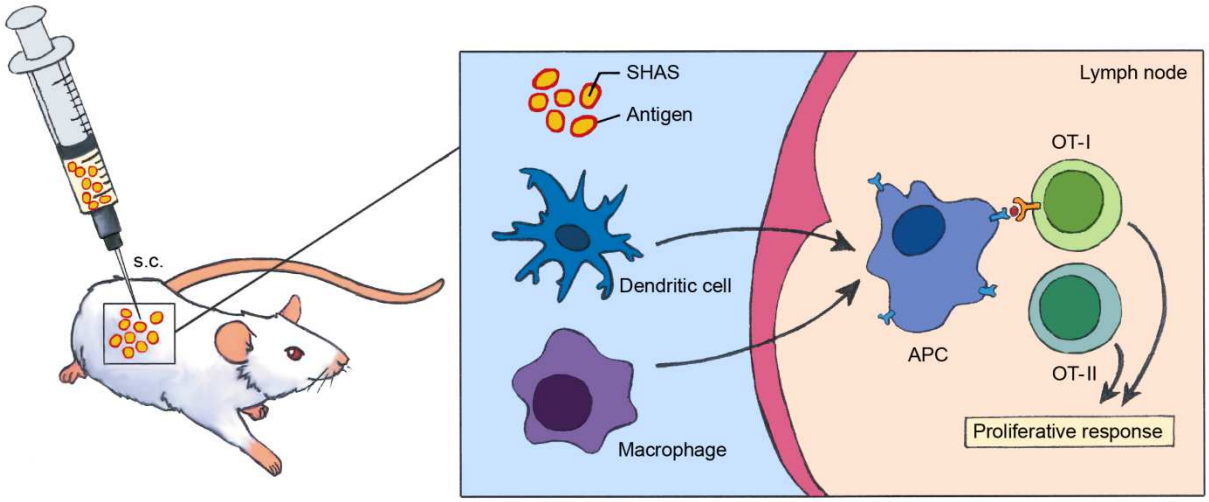
Abstract

The suitability of micro-sized hydroxyapatite (HA) spheres for the creation of a depot and the slow release of protein antigen relies on its protein-adsorbing properties. The production of homogeneous spherical HA materials is, however, still challenging, and few studies have rigorously investigated HA-associated antigen delivery *in vivo*.

In this study we report the development of a novel and economical manufacturing process for the production of homogeneous micro-sized strontium-doped HA porous spheres (SHAS), suitable for the subcutaneous delivery of protein antigens. After morphological and chemical characterization, SHAS were tested *in vivo* for antigen release and immunotherapeutic potential.

In a murine model of allergic inflammation we found that ovalbumin (OVA), loaded on SHAS and injected subcutaneously, was detectable several days longer in the draining lymph node in comparison with OVA injected in soluble form. Moreover, we identified $CD11b^+$ migratory dendritic cells as the subset of antigen presenting cells, which was mainly responsible for the persistent presentation of OVA epitopes in the lymph node and the subsequent sustained stimulation of both $CD4^+$ and $CD8^+$ T-cells. Furthermore, we found a low inflammatory profile for antigen-loaded SHAS. Thus, we conclude that SHAS constitute a suitable carrier for allergen-specific immunotherapy in allergy.

Graphical abstract



Introduction

Immunomodulatory interventions, such as vaccinations, play a key role in the control of infectious diseases and thereby contributed to save millions of human lives [1]. More recently, immunotherapy became customary for the treatment of other conditions like allergic and autoimmune diseases [2, 3] as well as for the prophylactic immunization against cancer [4, 5]. To enhance the triggered immune response many vaccine preparations require adjuvants. Their importance is known since almost one century [6], but mostly because of regulatory issues only aluminum and calcium salts are commonly used [7]. More recently, the oil in water emulsions MF59, AS03 and AF03, virosomes, and the monophosphoryl lipid A preparation AS04 have been approved for human use in the United States or in Europe [8]. Furthermore, many doubts have been expressed about the safety of the well-established adjuvant aluminum hydroxide (alum). In addition to well-known local and systemic reactions to alum including local irritation, hypersensitivity reactions, subcutaneous nodules, and the induction of unwanted TH2-driven immune responses [9], various studies demonstrated the neurotoxic effects of aluminum, which has been put in connection with neurodegenerative diseases such as Alzheimer's disease [10]. For this reasons the development of new, affordable and safe adjuvants that are able to shape the immune response is desirable.

The mechanisms by which micro- and nanoparticles enhance the immune response are diverse and have been reviewed before [11]. The administration of an antigen in a particulate form has been shown to enhance its stability and integrity and to create a so called depot effect, where the antigen is gradually released thus persistently challenging the immune system [12]. From a cellular point of view the close proximity of antigens on particles leads to enhanced cell-activation and enhanced uptake by antigen presenting cells (APCs). In this context it is important to mention that the subsets of APCs being targeted by the formulation are crucial for the outcome of the immunization. Antigen uptake by CD11b+ migratory DCs (in mice) or CD1c+ DCs (in humans) generally results in the priming of T-helper cells [13, 14] or the development of tolerogenic immune response involving the generation of T-regulatory cells [15], while uptake by the cross-presenting migratory Xcr1+ DCs (in mice) or CD141+/hi DCs (in humans) will mostly result in cytotoxic T-cell responses [16-18].

The addition of Strontium and an optimized synthesis procedure allowed the production of novel microparticles, very homogeneous in size, porosity and morphology. These particles are called strontium-doped hydroxyapatite porous spheres (SHAS) [19]. Similar spheres have been shown to bind consistent amounts (~0.37 mg/mg) of the antibiotics vancomycin and cephalothin, which were then slowly released over many hours in vitro [20]. In this study we evaluated the potential of SHAS as a matrix for a prolonged local delivery of protein antigen and evaluated their potential as tolerance-inducing adjuvant for immunotherapeutic interventions.

Material and methods

Preparation and characterization of SHAS

NaCl, KCl, Na_2HPO_4, and KH_2PO_4 were dissolved in water at a molar ratio of 137.0 : 2.7 : 8.1 : 1.5 to form a phosphate buffer solution. After addition of calcium chloride (1 mM), magnesium chloride (0.05 mM) and strontium nitrate (0.6 mM) the mixture was heated at 100°C under stirring. After 6 hours the precipitation was filtered and washed twice with room-temperature ethanol. All chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) at the highest purity grade available.

Several analytical techniques were used to study morphology, structure, and crystallinity of SHAS. The morphology of the spheres before and after OVA adsorption was analyzed by Field Emission Scanning Electron Microscopy (FE-SEM, LEO 1550). Powder X-ray diffraction (XRD, Siemens Diffractometer D5000, Siemens, Munich, Germany) using Cu Kα radiation (λ = 1.5418 Å) operated at 45 kV and 40 mA at a 2θ range of 5°–60° was used to analyze the crystallinity of SHAS. The surface area and the porosity of the materials were estimated from the N2 sorption isotherm, which was performed by Accelerated Surface Area and Porosimetry (ASAP 2020, Micromeritics, Norcross, GA, United States) in accordance to the Brunauer-Emmet-Teller principle. The ion composition was analyzed using inductive coupled plasma optical emission spectroscopy (ICP-OES, Optima 5300DV; Perkin-Elmer, Waltham, MA, United States).

Protein labeling and loading

Labeled OVA (OVA_{488}) was produced using Dylight 488 Amine-Reactive Dye (Thermo Scientific, Rockford, IL, United States) and Grade VI OVA (Sigma-Aldrich). OVA was resuspended in phosphate buffered saline (PBS) at a concentration of 2 mg/ml, sterilized by filtration and added to the vial containing the dye. After 3 h incubation at room temperature and 12 h at 4°C, the unreacted dye was removed by dialysis against PBS with a SpectraPor Membrane with a molecular weight cut-off of 6-8 kDa (Spectrum Labs, Roncho Domingues, CA, United States) and 4 buffer exchanges over 36 h.

1-5 mg of SHAS were resuspended in 500 µl PBS in an Eppendorf tube and put in a sonication bath five times for 10 s in order to obtain a homogeneous solution. Between each sonication burst the tubes were shaken by hand. The spheres were allowed to stand for 5 min, centrifuged (2000 x g, 2 min), and washed once with 500 µl PBS. Afterwards they were re-suspended in 300 µl of 1 mg/ml protein solution per mg of particles and left overnight under constant shaking. Thereafter the beads were collected by centrifugation (2000 x g, 2 min) and the protein concentration in the supernatant determined by Bradford (Biorad, Hercules, CA, United States) to calculate the amount of protein loaded. The major cat allergen Fel d 1, used as a protein loading control for SHAS, was produced in *E. coli* and purified as described [21].

In vitro release

2 mg of OVA-loaded spheres were re-suspended in 200 µl PBS or PBS-Tween20 (0.05%) and kept in suspension by rolling at room temperature. Spontaneous release was monitored by measuring the protein concentration in the supernatant over 20 h. The quality of the protein released was visualized by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Residual protein bound to the spheres was released by incubation for 5 min at 90°C in SDS-PAGE loading buffer (50 mM Trizma-HCl pH 8, 5% Glycerol, 2.5% β-mercaptoethanol, 50 µg/ml bromophenol blue, 10% dithiothreitol).

Mice

7-10 weeks old female C57/BL6 or BALB/c mice were housed under specific pathogen free (SPF) conditions and handled in accordance with French and European directives with ethical approval from the Centre d'Immunologie de Marseille-Luminy. OT-I and OT-II mice have been previously described [22, 23].

Preparation of monocyte-derived dendritic cells (mdDCs)

Human monocyte-derived dendritic cells (human mdDCs) were prepared from peripheral blood mononuclear cells obtained by ficoll density gradient centrifugation (Biocoll Separating Solution, Biochrom AG, Berlin, Germany) from healthy donors with written informed consent. The $CD14^+$ population was isolated by AutoMACS using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the instructions of the manufacturer and cultured at 1 mio/ml in 6-well plates in cRPMI (RPMI 1640, 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1x MEM vitamins, 1x MEM non-essential amino acids, 100 U/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml kanamycin, all from Sigma-Aldrich) in the presence of 1000 U/ml GM-CSF (PeproTech, Hamburg, Germany) and 1000 U/ml IL-4 (Novartis, Basel, Switzerland) for 5 days. The successful differentiation to human mdDCs was confirmed by flow cytometric CD11c staining together with the viability staining agent eFluor 780 (eBioscience, Vienna, Austria).

Mouse $CD11c^+$ DCs (mDCs) were isolated from the inguinal, brachial, cervical, and axillar LNs by AutoMACS, using mouse CD11c microbeads (Miltenyi Biotec) according to the manufacturer's instructions.

In vitro toxicity, apoptosis detection assays

Toxicity experiments were performed using human mdDCs as follows. 100, 10, or 1 µg of SHAS were added to 2×10^5 cells in 200 µl cRPMI in a 96-well plate. After 24 h incubation the plate was centrifuged at 380 x g for 5 min. The cell pellet was washed twice with PBS, and the cells were stained for Annexin and 7AAD with the PE Annexin V Apoptosis Detection Kit I (BD Biosciences, Erembodegen-Aalst, Belgium) and analyzed by flow cytometry according to the instructions. NF-κB activation experiments were performed in the same way but using the reporter cell line THP1-

Blue-CD14 (Invivogen, San Diego, CA, United States). Secreted alkaline phosphatase in the supernatant was detected by QUANTI-Blue (Invivogen). Stimulation with 1 μg/ml γ-irradiated Lipopolysaccharides (LPS) from *E.coli* 055:B5 (BioXtra, Sigma-Aldrich) was used as positive control.

Flow cytometry

Cells were stained and analyzed using a FACS LSRII or a Canto system with DIVA software (BD Biosciences). Cell viability was evaluated using Sytox (Molecular Probes, Invitrogen Detection Technologies, Eugene, USA) according to the manufacturer's protocol. Anti-NK1.1 (PK136), anti-CD3 (17A2), anti-Ly-6G (1A8), anti-CD19 (6D5), anti-CD64 (X54-5/7.1) were from Biolegend, San Diego, CA, USA. Anti-CD11c (N418), anti-MHC Class II (I-A/I-E) (M5/114.15.2), anti-CD45.2 (104), anti-CD45.1 (A20), anti-CD24 (M1/69), and anti-CD5 (53-7.3) were from eBioscience. Anti-Ly6C (AL21), anti-CD4 (RM4-5), and anti-CD8a (53-6.7) were from BD Pharmingen, San Diego, CA, USA.

After dead cells exclusion, B cells, T cells, NK cells, and neutrophiles were systematically gated out using a dump-channel corresponding to cells positive for B220, CD3, NK1.1, and Ly-6G. The lymphoid tissue-resident (LT) population of DCs was identified as $CD11c^{+}$ $MHC\text{-}II^{lo}$ and separated in $Xcr1^{+}$ LTDCs ($CD24^{+}$ $CD11b^{lo}$) and $CD11b^{+}$ LTDCs ($CD24^{lo}$ $CD11b^{+}$). The migratory population was identified as $CD11c^{lo}$ $MHC\text{-}II^{+}$, and separated according to the expression of CD24 and CD11b in $CD24^{+}$ migDCs, $CD11b^{+}$ migDCs and $CD11b^{+}$ migDCs. migMFs were identified as $CD11b^{+}$ $CD64^{+}$ cells. Analysis was performed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Antigen presentation in the inguinal lymph node

C57/BL6 mice were injected s.c. in the lumbar area with 200 μl of a solution containing 50 μg OVA_{488} bound or not bound to SHAS. The animals were sacrificed 1, 4 or 7 days after injection and lymphocytes were isolated from the inguinal LN and analyzed by flow cytometry as previously described [24, 25].

Preparation and adoptive transfer of labeled OT-I and OT-II cells

OVA-specific $CD8^{+}$ (OT-I) and OVA-specific $CD4^{+}$ (OT-II) T-cells were isolated from pooled spleens and LNs of OT-I and OT-II mice kept on a *Rag-2*$^{-/-}$ x B6 [CD45.1] background and grinded through a 70 μm strainer. After red blood cell lysis, $CD8^{+}$ (OT-I) and $CD4^{+}$ (OT-II) T-cells were isolated by Dynal negative selection (Life Technologies). Purity was determined by staining with CD4, CD8, CD5 and TCRVα2. The cells were stained with Cell Trace Violet (CTV, Life Technologies). Per mice 10^{6} OT-I and 10^{6} OT-II cells were injected intravenously (i.v.).

Determination of OT-I and OT-II cell proliferation

C57/BL6 mice were injected s.c. in the lumbar area with 200 μl of a solution containing 20 μg OVA in solution or bound to SHAS. After 3, 6 or 8 days, 10^{6} of both

CTV labeled OT-I and OT-II cells were injected i.v. Three days later mice were sacrificed, and the splenocytes were isolated and analyzed by flow cytometry as previously described [24, 25].

Immune response in vivo

To characterize the type of immune response to antigens delivered through SHAS wild-type BALB/c mice were sensitized using an adjuvant-free protocol as described [26], with 3 s.c. injections of 10 μg OVA in solution or bound to SHAS on days 0, 7 and 14. Negative control mice were injected with the corresponding amounts of SHAS or PBS and a positive control group of mice was injected i.p. with 10 μg OVA adsorbed to 500 μg Alum (Imject Alum, Thermo Scientific). Mice were challenged on days 26, 27, 28 with 1% OVA aerosols for 20 min and sacrificed on day 30. Blood samples were taken with the tail cut method [27] on days 0, 14, 26 and 30 and the concentration of OVA-specific IgE, IgG_1 and IgG_{2a} was determined by ELISA (see below, Fig. 5A).

Immunoglobulin ELISA

Nunc-Immuno Clear Flat-Bottom Maxisorp 96-well plates (Thermo Scientific) were coated by overnight incubation at 4°C with 100 μl of 10 μg/ml OVA in PBS. Unspecific bindings were avoided by 1 h incubation in 150 μl blocking buffer (1% BSA and 5% Sucrose in PBS, all from Sigma-Aldrich). After washing, 100 μl diluted sera (1:10 for IgE, 1:100 for IgG_{2a}, 1:100'000 for IgG_1) were added and incubated for 3 h. OVA-specific antibodies were detected with 100 μl of the corresponding biotinylated anti-isotype at 0.5 μg/ml and incubated for 2 h. After 45 min incubation with Streptavidin-HRP diluted 1:200 (R&D Systems, Minneapolis, MN, United States) bound antibodies were detected with OptiEIA TMB Substrate Reagent (BD Biosciences) according to the instructions of the manufacturer. If not otherwise mentioned incubation steps were performed at room temperature. Antibodies, sera and enzymes were diluted in PBS containing 1% BSA. Between each step, plates were washed 3 times with PBS-Tween (0.05%). The following anti-isotype antibodies were used: biotin anti-mouse IgE (RME-1), biotin anti-mouse IgG_1 (RMG1-1), and biotin anti-mouse IgG_{2a} (RMG2a-62; all from Biolegend). Serum concentrations were calculated by 4-parametric curve regression using the following antibodies as standards (50-0.4 ng/ml): mouse anti-OVA IgE (2C6; AbD Serotec, Raleigh, NC, USA), mouse anti-denatured OVA IgG_1 (6C8; Thermo Scientific), mouse anti-denatured OVA IgG_{2a} (6G2; Thermo Scientific).

Immunotherapy model

The possibility of using SHAS for immunotherapeutic purposes was investigated using a previously described allergy model [28]. Wild-type BALB/c mice were sensitized with two i.p. injections of 10 μg OVA precipitated together with 2.25 mg Alum (Imject Alum, Thermo Scientific) on days 0 and 7. Mice were then treated with two s.c. injections of 0.2 mg OVA, SHAS-OVA or protein-free SHAS on day 21 and 28.

The control immunotherapy group (OVA-ctrl) was treated with three s.c. injections of 1 mg OVA on days 21, 23, 25 [28]. On days 35, 37 and 39 mice were challenged with 1% OVA aerosols for 20 min and sacrificed on day 40 (Fig. 6A).

Bronchoalveolar lavage

Mice were sacrificed by i.p. injection of pentobarbital (150 mg/kg, Streuli Pharma AG, Uznach, Switzerland). Bronchoalveolar lavage (BAL) was performed by sealing the upper part of the trachea with medical tweezers and injecting 1 ml PBS containing the cOmplete proteinase inhibitor cocktail (Roche, Mannhein, Germany), using a 1 ml syringe with a bent 26G needle. Determination of cell type and content in BAL was determined by Romanowski stain (Diff-Quick, Medion Diagnostics, Düdingen, Switzerland) for cell subsets.

Behavioral observations

To assess pain or discomfort, mice were observed in an open-field test. After immunotherapeutic injections every mouse was allowed to rest for 45 min in the home cage. Afterwards it was transferred to a 30x50 cm cage divided into 10x10 cm squares and observed for 3 min. Following parameters were recorded and scored as follows: fur (1 point if crumpled, 2 points if very sweaty), posture (1 point if the mouse was crooked, 2 points for unseemly locomotion), movement (full body access to the 10 cm sectors, 1 point for only entering 4-10 sectors, 2 points for only entering 1-3 sectors), rearing behavior (1 point if absent), urination and defecation (1 point if absent). The cage was cleaned first with a humid cloth and then with a dry one before starting the experiment with another animal (adapted from [26, 29]).

Statistical analysis

The Wilcoxon-Mann-Whitney-Test was used to assess statistical significance. Probability values are expressed as the following: ***, $p<0.001$; **, $p<0.01$; *, $p<0.05$; NS, non significant.

Results

Physical characterization of SHAS

Scanning electron microscope (SEM) pictures revealed that the synthesis procedure developed for the production of SHAS results in a homogeneous population of spheres (Fig. 1A), mainly consisting of hydroxyapatite as shown by XRD analysis. Peaks were coherent with the joint committee on powder diffraction standards (JCPDS) data (JCPDS-09-0432/1996, Fig. 1B). SHAS possess a spherical and porous shape, with an average diameter of 1.8 μm (Fig. 1C). Further physical properties of SHAS are summarized on Table 1.

Table 1 Physical properties of SHAS

Size[1]	Pore Size[2]	Surface Area[2]	Composition (Molar Ratio)[3]
1800 nm	33.1 nm	95.5 m^2/g	1.00 Magnesium 2.15 Strontium 3.85 Calcium 5.42 Phosphate

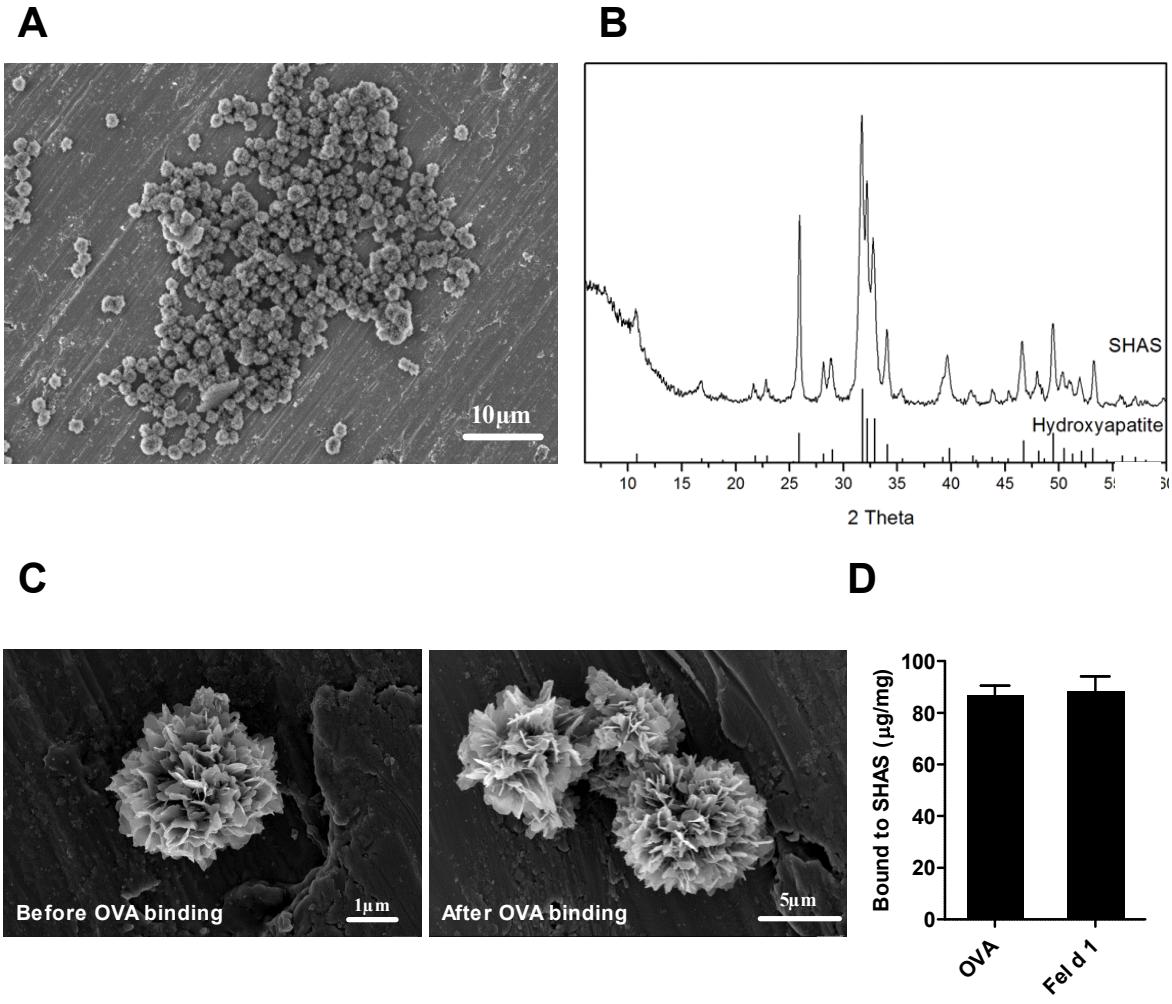
[1] SEM; [2] N_2 adsorption and desorption; [3] ICP-AES

Binding to and release of proteins from SHAS in vitro

Approximately 90 µg of OVA or of the major recombinant cat allergen Fel d 1, as an additional control, bound to 1 mg of SHAS (Fig. 1D). The morphology of SHAS was not influenced by OVA binding as demonstrated by electron microscopy pictures (Fig. 1C), and the particle-bound OVA did not appear degraded or modified after spontaneous or SDS-induced release (Fig. 1E). Observation of the release dynamics from SHAS in PBS showed a first release burst within one hour, followed by a sustained release, which is gradually slowed down by the presence of protein in the supernatant. An equilibrium is reached as about 10 µg of protein are released, corresponding to a concentration of 50 µg/ml in the supernatant. Resuspension in fresh buffer after 2 h clearly restored protein release (Fig. 1F). Interestingly, the protein release from SHAS is boosted by Tween20, suggesting polarity dependent binding mechanisms.

Figure 1 Characterization of SHAS and their protein binding properties

A The synthesis procedure developed for the creation of SHAS results in a homogeneous population of beads. **B** X-Ray diffraction pattern showing that SHAS consist of hydroxyapatite. Peaks were coherent with the pattern provided by the joint committee on powder diffraction standards **C** Representative SEM image of SHAS before (left) and after OVA binding (right). **D** Binding of OVA and the major cat allergen Fel d 1 to SHAS, determined by subtracting the amount of unbound protein in the supernatant after the adsorption process from the initial amount. Results are expressed in µg of protein that bound to 1 mg of SHAS (n=3, mean ± SEM). **E** SDS-PAGE before and after binding to SHAS. Line 1, OVA; line 2, SDS-released OVA; line 3, PBS-released OVA. **F** Spontaneous and time dependent release of SHAS-bound OVA in PBS (dotted line) or PBS-Tween20 (solid line). After two hours, as equilibrium between release and binding was reached, OVA-loaded SHAS were challenged with fresh buffer, triggering additional release of OVA. One out of 3 representative experiments is depicted.


A
10µm
B
SHAS
Hydroxyapatite
10 15 20 25 30 35 40 45 50
2 Theta
C
Before OVA binding
1µm
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KDa
97
64
51
39
28
14
F
Release of OVA (µg/mg)
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PBS Tween20
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0.0 0.5 1.0 1.5 2.0 5 10 15 20
Time (h)

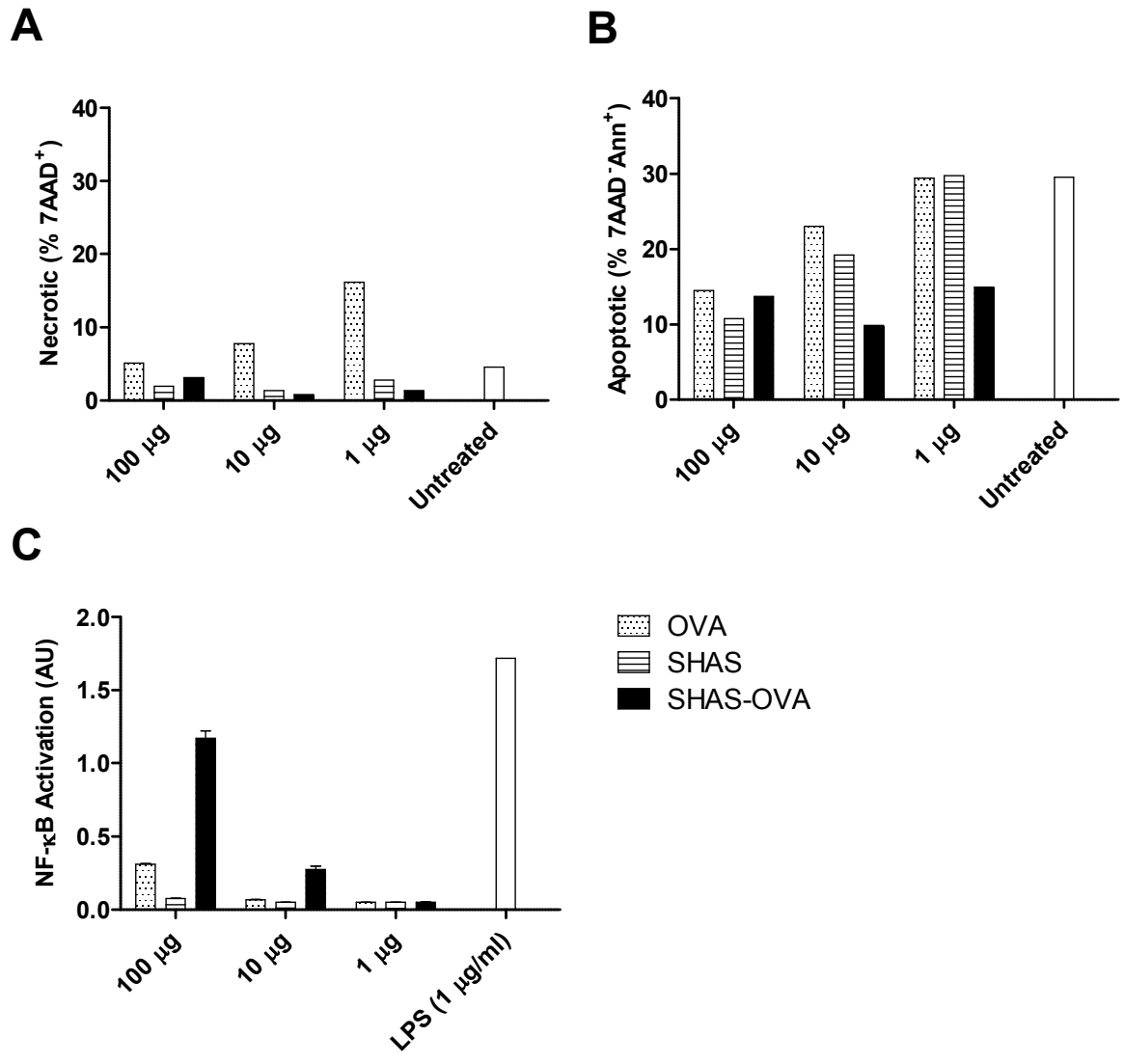


Figure 2 *In vitro* toxicity and NF-κB activation of empty and antigen-loaded SHAS particles

Percent of necrotic (**A**) and apoptotic (**B**) human mdDCs after 24 h contact with OVA, SHAS or SHAS-OVA as determined by 7AAD and anti-annexin staining by flow cytometry. **C** NF-κB activation in THP1 monocytes after 24 h contact with the spheres *in vitro* and LPS as control; determined through the secreted alkaline phosphatase reporter gene and QUANTI-Blue colorimetric assay (n=3).

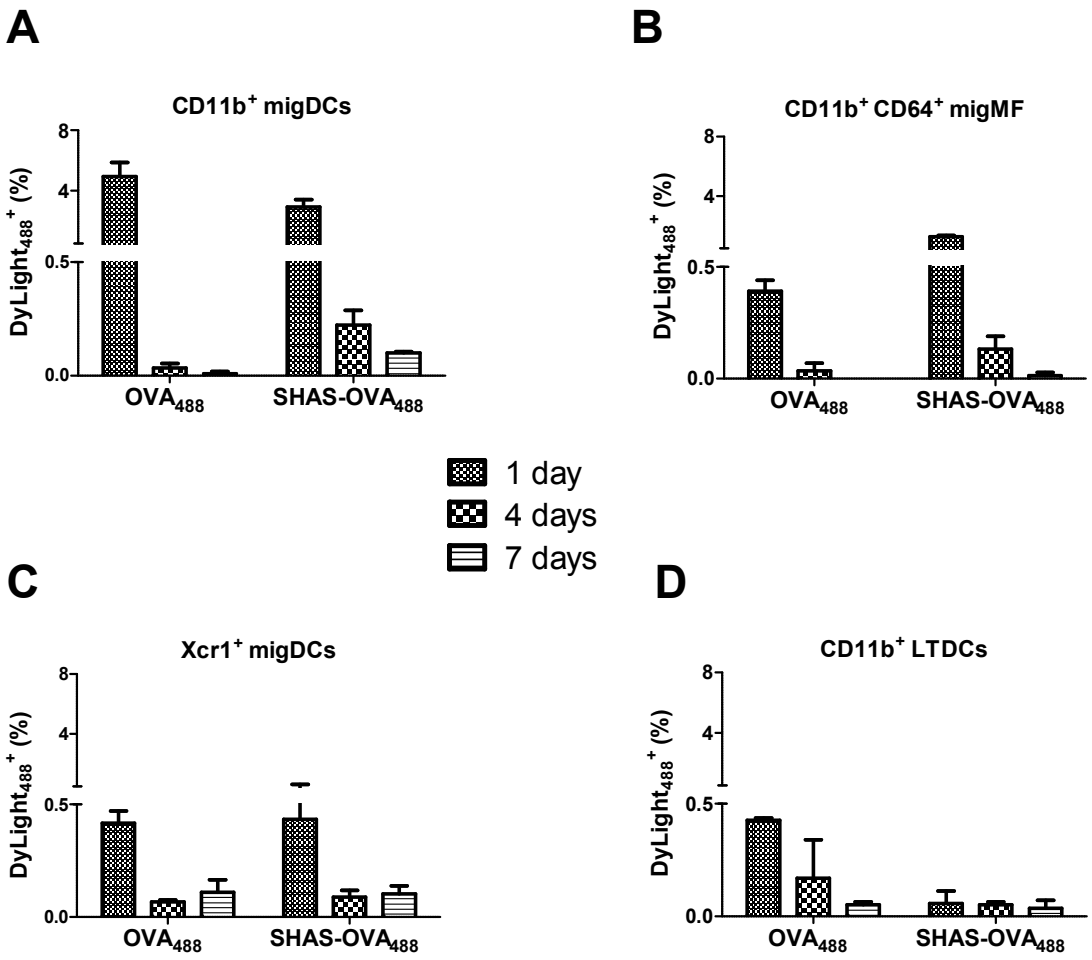


Figure 3 Persistence of free and SHAS-bound OVA_{488} after s.c. administration

OVA_{488} positive cells in the draining inguinal LN 1, 4 and 7 days after s.c. injection of OVA_{488} or SHAS-OVA_{488} in the lumbar area of the mouse back. Single cell suspensions from draining inguinal LN were analyzed by flow cytometry for the determination of the percentage of $Dylight_{488}$ positive cells among the $CD11b^+$ migDCs (**A**), $CD11b^+$ $CD64^+$ migMFs (**B**), Xcr1+ migDCs (**C**), and $CD11b^+$ lymphoid tissue resident (LT) DCs (**D**) (n=2). The populations showed here result from a gating strategy that excludes dead cells, B-cells, T-cells, NK-cells and neutrophils, and further divides phagocytic and antigen presenting cells by the expression of CD11c, MHC-II, CD24, CD11b and CD64.

Toxicity, apoptotic and pro-inflammatory effects of OVA-loaded SHAS in vitro

The addition of OVA-loaded SHAS to human mdDCs did not have any significant necrotic or apoptotic effects even at high densities, where SHAS formed a proper layer at the bottom of the 96-well plate. On the contrary the addition of protein-free SHAS to the medium appeared to protect from the apoptotic and necrotic events occurring in untreated cells and, interestingly, in cells treated with OVA (Fig. 2A-B). In the reporter monocyte cell line THP1-Blue-CD14, SHAS-OVA triggered a moderate NF-κB activation (Fig. 2C).

Prolonged detection of SHAS-bound antigen in the draining lymph node

One day after s.c. application in the lumbar area of the mouse back, OVA_{488} could be detected in the draining LN, carried by a variety of DC subtypes and macrophages. This was observed both for the application of OVA_{488} in SHAS-bound form or in solution. The depot effect accompanying SHAS administration was clearly visible by looking at $CD11b^+$ migratory DCs (migDCs). In particular, OVA positive migDCs were detectable for up to 7 days in the draining LN, indicating a continuous migration from the tissue (Fig. 3A). Notably, also a marked persistence of antigen-bearing $CD11b^+$ $CD64^+$ migratory macrophages (migMFs) could be detected (Fig. 3B). The presence of SHAS did not influence antigen transport by $Xcr1^+$ migDCs, which could be detected in the LN only up to one day after antigen administration (Fig. 3C).

Interestingly, OVA_{488}-positive $CD11b^+$ lymphoid-tissue resident DCs ($CD11b^+$ LTDCs) could also be detected, but only when OVA was injected in soluble form (Fig. 3D). These cells probably picked up the antigen directly from the lymph or from migratory cells. These data indicate that OVA_{488} injected bound to SHAS persists longer and do not flow freely through lymph vessels, as observed for OVA_{488} injected in solution.

Sustained presentation of SHAS-bound antigen to OT-I and OT-II cells

The prolonged permanence of the antigen in the LN, consequential to a SHAS-bound application, results in a sustained antigen presentation. Proliferation of OT-I and OT-II cells could be induced and detected in the spleen 8 days after s.c. injection of OVA loaded SHAS. In contrast, the same cell subsets showed only baseline proliferation in animals that were injected with OVA in solution (Fig. 4). These data show that OT-I and OT-II cells still recognize OVA peptides presented on MHC molecules more than one week after administration of SHAS-OVA.

Immune responses to SHAS-bound antigen are comparable with those obtained with soluble antigen

Three s.c. administrations of 10 μg SHAS-OVA lead to IgE and IgG_{2a} levels, which were comparable with the ones obtained with a standard sensitization protocol (Alum-adjuvated i.p. injections). In contrast, IgG1 levels were significantly higher following Alum-adjuvated sensitization (Fig. 5B-D). Besides slightly increased IgE

levels, the immunoglobulin profile induced by SHAS-OVA was therefore indistinguishable from the one induced by soluble OVA, indicating that SHAS coupling does not alter the way in which OVA is recognized. Moreover, following aerosol allergen challenge, the amount of eosinophiles in the BAL fluid was comparable for all protocols, indicating analogous levels of allergic sensitization (Fig. 5E).

Evaluation of SHAS in a mouse model for immunotherapy

The slow and sustained protein release from SHAS may lead to tolerance. For this reason we assessed the potential of SHAS as antigen-carrier for immunotherapeutic interventions for allergic diseases. With our rush immunotherapy protocol OVA bound to SHAS was equivalent to OVA in solution with regard to some of the most common symptoms of allergic asthma in mice. In fact, the number of eosinophiles in BAL fluid (Fig. 6B) and the therapy-induced IgG_1 (Fig. 6C) were equivalent between the two treatments. Importantly, the symptoms following both the first and the last immunotherapeutic injection were reduced using SHAS-OVA (Fig. 6D-E), suggesting that SHAS make the injected therapeutic antigen less accessible to the immune system and hence result in reduced inflammation.

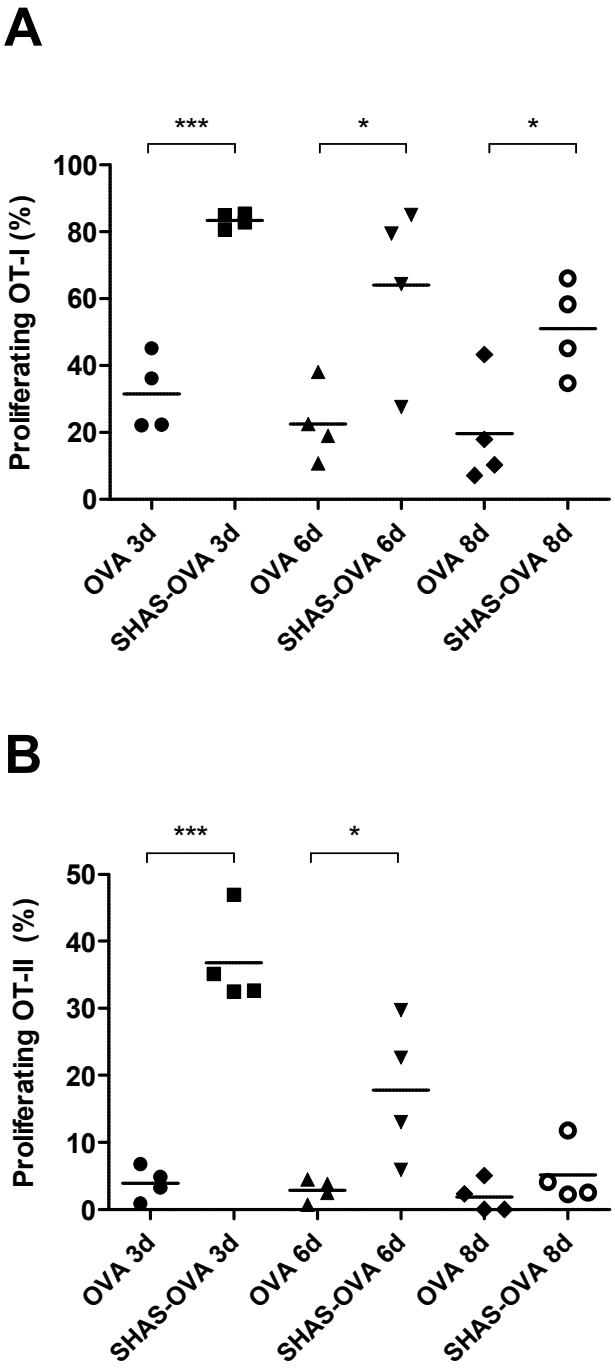


Figure 4 Functional antigen presentation to OT-I and OT-II cells in the spleen

CTV-labeled OT-I ($CD8^+$) and OT-II ($CD4^+$) T cells were adoptively transferred by intravenous injection in WT C57/BL6 mice 3, 6 or 8 days after the s.c. injection of OVA_{488} or SHAS-OVA_{488}. The proliferation was assessed in the spleen 3 days later by flow cytometry. After gating on $CD45.1^+$ $CD3^+$ $TCRV\alpha2^+$ $CD8^+$ or $CD4^+$ cells, proliferation was assessed by the dilution of CTV. Percentage of proliferating OT-I (**A**) and OT-II T cells (**B**) is depicted (n=4). Significant differences among the groups are marked (*, $p < 0.05$; **, $p < 0.01$; Two-tailed Mann-Whitney test).

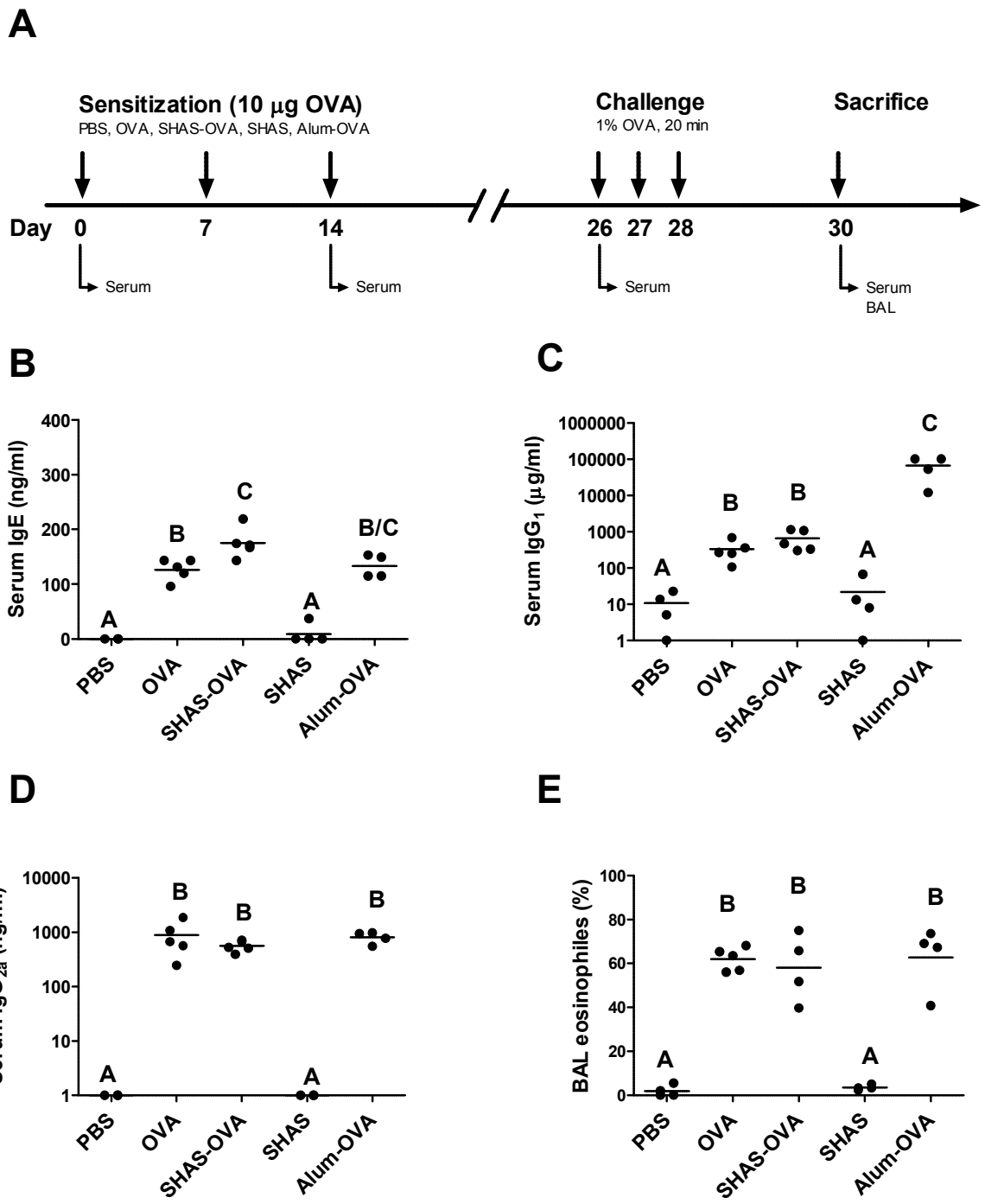
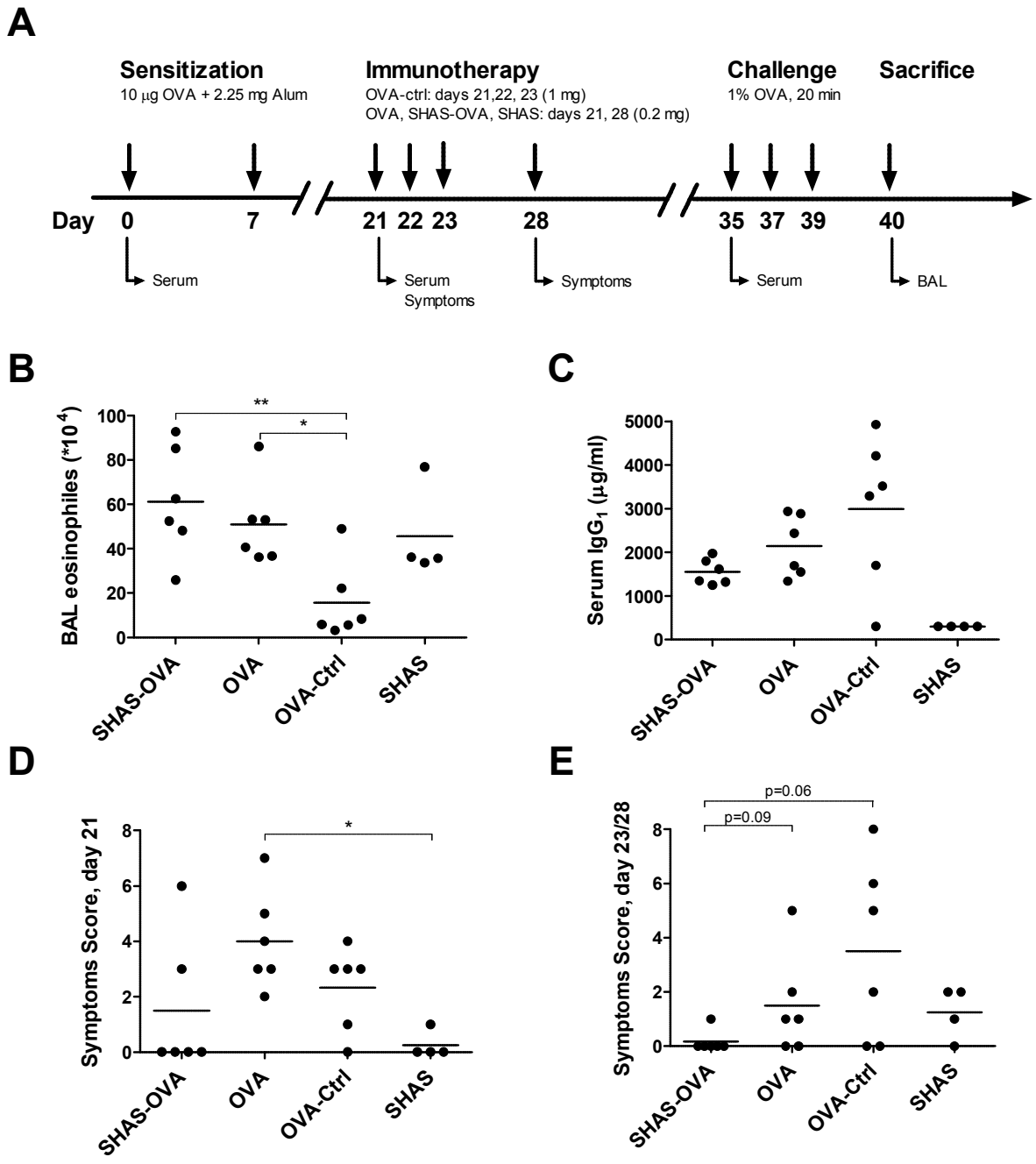


Figure 5 Immune responses triggered by the delivery of small amounts of antigen bound to SHAS

A Sensitization and challenge protocol; mice were sensitized s.c. with PBS, OVA, SHAS-OVA or SHAS, or i.p with Alum-OVA (10 μg OVA). The appearance of immunoglobulins in serum was monitored over 26 days, as well as the number of eosinophiles in the lung after OVA challenge as an indicator of inflammation **B-D** IgE, IgG1 and IgG_{2a} immunoglobulin levels as determined by ELISA, 26 days after the first injection. **E** Eosinophiles levels in BAL fluid after OVA challenge. Different capital letters indicate significant differences among the groups ($p < 0.05$, Two-tailed Mann-Whitney test). Each group consisted of 6 animals, except for the control groups, which consisted of 4 animals.

Figure 6 SHAS immunotherapy has reduced side effects

A Schematic immunotherapy model; after sensitization with Alum adsorbed OVA mice were treated with two s.c. injections of OVA, SHAS or SHAS-OVA (0.2 mg OVA) on days 21 and 28. As a control higher amounts were injected three times every second day (1 mg OVA). Serum samples were collected throughout the experiment as well as the BAL fluid after sacrifice. **B** Absolute number of eosinophiles in BAL fluid after OVA sensitization, immunotherapy and aerosol challenge (day 40). **C** Serum concentration of IgG_1 before the challenge, i.e. at the end of immunotherapy phase (day 35). **D** Symptoms score 45 min after the first immunotherapeutic injection (day 21). **E** Symptoms score 45 min after the last immunotherapeutic injection (day 23 for the OVA-ctrl group, day 28 for the other groups). Symptoms were scored according to the following occurrences: fur (1 point if crumpled, 2 points if very sweaty), posture (1 point if the mouse was crooked, 2 points for unseemly locomotion), movement (full body access to the 10 cm sectors, 1 point for only entering 4-10 sectors, 2 points for only entering 1-3 sectors), rearing behavior (1 point if absent), urination and defecation (1 point if absent). Significant differences among the groups are marked (*, $p < 0.05$; **, $p < 0.01$; Two-tailed Mann-Whitney test). Each group consisted of 6 animals, except for the SHAS control group, which consisted of 4 animals.


A
Sensitization
10 μg OVA + 2.25 mg Alum
Immunotherapy
OVA-ctrl: days 21,22, 23 (1 mg)
OVA, SHAS-OVA, SHAS: days 21, 28 (0.2 mg)
Challenge
1% OVA, 20 min
Sacrifice
Day 0 7 21 22 23 28 35 37 39 40
Serum
Serum
Symptoms
Symptoms
Serum
BAL
B
BAL eosinophiles (*10^4)
SHAS-OVA
OVA
OVA-Ctrl
SHAS
C
Serum IgG1 (μg/ml)
SHAS-OVA
OVA
OVA-Ctrl
SHAS
D
Symptoms Score, day 21
SHAS-OVA
OVA
OVA-Ctrl
SHAS
E
Symptoms Score, day 23/28
p=0.06
p=0.09
SHAS-OVA
OVA
OVA-Ctrl
SHAS

Discussion

In this study we report a cheap and robust strategy for the production of homogeneous Sr-doped hydroxyapatite porous spheres (SHAS) for the subcutaneous delivery of antigens. We show how the application of proteins bound to SHAS induces a long lasting presence of antigen-loaded $CD11b^+$ migDCs in the draining LN, resulting in a prolonged antigen-presentation to $CD8^+$ and $CD4^+$ T-cells.

In the last years, a few studies evaluated the potential of HA-spheres for the sustained delivery of trace elements [19] and various loaded compounds, including antibiotics [20, 30], lipophilic drugs [31], and proteins [31, 32]. These publications nicely showed sustained release from HA-particles, both *in vitro* and *in vivo*, and evaluated the subcutaneous persistence of HA-spheres. Although comparisons between the different studies are hampered by the great diversity of HA-particles formulations used, our study permits to enlighten some of the cellular aspects of HA-spheres-based protein delivery.

First, the s.c. delivery of proteins in a SHAS-bound formulation in mice maintained antigen presentation to antigen specific $CD4^+$ (OT-II) and $CD8^+$ (OT-I) T-cells for 6 and 8 days, respectively. This prolonged presentation correlated with the presence of $CD11b^+$ migratory DCs ($CD11b^+$ migDCs) in the LN. Hence, of all the DCs populations tested, we identify $CD11b^+$ migDCs as the main antigen presenters in SHAS mediated delivery. These cells have been shown to induce T_H2 or T_{reg} dominated immune responses [15] and may therefore contribute to the development of tolerogenic responses.

Second, mice sensitization with SHAS-OVA and the resulting humoral response and hypersensitivity is not substantially different from the one obtained with soluble protein. This is partially confirmed by *in vitro* studies showing the absence of toxicity or apoptosis and modest NF-κB activation by challenging cells with SHAS. The low toxicity may be related to the regular round shape of SHAS. In fact, spherical and rod-shaped hydroxyapatite materials have been shown to be less toxic than particles with other morphologies [33].

Allergic mice challenged s.c. with allergens bound to SHAS showed reduced side effects in comparisons with mice that were challenged with soluble antigen. We speculate that SHAS-bound antigens are less accessible for the cell subsets responsible for allergic reactions, such as mast cells and basophiles.

Conclusions

Our results show that SHAS possess the potential to be employed in immunotherapeutic interventions as a low side-effects carrier material for sustained protein release. We showed how SHAS are able to adsorb and slowly release proteins, presenting them for over one week to migratory DCs and macrophages, which in turn stimulate the immune system over a prolonged period of time.

Acknowledgments

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CHAPTER 2

Sequestration of DCpep targeted constructs by macrophages and monocytes

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Abstract

It has been 10 years since DCpep, the first targeting peptide binding to human and mouse dendritic cells, was discovered. Despite convincing and very promising initial results, DCpep never hit the big time and its use was never implemented for pharmacological approaches.

In this study, we aimed to evaluate the future usability of DCpep. Therefore, we looked into its binding properties in vivo, focusing on the identification of the targeted cell subsets in the mouse skin, and drew parallels with human cell subsets differentiated in vitro.

Our findings confirmed that DCpep represents an easy and elegant way to enhance antigen delivery to dendritic cells in vitro. However, we observed that the targeting benefit disappears in vivo, after application of DCpep fusion proteins by laser microporation in the mouse skin. We further identified a lack of specificity for professional antigen presenting cells as the most probable cause. In fact, DCpep did not only enhance the binding to the analyzed mouse dendritic cell subpopulations, but it also bound to dermal cells with poor antigen-presenting properties, such as monocytes and macrophages. These cells most likely act as a sort of antigen sink, efficiently removing DCpep fusion proteins from the application site.

Taken together, these results suggest that vaccines targeting dendritic cells in vivo are preferentially phagocytosed by cells with poor antigen-presenting properties, thereby counter-acting the targeting benefit observed in vitro.

Introduction

Ten years ago Curiel and co-workers identified by phage display three 12-mer peptides able to specifically target human monocyte derived Dendritic Cells (mdDCs) and to enhance their ability to activate autologous CD4 and CD8 T-cells in an antigen specific manner. One of them, DCpep (FYPSYHSTPQRP), bound to $CD11c^+$ $I\text{-}A^+$ DCs derived from mouse bone marrow as well [1]. In more recent *in vivo* experiments DCpep was shown to improve the efficacy of *Lactobacillus acidophilus* mediated vaccination against *Bacillus anthracis*. Oral vaccination of mice with *L. acidophilus* expressing the *B. anthracis* protective antigen (PA) fused with DCpep resulted in higher and more protective antibody titers and a different cytokine pattern compared with PA alone or fused with a control peptide [2]. DCpep was also used in combination with the invariant chain (Ii)-derived peptide Ii-key to achieve higher levels of antigen presentation from DCs and the activation of high-avidity antigen-specific CD4 T-cells *in vitro* [3], thereby combining DC-targeting and the enhanced MHC-II presentation promoted by Ii [4].

The data showing increased targeting and presentation *in vitro* and the increased functionality of DCpep-antigen pulsed DCs *in vitro* and *in vivo* are convincing. However, to the best of our knowledge, no studies dealt with a direct DCpep-mediated targeting *in vivo* with soluble constructs. Moreover, since the receptor for DCpep on DCs is still unknown, it is not possible to predict which cells and, more specifically, which DCs subsets will be targeted by DCpep constructs. Yet this information would be helpful, because the various DC subsets might or might not express a DCpep receptor and possess distinct functions. Hence targeting the cross-presenting $Xcr1^+$ conventional DCs (cDCs) (reviewed in [5]) would mostly result in $CD8^+$ T-cell activation, targeting of $CD11b^+$ cDCs is expected to influence mostly T_H2 and regulatory T-cell responses [6], and specific targeting of Langerhans cells (reviewed in [7]) may contribute to a reduction of symptoms in allergy immunotherapy through allergen delivery to the epidermis [8].

Using the skin as site of vaccination has obvious advantages. The skin is easily accessible and is densely populated by numerous DC subsets with different function and specialized in the uptake, processing and presentation of antigens in the lymph node [9]. A precise and standardizable route to deliver antigens into the skin is laser microporation [10]. Combining this method with immunization approaches targeting particular cell subsets and the right adjuvation stimuli may offer the opportunity to shape immune responses towards the desired phenotype. For this reason we sought to investigate skin delivery of DCpep-fusion proteins to evaluate the efficacy of the approach, together with an exact identification of the cell subsets targeted *in vitro* and *in vivo*.

Material and methods

Protein cloning and production

The multiple cloning site of the expression vector pET17b (Merck Millipore, Billerica, MA, United States) was modified by cloning a synthetic oligonucleotide (GeneArt, Life Technologies, Grand Island, NY, United States) coding for DCpep (FYPSYHSTPQRP) and preceded by the start codon ATG. Downstream of DCpep restriction sites and spacers allowed for the cloning of proteins and protein domains of interest. For protein purification a hexahistidine tag was placed at the N-terminus (Suppl. Fig. 1A)

Recombinant proteins were produced in 1.2 L 2xYT medium (MP Biomedicals, Solon, OH, United States) in 2 l Erlenmeyer flasks at 37˚C shaken at 220 rpm using the *E.coli* strain BL21 (DE3) Star pLyss (Life Technologies). Cultures were grown until OD 0.7-0.8 and induced with 1 mM IPTG (Thermo Scientific, Rockford, IL, United States). Bacteria were harvested 3-5 h later by centrifugation at 7000 x g, 15 min, 4˚C and stored at -80˚C after resuspension in 20 mM Tris-HCl pH 8 / 6 M guanidinium-HCl / 0.5 M NaCl / 5 mM imidazole (OVA) or 50 mM NaH_2PO_4 pH 8 / 0.3 M NaCl / 10 mM imidazole (Bet v 1). All chemicals were obtained from Sigma-Aldrich, Steinheim, Germany.

Purification of OVA constructs

Bacterial lysates were thaw, sonicated three times for 30s and centrifuged at 50'000 x g for 30 min at 4˚C. Low-molecular-weight compounds were removed by gel filtration over a 30 cm / 240 ml column packed with Sephadex G-25 Medium (GE Healthcare, Uppsala, Sweden) [11] and the protein fraction was loaded onto a 5 ml nickel charged HisTrap FF crude column (GE Healthcare). The column-bound protein was first washed with 50 Column Volumes (CV) of 20 mM Tris-HCl pH 8 / 8 M urea / 0.5 M NaCl / 5 mM Imidazole / 0.1% Triton X-100 / 0.05% Triton X-114 ([12], adapted) for endotoxin removal and then with 20 CV of 20 mM Tris-HCl pH 8 / 8 M Urea / 0.5 M NaCl / 20 mM imidazole to remove unspecifically bound proteins and detergents. Finally, elution was achieved by increasing the imidazole concentration from 20 to 500 mM. Proteins were diluted to 150 µg/ml, dialyzed against 5 mM carbonate-bicarbonate buffer / 5 mM NaCl with a 6-8 kDa MWCO membrane (Spectrum Labs, Roncho Dominguez, CA, United States), concentrated ten times by evaporation (DNA110 Speed Vac, Savant, Farmingdale, NY, United States), supplemented with glycerol at 10% end concentration and stored at -80˚C. All chemicals were obtained from Sigma-Aldrich.

Purification of Bet v 1 constructs

Bacterial lysates were thaw and incubated for 30 min on ice after addition of 1 mg/ml chicken egg Lysozyme, sonicated three times for 30 s and centrifuged at 50'000 x g for 30 min at 4˚C. Supernatants were loaded onto a 5 ml nickel charged

HisTrap FF crude column (GE Healthcare Amersham Biosciences AB, Uppsala, Sweden). The column-bound protein was first washed with 50 CV of 50 mM NaH_2PO_4 pH 8 / 0.3 M NaCl / 5 mM imidazole / 0.05% Triton X-114 (protocol adapted from ref. [12]) for endotoxin removal and then washed with 20 CV of 50 mM NaH_2PO_4 pH 8 / 0.3 M NaCl / 20 mM imidazole. Elution was achieved by increasing the imidazole concentration to 300 mM. Proteins were dialyzed against 50 mM NaH_2PO_4 pH 8 / 0.3 M NaCl with a 6-8 kDa MWCO membrane (Spectrum Labs), supplemented with glycerol at 10% end concentration and stored at -80°C. All chemicals were obtained from Sigma-Aldrich.

Endotoxin content determination

Endotoxin contamination was quantified using PyroGene rFC Assay (Lonza, Walkersville, MD, United States) and the endotoxin content of all protein solutions was normalized by spiking with cell-culture grade Lipopolysaccharides (LPS) from *E.coli* 0111:B4 (Sigma-Aldrich).

Fluorescent labeling of proteins

Fluorescent labeling was performed using Dylight 488 Amine-Reactive Dye (Thermo Scientific) according to the instructions of the manufacturer and quantified by measuring the absorption at 493 nm using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Construct binding to mouse cells in vitro

2 mio cells extracted from the mouse ear as described [13] were resuspended in 150 µl culture medium (RPMI, 2% FCS, 30 mM HEPES, 100 U/ml Penicillin, 100 µg/ml Streptomycin, all from Sigma-Aldrich) containing 26.7 µg/ml of the different protein constructs. In order to account for the variable degree of labeling and the resulting differences in brightness, the amount of protein was corrected according to the absorption at 493 nm. After 1 h incubation at 37°C cells were washed with PEF (PBS, 2 mM EDTA, 2% FCS) and analyzed by flow cytometry as described [14]. Briefly, dead cells (Sytox blue, Molecular Probes, Invitrogen Detection Technologies, Eugene, United States), T cells (anti-CD3, clone 17A2, Biolegend, San Diego, CA, United States), natural killer cells (anti-NK1.1, clone PK136, Biolegend), B cells (anti-B220, clone RA3-6B2, Biolegend), and neutrophils (anti-Ly-6G, clone 1A8, Biolegend) were excluded through a dump channel (lin^+). $CD24^{low}$ $CD11b^+$ cells were then selected within the remaining $CD45.2^+$ population and the dermal DCs excluded by gating out Ly-$6C^-$ $CD64^-$ cells. Within this population monocytes were defined as $CCR2^+$ Ly-$6C^{hi}$ MHC-II^- and macrophages as $CCR2^-$. Following antibodies were used: anti-CD24 (clone M1/69, eBioscience, Vienna, Austria), anti-CD11b (clone M1/70, Biolegend), anti-CD45.2 (clone 104, eBiosciences), anti-Ly6C (clone AL21, BD Pharmingen, San Diego, CA, United States), anti-CD64 (clone X54-5/7.1, Biolegend), anti-MHC-II (I-A/I-E, clone M5/114.15.2, eBioscience), anti-CCR2 (clone 48607, BD Pharmigen).

For fluorescence microscopy analysis cells were purified by AutoMACS using CD11c MicroBeads (Miltenyi Biotec), challenged with the constructs as described above, transferred to a microscope slide by cytospin (500 rpm, 2 min), and fixed with 4% Paraformaldehyde for 5 min. After washing with PBS the cells were covered with Prolong Gold Antifade Reagent with DAPI (Life Technologies) and visualized by a Leica TCS SPE confocal microscope (Leica Microsystems, Heerbrugg, Switzerland).

In vitro presentation

Spleno- and lymphocytes were isolated from mouse spleens and lymph nodes as described [15]. After pooling them, DCs were isolated by AutoMACS using CD11c MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). 90'000 $CD11c^+$ cells were resuspended in 90 µl of culture medium containing (unlabeled) protein constructs, incubated for 45 min, washed with culture medium and split into three wells of a 96 well plate, each containing 25'000 OT-I and 25'000 OT-II cells labeled with Cell Trace Violet (Life Technologies). After 4 days incubation at 37°C and 5% CO_2 proliferation and T-cell activation were assessed by flow cytometry as described above.

Mice

7-10 weeks old female C57/BL6 or BALB/c mice were housed under specific pathogen free (SPF) conditions and handled in accordance with French and European directives with ethical approval from the Centre d'Immunologie de Marseille-Luminy.

Dendritic cells targeting in vivo by laser microporation

C57/BL6 mice were anesthetized with ketamine and their ears immobilized with a double-sided tape on the lid of a 96-well plate. After laser-microporating 5% of the surface of the backside of the ear (11.9 J/cm^2) with a Precise Laser EpidermAl SystEm device (P.L.E.A.S.E, Pantec Biosolutions, Ruggell, Liechtenstein), 20 µl fluorescent protein solution (0.5 mg/ml, 340 EU/ml of LPS) were applied [16]. After 24 h mice were sacrificed, DCs were enriched from the dermis of the ear and analyzed by flow cytometry as described [13].

Preparation and adoptive transfer of labeled OT-I and OT-II cells

OT-I and OT-II T-cells were isolated from pooled spleens and lymph nodes grinded through a 70 µm cell strainer. After red blood cell lysis, $CD8^+$ (OT-I) and $CD4^+$ (OT-II) cells were isolated by Dynal negative selection (Life Technologies) and stained with Cell Trace Violet (CTV, Life Technologies). Per mice 1 mio OT-I and 1 mio OT-II cells were injected i.v. retro-orbitally.

In vivo presentation

24 h after adoptive transfer of CTV labeled OT-I and OT-II cells, 70 nmol of every construct were applied on the mouse ear as described above. 62 h later mice were

sacrificed. CTV labeled OT-I and OT-II cells were extracted from the superficial cervical lymph nodes and analyzed by flow cytometry using the following surface markers: CD5, CD8, and CD4 for the identification of helper and cytotoxic T-cells, TCRvα2 and CD45.1 for the identification of the injected T-cells only. Dead cells were excluded with ToPro (Life technologies). All antibodies were from Biolegend.

Experiments with human cells

Peripheral Blood Mononuclear Cells were obtained by ficoll density gradient centrifugation (Biocoll Separating Solution, Biochrom AG, Berlin, Germany) from healthy donors with written informed consent. For the generation of monocyte derived Dendritic Cells (mdDC) or macrophages (mdMF) the $CD14^+$ population was isolated by AutoMACS using CD14 MicroBeads (Miltenyi Biotec) according to the instructions of the manufacturer and cultured at 1 mio/ml in well plates in presence of 1000 U/ml GM-CSF (PeproTech, Hamburg, Germany) and 1000 U/ml IL-4 (Novartis, Basel, Switzerland) for mdDCs, 1000 U/ml GM-CSF for the generation of GM-CSF macrophages, and 100 U/ml M-CSF (eBioscience) for the generation of mdMF. All differentiations were performed over 5 days. Two days before harvest M-CSF generated mdMF cultures were supplemented with either 1000 U/ml IL-4 or 50 ng/ml IFNγ (PeproTech) for further polarization (adapted from [17]). The successful differentiation to mdDCs or mdMFs from monocytes was confirmed by CD11c, CD14, CD68, CD80, CD163 expression. Cells were fixed and permeabilized with the Intracellular Fixation & Permeabilization Buffer Set (eBioscience) and stained with the respective antibodies (all from Biolegend).

Results

DCpep enhances uptake by DCs in vitro

We started our investigation by ensuring that DCpep enhances protein binding to murine DCs *in vitro*, coupled with the model allergen Ovalbumin (OVA). For this purpose we used 3 different constructs i) recombinantly produced OVA, ii) DCpep-OVA fusion, iii) $DCpep_4$-OVA consisting of 4 repetitions of DCpep fused to OVA (Suppl. Fig. 1B). In contrast to the original publication [1], we investigated in detail the binding to the most relevant DC populations. Challenge of DCs from the mouse ear with the constructs showed an increased binding of $DCpep_4$-OVA to all the populations that were analyzed, indiscriminately. On the other hand, binding of DCpep-OVA resulted in the appearance of a highly positive subpopulation of $CD24^+$ and $CD11b^+$ dermal DC ($CD24^+$ DDC and $CD11b^+$ DDC) but not of Double-Negative DC (DNDC) and Langerhans Cells (LC) (Fig. 1A-B). The same results were obtained with cells from the mouse auricular lymph node (not shown). The enhanced binding of $DCpep_4$-OVA to $CD11c^+$ DCs also became evident by fluorescence microscopy imaging (Fig. 1C).

DCpep constructs applied intradermally do not show enhanced uptake or presentation by DCs in vivo

To broaden our knowledge on the efficacy and the specificity of DCpep *in vivo* we applied fluorescently labeled constructs to the densely DCs-populated dermis of the mouse ear after laser microporation. This application route has been recently shown to be comparable to an intra-dermal injection for the challenge of antigen presenting cells with proteinaceous constructs [10, 16]. After 48 h construct-positive DCs could be detected in the dermis but no benefit of the targeting domains was observed. Contrarily, the uptake of native OVA was superior in some DC subsets (Fig. 3A). Importantly, MFs and monocytes populations [14] appeared very active in the uptake of the applied constructs (Fig. 3B). We then investigated if DCpep could improve antigen presentation to the OVA-specific T-cells OT-I ($CD8^+$) and OT-II ($CD4^+$) upon dermal application. In accordance with the targeting experiment described above, DCpep-OVA fusions resulted in equal or reduced proliferation when compared to OVA alone (Fig. 3C).

DCpep enhances binding to a broad range of phagocytic cells

Why is DCpep failing *in vivo*? A possible explanation is the absence of specificity for DCs that would lead to absorption by non-presenting cells and the consequent loss of the targeting benefit. We indeed observed that DCpep binds to a broad range of mouse MF and monocyte populations, which consistently showed enhanced binding that in some cases outperformed the binding to DCs (Fig. 4). The staining intensity followed the same ranking for mdDCs and mdMFs, i.e. $DCpep_4$-Betv1 > DCpep-Betv1 > Betv1, Betv1-DCpep (Suppl. Fig. 2).

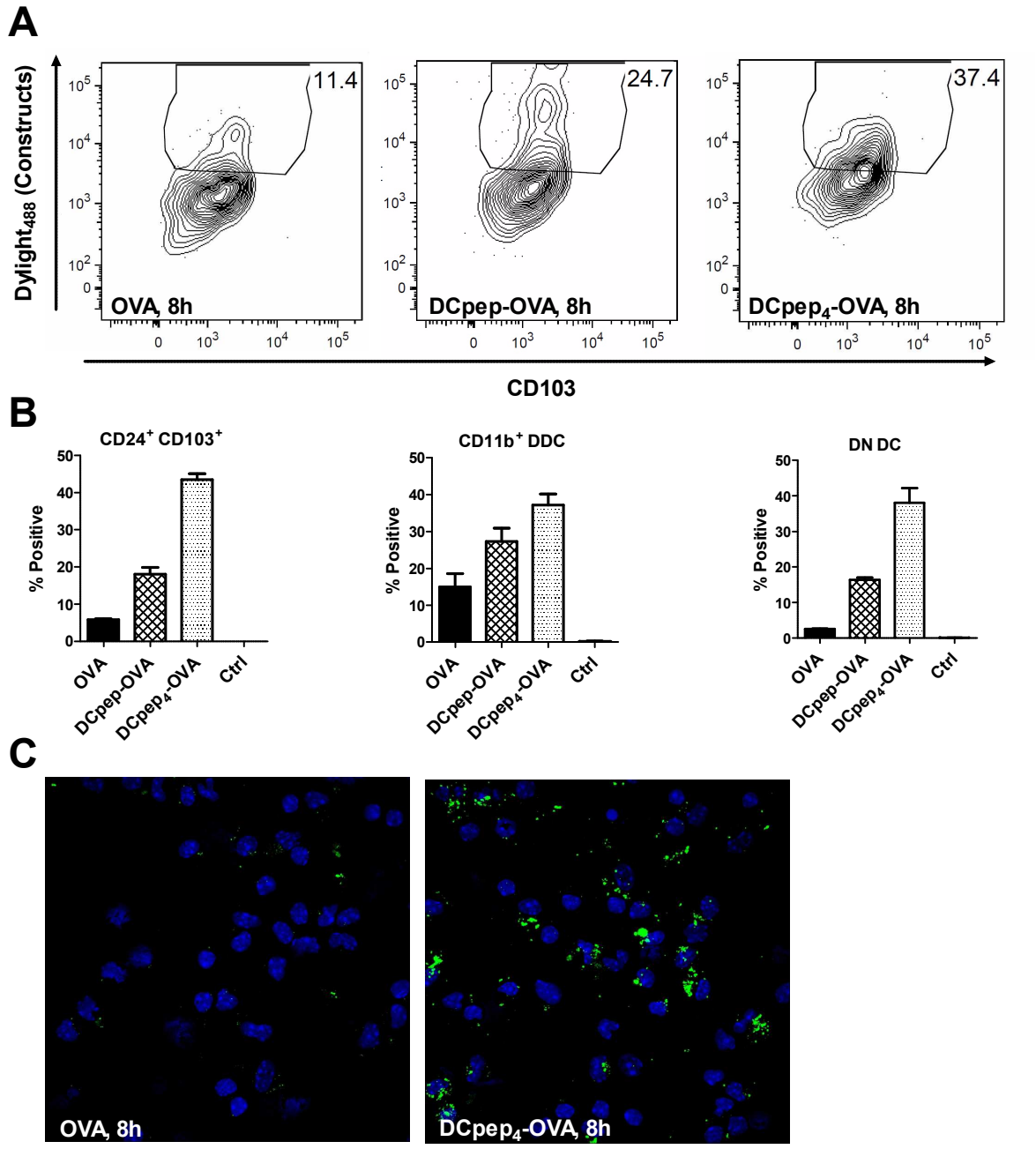

Figure 1 DCpep and DCpep$_4$ protein fusions enhance binding to mouse DCs *in vitro*

A-B Flow cytometry of the DCs-enriched cell fraction of the mouse ear challenged for 45 min at 37°C with Dylight$_{488}$ fluorescently labeled OVA, DCpep-OVA or DCpep$_4$-OVA. After staining with the constructs the cells were labeled with surface antibodies and analyzed by flow cytometry. This data show the enhanced binding triggered by DCpep or DCpep$_4$, which results in larger populations of Dylight$_{488}$ positive cells. Gating for positive cells is shown as exemplified by the dermal DCs population (**A**) and quantified as % of gated positive cells for the selected DC populations CD24$^+$ CD103$^+$ DCs, CD11b$^+$ dermal DCs (DDC), and double-negative DCs (DN DC) (**B**). Error bars represent s.e.m. n=3. **C** Fluorescence microscopy image showing that after 8 h of incubation the uptake of fluorescently labeled DCpep$_4$-OVA by CD11c$^+$ cells is significantly enhanced compared to OVA.

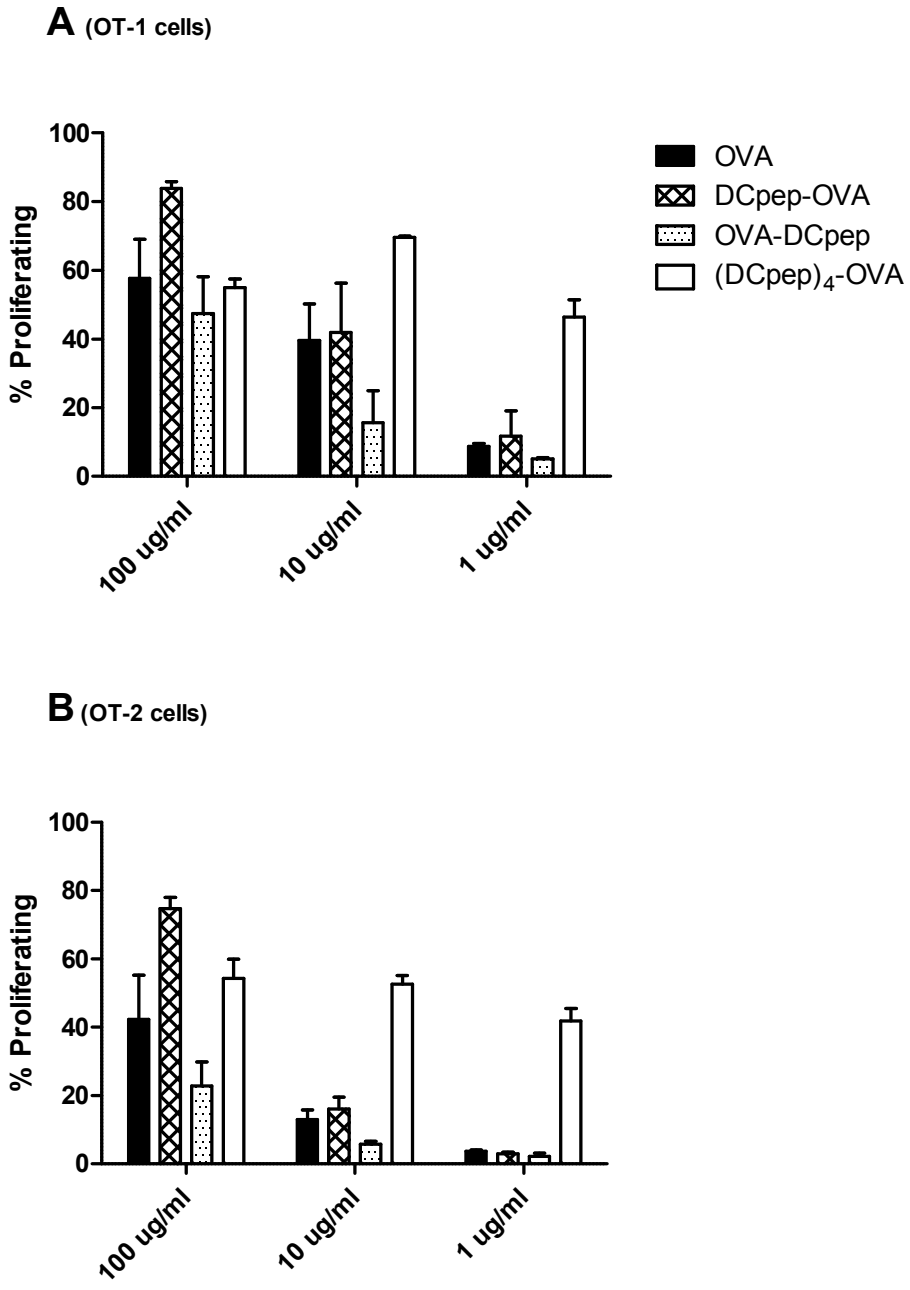

Figure 2 Solely DCpep$_4$-OVA results in enhanced presentation *in vitro*

A-B CD11c$^+$ DCs obtained from grinded spleens and lymph nodes by magnetic-beads isolation and pulsed with DCpep$_4$-OVA for 45 min at 37°C are superior in triggering the proliferation of CTV labeled T-cells at low concentrations. No major differences are observed by comparing CD11c$^+$ DCs pulsed with all the other constructs under the same conditions. Histograms represent the percent of proliferating (i.e. with reduced CTV fluorescence) OT-I cells (**A**) and OT-II cells (**B**) as measured by flow cytometry. Error bars represent s.e.m.

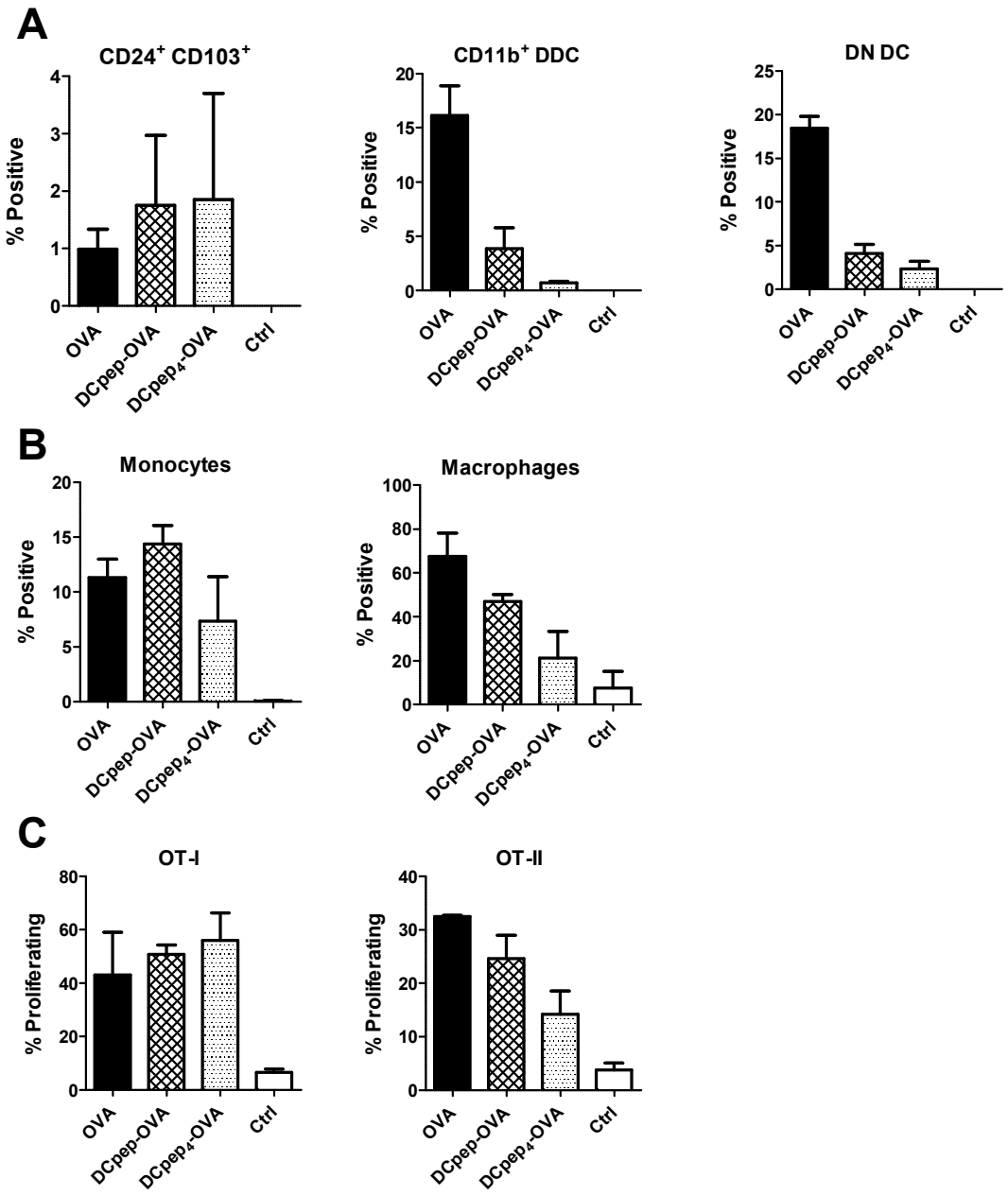

Figure 3 DCpep does not enhance uptake and antigen presentation through the mouse dermis *in vivo*

Following laser microporation fluorescently labeled constructs (OVA, DCpep-OVA or $DCpep_4$-OVA) are applied on the mouse ear. After 24 h of incubation dermal cells are extracted from the dermis of the ear and analyzed by flow cytometry. **A** The analysis shows how the uptake of protein constructs by $CD24^+$ $CD103^+$ DCs, $CD11b^+$ Dermal Dendritic Cells (DDC) and $XCR1^-$ $CD11b^-$ double negative DC (DN DC) is not enhanced by DCpep. On the contrary the uptake of OVA alone is superior. **B** A significant fraction of dermal monocytes and macrophages populations were positive for the applied constructs, suggesting high phagocytic activity **C** DCpep-OVA fusions were not superior in triggering the proliferation of CTV labeled OT-I and OT-II cells. For this analysis the constructs were applied by laser microporation on both ears 24 h after the i.v. injection of OT-I and OT-II cells. Mice were sacrificed after 62 h and proliferation was assessed in the superficial cervical lymph node by flow cytometry. Error bars represent s.e.m.

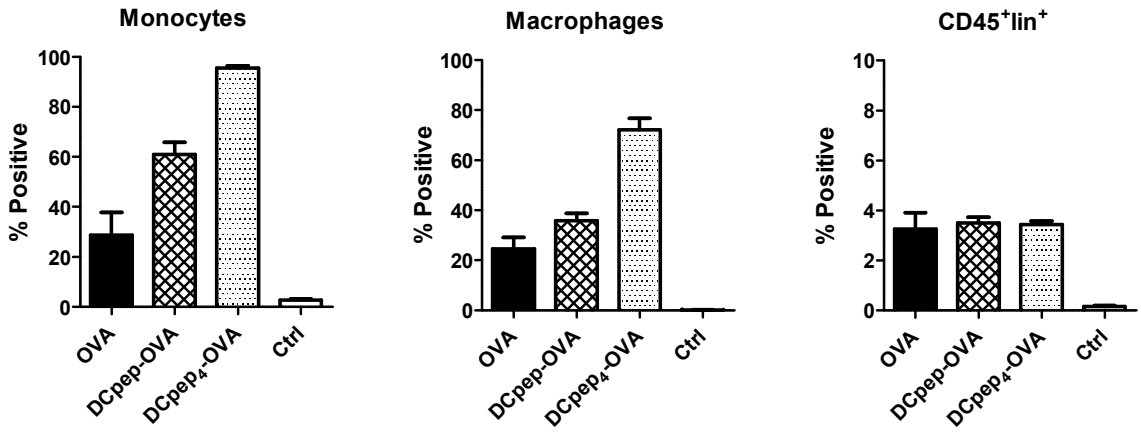

Figure 4 DCpep and DCpep$_4$ fusions enhance protein binding to mouse macrophages and monocytes

After 1 h of incubation at 37°C in complete medium together with a mixed-cells population extracted from the dermis of the ear, fluorescently labeled DCpep and DCpep$_4$ fusions with OVA showed enhanced binding to monocytes and MFs, but not to the remaining non-phagocytic cell populations ($CD45^+$ lin^+). Monocytes and MFs have been distinguished by flow cytometry according to previous publications [14]. Briefly, dead cells, T cells, NK cells, B cells and neutrophils were excluded through a dump channel (lin^+). $CD24^{low}$ $CD11b^+$ were then selected within the remaining $CD45^+$ population and the dermal DCs excluded by gating out Ly-$6C^-$ $CD64^-$ cells. Within this population monocytes were defined as $CCR2^+$ Ly-$6C^{hi}$ MHC-II^- and macrophages as $CCR2^-$. Error bars represent s.e.m.

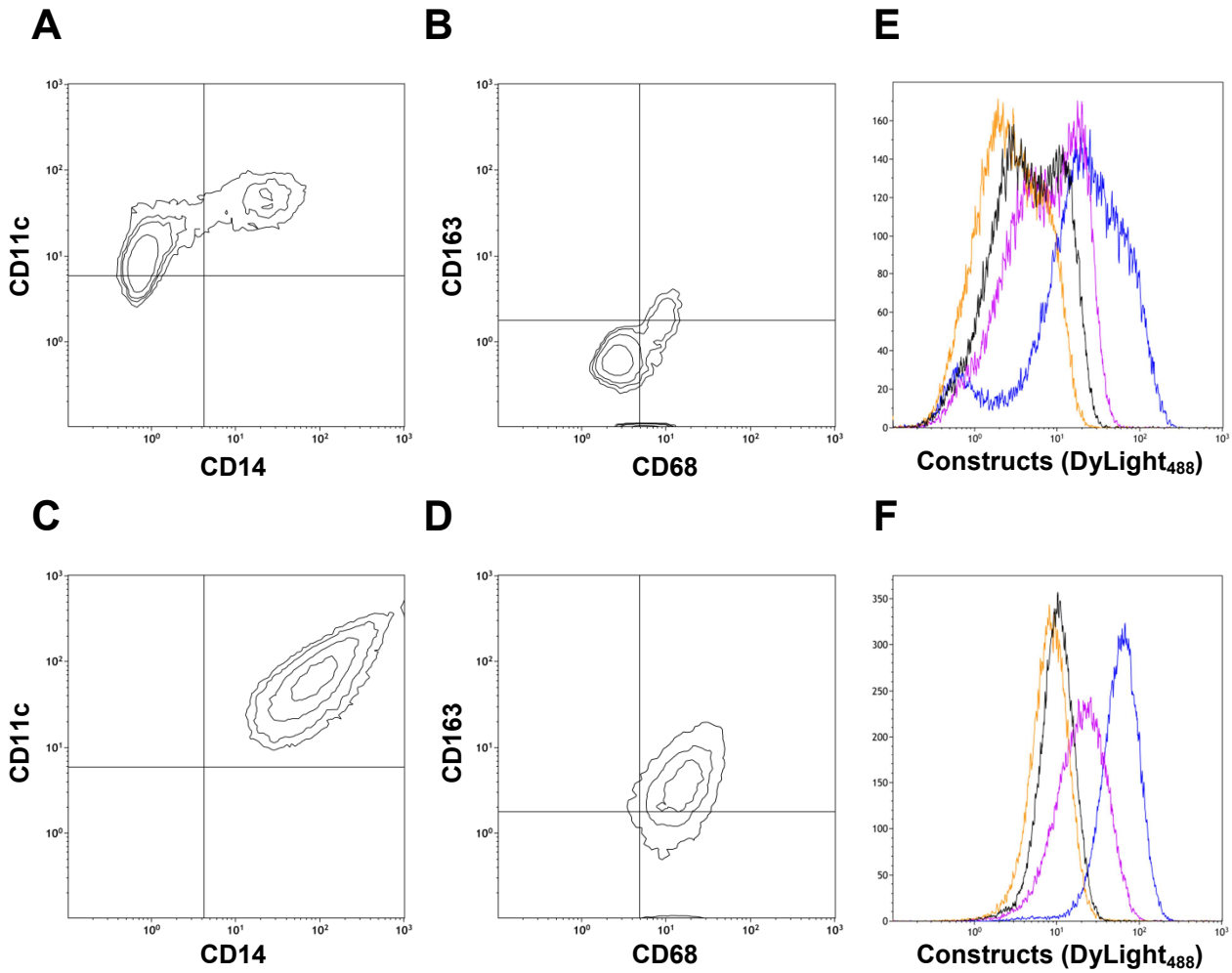

Figure 5 DCpep enhances antigen uptake in human mdDCs and mdMF.

A-D The differentiation of mdDCs (**A-B**) and mdMF (**C-D**) from $CD14^+$ monocytes as confirmed by flow cytometry; mdDCs lost CD14 expression and upregulated CD11c, but not CD68 and CD163. On the other hand M-CSF generated mdMFs maintained CD14 expression and upregulated CD11c, CD68, CD163. **E-F** Construct bearing DCpep at the N-terminus, either once (DCpep-Betv1, violet), or repeated four times ($DCpep_4$-Betv1, blue) showed increased binding to both mdDCs (**E**) and mdMFs (**F**). Positioning DCpep between Bet v 1 and his-tag (Betv1-DCpep, orange) didn't result in any improvement of the binding compared to Bet v 1 alone (black).

DCpep enhances binding to in vitro derived human macrophages

Since DCpep may have different specificities in human and mouse cells we decided to investigate if the binding to MFs can be reproduced *ex vivo* using human cells. For this purpose we used monocyte derived macrophages (mdMF) and DCs (mdDCs) that were challenged with the birch allergen Bet v 1, which is a relevant for allergic reaction in humans (Suppl. Fig. 1C). The data confirmed the observations that were made in mice: DCpep appreciably enhanced the binding not only to mdDCs but also to mdMF. The presence of four repetitions of DCpep resulted in a stronger enhancement of the binding. Interestingly, the localization of DCpep was crucial for the efficient binding of Bet v 1 constructs. Positioning the peptide C-terminally between Bet v 1 and the hexahistidine tag resulted in the loss of the binding benefit (Fig. 5). The enhanced binding triggered by DCpep to mdMFs was not dependent on the differentiation protocol that was used (M-CSF or GM-CSF) and on the polarization stimuli IL-4 and IFNγ that were given two days before the binding assay.

Discussion

This study confirmed the functionality of DCpep *in vitro*. DCpep significantly improved construct binding to mouse DCs, without a clear preference for a particular subset among all subsets analyzed. The binding to MFs appears stronger than the one observed for DCs, and even in this case no preferential binding to particular subpopulations could be observed. The enhanced binding through DCpep obtained *in vitro* correlated with increased antigen presentation to antigen-specific T-cells, indicating that the bound antigen is internalized, processed and presented. Notably, we could detect an improvement in antigen presentation at low concentrations only for the constructs where DCpep was repeated four times ($DCpep_4$). Peptide multimerization is a well known and simple way to enhance the affinity of targeting peptides by increasing avidity and local concentration [18], and in our hands, for DCpep, the benefits in terms of binding were essential for the enhancement of antigen presentation.

For the first time we investigated the use of the DCs binding peptide DCpep to improve antigen delivery to skin DCs *in vivo*. The approach, consisting of a dermal application on the mouse ear after laser microporation, failed. DCpep couldn't enhance DCs binding and presentation to antigen-specific T-cells. Instead, we observed significant amount of antigen-positive MFs after the application of the fluorescent construct. Binding to MFs is expected to be detrimental for immunization approaches, because dermal MFs have poor antigen-presenting capacities and do not migrate to the draining lymph node in mice [14]. For this reason MF-bound DCpep constructs are expected to prematurely disappear from the dermis and to be degraded without being presented to T-cells. We speculate that this mechanism may be at the basis of the observed elimination of the benefits that DCpep has previously been shown to provide [1-3].

Another relevant phenomenon that becomes apparent by looking at the results obtained in this study is that the position of DCpep can be crucial for its functionality. In our hands, the original construction [1] where DCpep was positioned C-terminally between antigen and his-tag was ineffective for targeting. We suspect antigen-related steric hindrance to be responsible for this effect and strongly suggest the testing of alternative constructions to identify the best binding protein fusions.

Our data show that DCpep remains a powerful tool for the induction of antigen presentation by DCs. We, however, detect some limitations of its use *in vivo*. In our opinion, the most probable cause for the problems encountered *in vivo* is the enhanced binding to MF, leading to premature clearance of the applied constructs. Another possible explanation is a conflict of the targeting approach with the application by laser microporation. Other work clearly showed that microporation is a valuable approach for the delivery of proteinaceous antigens [10, 16], however a construct-specific incompatibility for skin penetration through laser generated pores could not be completely ruled out.

Recently, DCpep has been shown to bind DCs from a broad range of species, including feline, avian, canine and equine DCs ([19], A. Ziegler unpublished results). It cannot be excluded that the specificity of DCpep may vary among the species and that the limitations that have been identified in this study will apply to all target organisms. It has, however, to be pointed out that *in vitro* results obtained in this study using human monocyte derived DCs and MFs went in the same direction as the ones obtained in mouse. It is important that future studies on DCpep will focus on the determination of its binding partner. In this way we will be able to better understand its potential as antigen-delivery peptide *in vitro* and draw a more complete picture of its potential for targeted delivery to APC. The same shall apply to all recently discovered DCs binding peptides, e.g. the NW peptide [20]. Particular care should be taken in the determination of the binding potential to MFs, which may be crucial for future *in vivo* applications.

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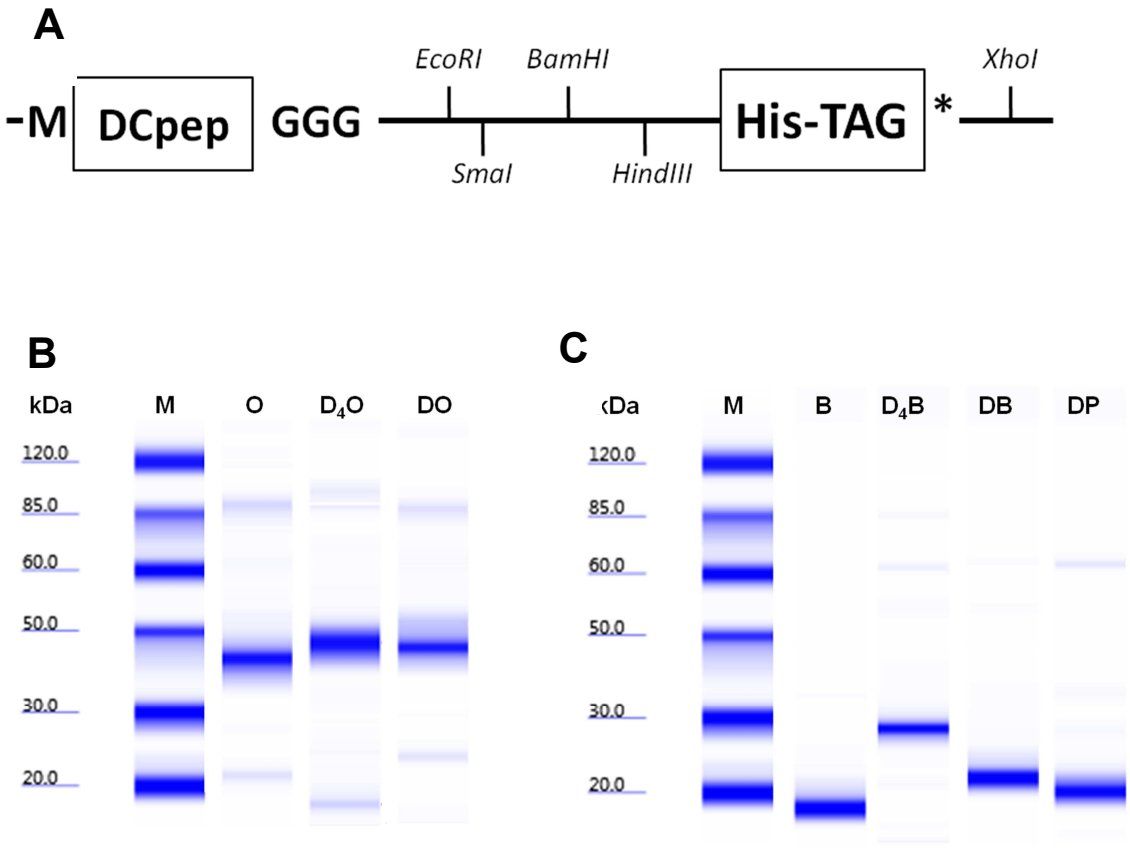
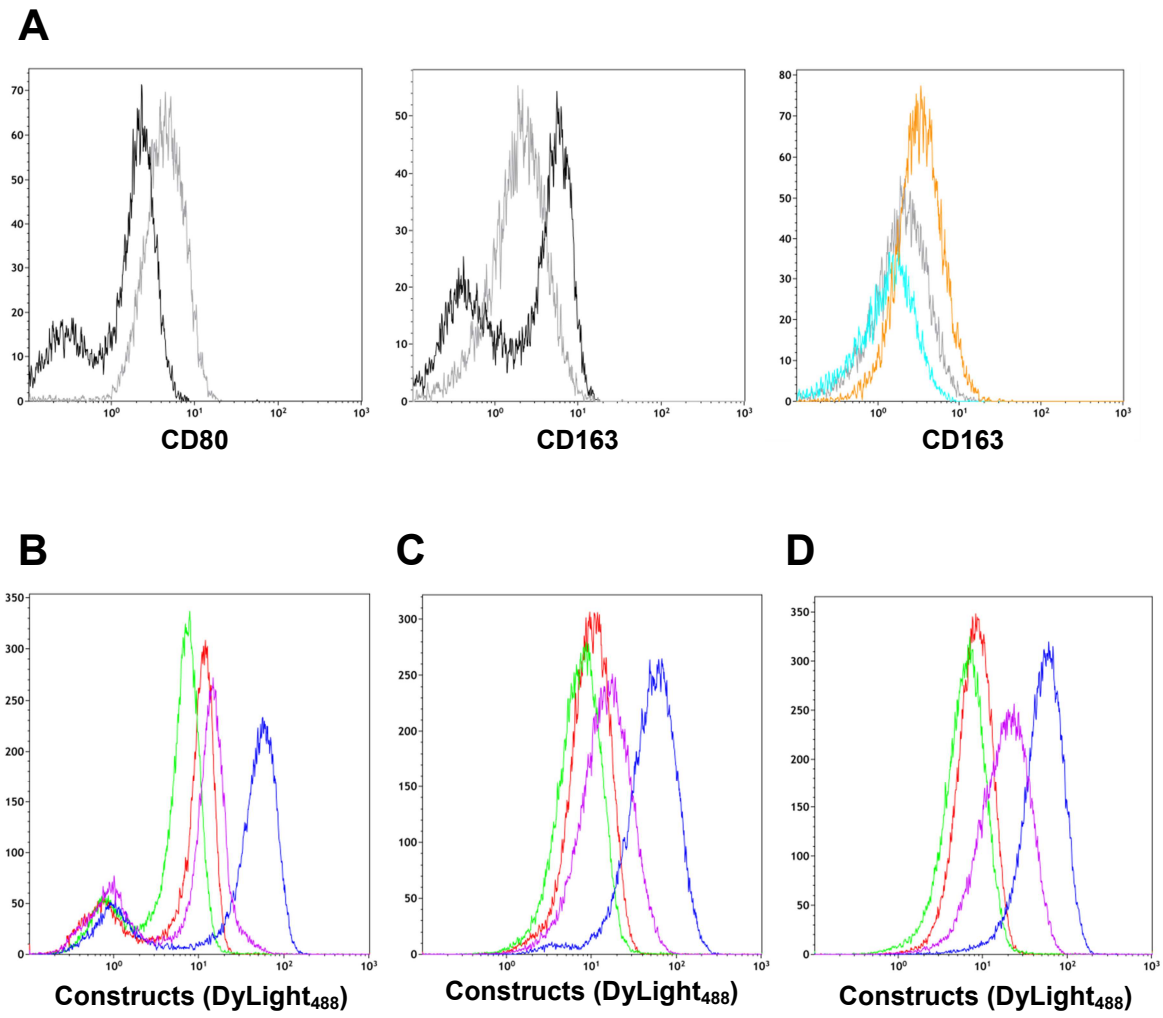


Suppl. Figure 1 Construct design and protein purity

A pET17b modification with the DNA sequences encoding for DCpep and His-Tag that was used for the cloning of OVA and Bet v 1 constructs. M, Mehionine; G, Glycine; *, Stop-codon; restriction enzymes are shown in italic **B-C** Purity of the recombinantly produced OVA and Bet v 1 assessed with the 2200 TapeStation (Agilent technologies, Santa Clara, CA, United States). kDa, KiloDalton; M, Marker; O, OVA; D, DCpep; B, Bet v 1



Suppl. Figure 2 Binding to polarized and GM-CSF differentiated mdDCs

A Additional MF populations tested. Comparison of CD80 and CD163 expression of alternatively differentiated human MFs. M-CSF (grey), GM-CSF (black), M-CSF + IL-4 (sky-blue), M-CSF + IFNγ (orange) **B-D** Binding of fluorescently labeled constructs to MFs that were differentiated with alternative strategies: GM-CSF (**B**), M-CSF + IL-4 (**C**), M-CSF + IFNγ (**D**). Similarly to mdDCs and mdMF, $DCpep_4$-Betv1 (blue) shows the highest binding, followed by DCpep-Betv1 (purple). No benefit could be observed with C-terminal DCpep (Betv1-DCpep, red) compared to Betv1.

CHAPTER 3

On-cell interactions of antibodies with cell-bound markers assessed by Quartz Crystal Microbalance (QCM) biosensing

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Abstract

The characterization of the binding properties for soluble proteins is currently performed by binding them to the corresponding binder with methods like Surface Plasmon Resonance (SPR). This approach is hampered for membrane proteins, because the purification is challenging, not always successful, and often requires laborious procedures. Additionally, it frequently results in protein denaturation, which may lead to biased binding properties with the interaction partners. In this context, the extended sensitivity range of Quartz Crystal Microbalance (QCM) technology may represent a useful tool, because it could be used to determine the binding properties of proteins by directly measuring the binding affinity on the cell surface, instead of isolating and purifying them.

We investigated the possibility to measure the affinity of antibodies for epitopes located on the cell surface with the Attana Cell™ 200 instrument (Attana AB, Stockholm, Sweden). Using HeLa cells transfected with the C-type lectin CLEC7A/Dectin-1 as a model system we provide general protocol guidelines for the optimization of binding assays. Additionally, we showed the importance of complementing "standard" with "on-cell" affinity measurements. This was achieved by comparing the results of the purified extracellular domain of human Dectin-1, with the results obtained with on-cell measurements for transfected Dectin-1; for two commercially available anti-Dectin-1 antibodies. Finally, we identify and discuss the current limitations of this approach, which will be decisive for future developments.

Introduction

The use of the piezoelectric property of quartz crystals to measure changes of mass (Quartz Crystal Microbalance, QCM) was first proposed in 1957 by G. Sauerbray [1] who observed a correlation between mass and change of frequency while depositing metal layers on an oscillating quartz crystal. Later, the observation of Nomura and Okuhara that oscillating piezoelectric quartz crystals can be operated in liquids [2] allowed the application of the QCM technology to biological samples [3].

Since then the continuous improvements of QCM instruments has allowed the development of various different biological assays. In the field of immunology QCM technology is used clinically for diagnostic purposes, e.g. for the detection of the auto-antibodies involved in the pathogenesis of celiac disease [4], or in R&D to determine affinity constants of macromolecular interactions. QCM devices are also widely used in assays involving interactions among small molecules, synthetic polymers, particles, viruses, carbohydrates, lectins, lipids and membranes, proteins, oligonucleotides, and cells [3].

The measurement of label-free protein-protein interactions has been possible since more than 30 years by the use of Surface Plasmon Resonance (SPR) based devices. The first immunoassay being the one proposed by Liedberg and co-workers in 1983 for the detection of anti-human IgG [5]. SPR technology does not allow direct measurement of on-cell kinetics and affinities. The main reason is that cells constitute a thick layer on the biosensor chip that impairs SPR measurements. QCM technology offers the opportunity to measure affinities over thicker layers of interaction partners, allowing direct label-free measurements of affinities on cells.

In the last few years reports have demonstrated that QCM devices are able to measure kinetics and affinity of lectin binding to the surface of cancer cell lines [6-8]. However, to the best of our knowledge, no publications in peer-reviewed journals dealt with the direct measurement of antibody interactions on cell surface receptors. This is a big deficit considering that nowadays research on antibodies occupies a central position in the pharmaceutical industry. In fact, interactions between antibodies and proteins or cells are crucial in many biological processes and for the development and characterization of novel biologicals, such as therapeutic or diagnostic monoclonal antibodies [9, 10].

We report and discuss the use of the QCM cell biosensor Attana Cell™ 200 for the determination of antibody kinetics and affinity for molecules on cell surfaces and provide comparisons between the on-cell results and the affinity constants obtained using recombinant proteins directly coating the biosensor chip. As a model system we employed the dendritic cell associated C-type lectin-1 receptor (CLEC7A/Dectin-1, Dec1) either expressed on HeLa cells by transfection or recombinantly produced in a human cell line as soluble extracellular domain, and two different monoclonal antibodies.

Dec1 is a trans-membrane protein expressed predominantly by myeloid cells such as monocytes, macrophages, neutrophils and dendritic cells. Limited expression has also been reported on other human cell subsets including eosinophils and various populations of lymphocytes [11]. We decided to use Dec1 because of the wide range of ligands, which may be investigated in further studies. The natural ligands of Dec1 include β1,3-linked glucans and carbohydrates that are found in fungal cell walls, bacteria, and plants as well as some yet unidentified molecules [11-13]. Dec1 exserts also relevant immunoregulatory functions both in innate and adaptive immunity: it can stimulate a variety of cellular responses including phagocytosis, respiratory burst, the production of lipid mediators [14], and of inflammatory chemokines and cytokines (CCL2, CCL3, CXCL2, TNF-α, IL-1β, IL-6, IL-10, and IL-23). Newer experimental data showed that Dec1 is also involved in the development of T_H17 responses [15, 16].

For these reasons Dec1 may represent an appropriate immunotherapeutic target. In fact, antibody mediated targeting of the OVA antigen to Dec1 resulted in enhanced $CD4^+$ T-cell and antibody responses [17], and the blockade of Dec1 was suggested to prevent the development of β-glucan-dependent autoimmune diseases [16, 18]. Deeper knowledge of the affinity of ligands and antibodies for Dec1 may therefore provide new insights facilitating the future development of immunotherapies.

With Dec1 and HeLa cells as a model system we aim at providing a new tool for an accurate measurement of kinetic rate constants and affinity of antibody-epitope interactions on membranes in a physiologically relevant environment (the cell surface), and new insights on how QCM measurements of antibody affinity on cells can be optimized, avoiding the laborious procedures needed for the purification of membrane proteins.

Material and methods

Construction of Dec1 expression plasmid

Human Dec1 (Uniprot Q9BXN2) was shuffled into a pUNO vector (Invivogen, San Diego, CA) by PCR amplification from previous cloning work. Briefly, human Dectin-1 was PCR amplified from a human cDNA sample derived from dendritic cells, cloned in frame into a bacterial expression vector with N-terminal hexahistidine tag, sequenced, and finally shuffled into pUNO. pUNO:Dec1 was propagated in *E. coli* NovaBlue (Merck Millipore, Zug, Switzerland) and purified to eliminate endotoxin contaminations with a Nucleobond Xtra Maxi EF kit (Macherey-Nagel, Düren, Germany). pUNO:Dec1 was linearized using the FastDigest restriction enzyme *Not*I (Thermo Scientific, Rockford, IL, United States) and purified on agarose gel.

Cell culture and transfection

50'000 HeLa cells were seeded into a 24-well plate in 1 ml cRPMI medium (RPMI 1640, 10% FCS, 2 mM L-Glutamine, 1 mM Sodium pyruvate, 1x MEM Vitamins, 1x

MEM non-essential amino acids, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 100 µg/ml Kanamycin, all from (Sigma-Aldrich, Steinheim Germany) and incubated for 24 h at 37°C, 5% CO_2.

0.5 µg of linearized pUNO:Dec1 were diluted in 50 µl Opti-MEM (Life Technologies, Grand Island, NY, USA) and mixed with 1 µl of TurboFect transfection reagent (Thermo Scientific). After 20 min incubation at room-temperature the mixture was added dropwise to the HeLa cells. The culture medium was changed after 4 h and incubated for 16 h at 37°C, 5% CO_2. Cells were then trypsinized (0.05% Trypsin, 0.02% EDTA), diluted 1:10, 1:100 and 1:1000 in cRPMI medium, and transferred into Nunclon Surface Petri dishes (Thermo Scientific). After 24 h 10 µg/ml Blasticidin (Invivogen) was added for the selection of transfected cells.

Cells were maintained in blasticidin selection medium for 10-14 days. Colonies were then harvested by trypsinization followed by gentle scratching and transfer into a disposable 1.5 ml plastic tube. After resuspension cells were transferred into a 12-well plate for further growing in cRPMI/Blasticidin and tested for Dec1 expression by fluorescence microscopy and flow cytometry.

Fluorescence microscopy

60'000 HeLa cells per chamber were seeded in an 8-well culture slide (BD Bioscience, Aalst, Belgium) in cRPMI and incubated for 16 h at 37°C, 5% CO_2. The cells were washed twice with PBS and fixed for 5 min with 4% Paraformaldehyde (PFA) (Sigma-Aldrich) in PBS. After washing with PBS-Tween (0.05%) the cells were blocked with 10% goat serum in PBS-Tween for 30 min and incubated with either 1 µg/ml mouse-IgG2a anti-human Dec1 (clone 15E2, Biolegend, San Diego, CA, United States) or 1 µg/ml mouse-IgG2b anti-human Dec1 (clone 259931, R&D Systems, Abingdon, UK) for 30 min. After three additional washes with PBS-Tween the cells were stained with 0.5 µg/ml Alexa Fluor 488 highly cross-adsorbed goat anti-mouse IgG (H+L) (Life Technologies) for 40 min, washed again with PBS-Tween and PBS, and fixed with 1% PFA for 5 min at room temperature. After a final wash with PBS the chambers were removed, and the cells covered with a droplet of Prolong Gold Antifade Reagent with DAPI (Life Technologies) and visualized after 24h by a Leica TCS SPE confocal microscope (Leica Microsystems, Heerbrugg, Switzerland).

Flow cytometry and cell sorting

Transfected HeLa cells (HeLa-Dec1) were harvested by trypsinization and washed with cRPMI and PBS. Dec1 stainings were performed using 0.2 µl of PE mouse-IgG2a anti-human Dec1 (clone 15E2, Biolegend) in 40 µl MACS buffer. If not otherwise mentioned the cells were incubated with the antibody for 15 min at room temperature.

The number of antibodies bound per cell, which corresponds to the number of expressed Dec1 molecules, was estimated using Quantibrite PE beads (BD Bioscience). 500'000 HeLa-Dec1 cells were stained for 45 min at room temperature

in 50 µl MACS buffer containing 1 µl of PE mouse-IgG2a anti-human Dec1 (clone 15E2), washed and analyzed by flow cytometry together with freshly resuspended Quantibrite PE beads. The number of Dec1 molecules was calculated by linear regression of $\log_{10}$(PE-fluorescence) x $\log_{10}$(PE-molecules per bead), as described [19].

To test the best buffer and regeneration conditions for affinity measurements, HeLa-Dec1 cells were fixed for 7 min in 1% PFA under constant agitation at a concentration of 2 mio/ml and filtered through a 40 µm cell strainer (BD Falcon). For stability tests of membrane-bound Dec1 the cells were subjected to different regeneration conditions for 20-30 times the usual regeneration time, washed, and then stained and analyzed as described above. To assess the effectiveness of regeneration, cells were first stained as described above and then treated with regeneration solution for the equivalent of a standard regeneration cycle (see "Affinity measurements and data analysis"), washed with MACS buffer (PBS, 2mM EDTA, 0.5% BSA) and analyzed by flow cytometry.

Cell sorting was performed with a BD FACSAria II cell sorter (BD Bioscience), and the analysis was performed using BD FACSDiva™ software v.6.1.3 (BD Bioscience). 20 mio HeLa-Dec1 cells were stained with 4 µl PE mouse-IgG2a anti-human Dec1 (clone 15E2) in 400 µl MACS buffer for 15 min at room temperature. After sorting, highly positive cells (Suppl. Fig. 2) were cultured in cRPMI for 3-4 days prior to use.

Estimation of the maximum achievable frequency shift by QCM

The maximal frequency shift that can be reached on the Attana Cell™ 200 was estimated by the following calculation; according to the Sauerbrey equation [1] and the fact that 0.7 ng of binding induces a shift of approximately 1 Hz (S. Altun, Attana AB, personal communication).

$$\Delta Hz_{max} = n_c \cdot \left(\frac{n_e}{2N_A}\right) \cdot \frac{M(Ab)}{k}$$

n_c, maximum number of cells on the chip (80'000); n_e, number of estimated epitopes per cell; N_A, Avogadro constant ($6.022*10^{23}$); M(Ab), molar mass of the antibody (180'000 Da); k, the Sauerbrey constant defining the correlation between frequency shift and mass shift for a 10 MHz quartz crystal ($7 \cdot 10^{-10}$ g/Hz). The number of estimated epitopes per cell is additionally divided by two, because only about half of every cell is exposed to the analyte.

Preparation of cell-biosensor chips (COP-1)

HeLa or HeLa-Dec1 cells were harvested by trypsinization, washed with cRPMI and PBS, and counted with a Neubauer Improved chamber (Assistant, Sondheim v. d. Rhön, Germany). 60'000 cells in 100 µl PBS were applied to the crystal surface by cytospin: the Attana COP-1 chip (Attana AB) was disassembled and the crystal transferred to a microscope slide. The cytospin assembly was carefully mounted and spun for 2 min at 500 rpm after transferring the cells in the cytospin funnel. The cells on the crystal were immediately fixed with 4% PFA for 4 min. After two

additional PBS washes using a 200 µl pipet the sensor chip was re-assembled and transferred into the biosensor device. After the affinity measurements the coverage of the chip was confirmed by staining the cell-nuclei with a 5 min injection of a 10 µM Höchst 33342 solution (Life Technologies) at 5 µl/min. The cells were visualized on an inverted fluorescence microscope (Zeiss Observer.A1, Carl Zeiss AG, Oberkochen, Germany) equipped with an AxioCam color camera (Carl Zeiss AG, Oberkochen, Germany). Images were acquired with the AxioVision software v.4.8.2 (Carl Zeiss Micro Imaging GmbH, Oberkochen, Germany).

Preparation of protein-biosensor chips (LNB)

The carboxyl-groups bearing surface of the Low Non-specific Binding (LNB)-Carboxyl sensor chip (Attana AB) was charged with the soluble recombinant extracellular domain of Dec1 (expressed in human cell line, NP072092.2, Thr66-Met201, Sino Biologicals, Beijing, China) or recombinantly produced Ovalbumin (Addgene plasmid 25098, [20]) by EDS/sulfo-NHS chemistry using the Amine Coupling Kit (Attana AB) according to the instructions of the manufacturer.

Affinity measurements and data analysis

Antibody affinity for cells and recombinant proteins was determined using the Attana Cell™ 200 biosensor at 23˚C and a flow rate of 20 µl/min. Measurements were started after system equilibration with the running buffer HBS-T (10 mM HEPES, 150 mM NaCl, 0.05% Tween 20, pH 7.4, Attana AB) until the frequency signal shift was below 0.2 Hz/min. Blank injections preceded every affinity measurement. Regeneration was accomplished by 10 s injections with 100 mM Glycine, pH 2.4 or 12.5.

For the kinetics and affinity determination, binding curves using 3 different antibody concentrations (27.8 nM, 55.6 nM, 83.4 nM) were generated in triplicates by injecting the antibody solution onto a LNB-carboxyl chip with immobilized Dec1 (Channel A) or OVA (Channel B) for 60 s. Unfortunately, it was not possible to optimize the regeneration procedure to completely avoid signal loss. It was therefore not possible to obtain curve replicates from the same chip surface used for measurements performed on cells. For this reason only 4 curves could be obtained by injecting the antibodies (6.95 nM, 13.9 nM, 27.8 nM, 41.7 nM) on a HeLa-Dec1-loaded (Channel A) and a HeLa-loaded COP-1 chip (Channel B) for 60 s. After binding, antibody dissociation was recorded over a period of 120 s before surface regeneration.

The curves obtained on every channel were referenced for unspecific signal by subtracting the leading blank injection curve. Off-target interaction with unrelated ligands was referenced by subtracting the channel B curves (OVA or HeLa) from the channel A curve (Dec1 or HeLa-Dec1). The referenced curves were analyzed with TraceDrawer (Ridgeview instruments AB, Uppsala, Sweden) using a 1:1 kinetic model compensated for mass-transport limitation. This model was chosen because of the

superior curve fit, as determined by R-squared analysis (not shown). Because of the observed loss of signal in subsequent measurements the maximum binding signal (B_{max}) was set to local, i.e. it was not assumed that every measurement can reach the same maximum signal.

Results

Stable Dec1 expression on HeLa cells

Stable HeLa cells clones transfected with the plasmid pUNO:Dec1 (HeLa-Dec1) were tested for surface expression of Dec1 by flow cytometry (Fig. 1A) and by fluorescence microscopy using two different monoclonal antibodies (Fig. 1C). Notably, substantial differences were observed in positivity, i.e. Dec1 expression on the cell surface for every HeLa-Dec1 clone (Fig. 1A). To ensure the suitability of the HeLa-Dec1/anti-Dec1 model, kinetic of antibody binding was analyzed by flow cytometry. The observed binding of the anti-Dec1 antibody to fixed and non-fixed cells was fast and practically independent from fixation, reaching 50% of the mean-fluorescence intensity within 86 s and 87 s, respectively (Fig. 1B).

Screening of running and regeneration buffers

Screening for the ideal running and regeneration buffers is a laborious procedure when performed directly on the Attana Cell™ 200. Moreover, some buffers compromise the integrity of the epitopes on the chip, which has to be substituted resulting in additional costs. For this reason we established a flow cytometry-based method, which allowed a fast and inexpensive screening of the initial assay conditions and the regeneration buffers for the removal of bound antibodies from the chip surface (see the “Material and methods” section). This test revealed how the binding of anti-Dec1 antibodies (clone 15E2) to fixed cells is comparable for a variety of commonly used buffers (Fig. 2A). Therefore, we choose to proceed with the standard assay buffer HBS-T (Attana AB, Stockholm, Sweden) as running buffer. The analysis of the effectiveness of different regeneration buffers as well as the analysis of their effects on the Dec1 epitope was performed in a comparable way (see the “Material and methods” section). This analysis showed the ideal regeneration buffer being 100 mM Glycine at pH 2.4 or 12.5. Regeneration under this conditions ensured efficient removal of the bound antibody without excessive harm to the Dec1 epitope (Fig. 2B-C).

Cytospin is a reliable way to transfer adherent and non-adherent cells to the surface of the COP-1 chip

The sterile and polystyrene coated COP-1 chip is designed to work with adherent cells, directly grown on the chip and fixed before kinetic and affinity measurements. In this context it has to be pointed out that, so far, it has not been possible to avoid

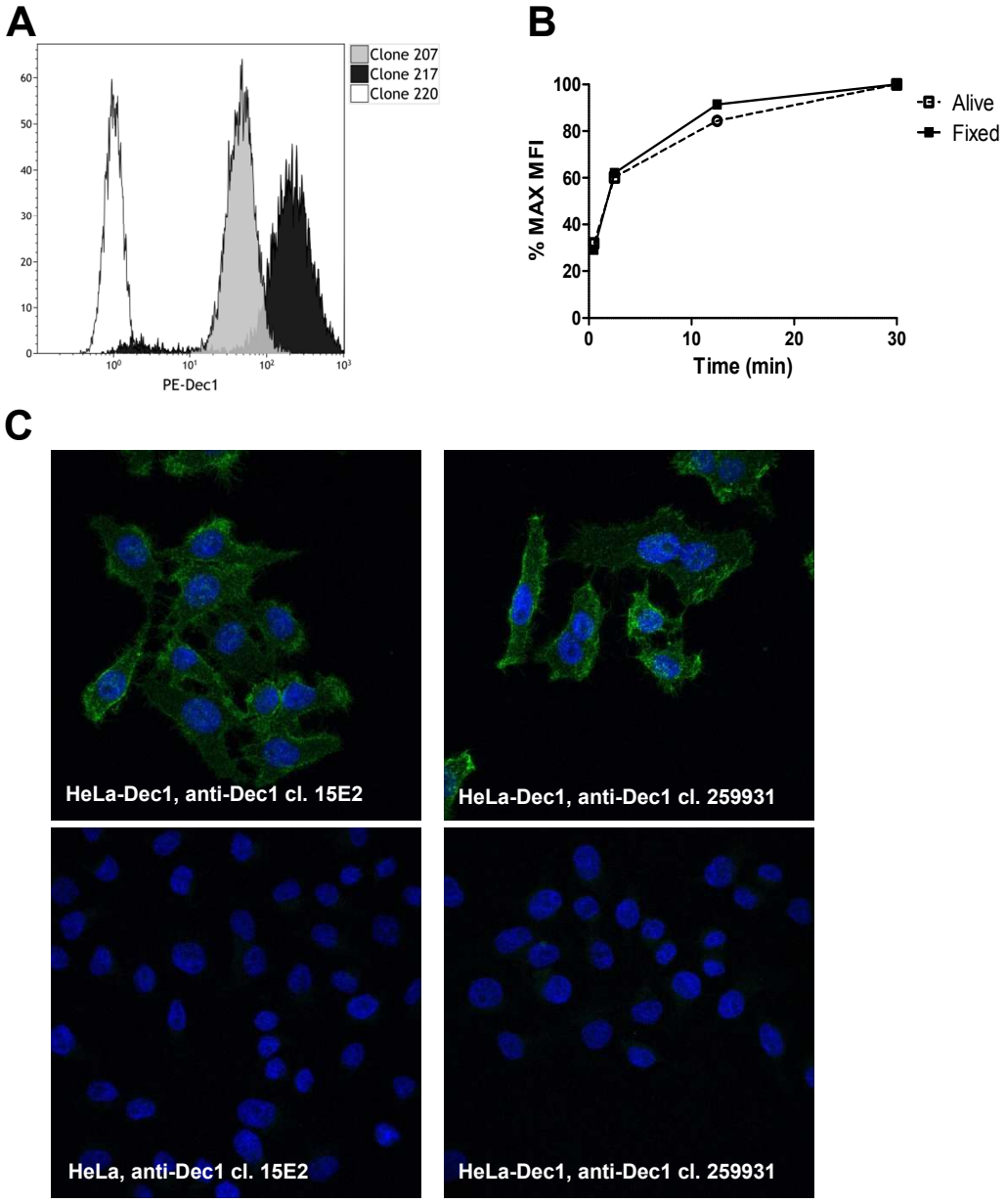
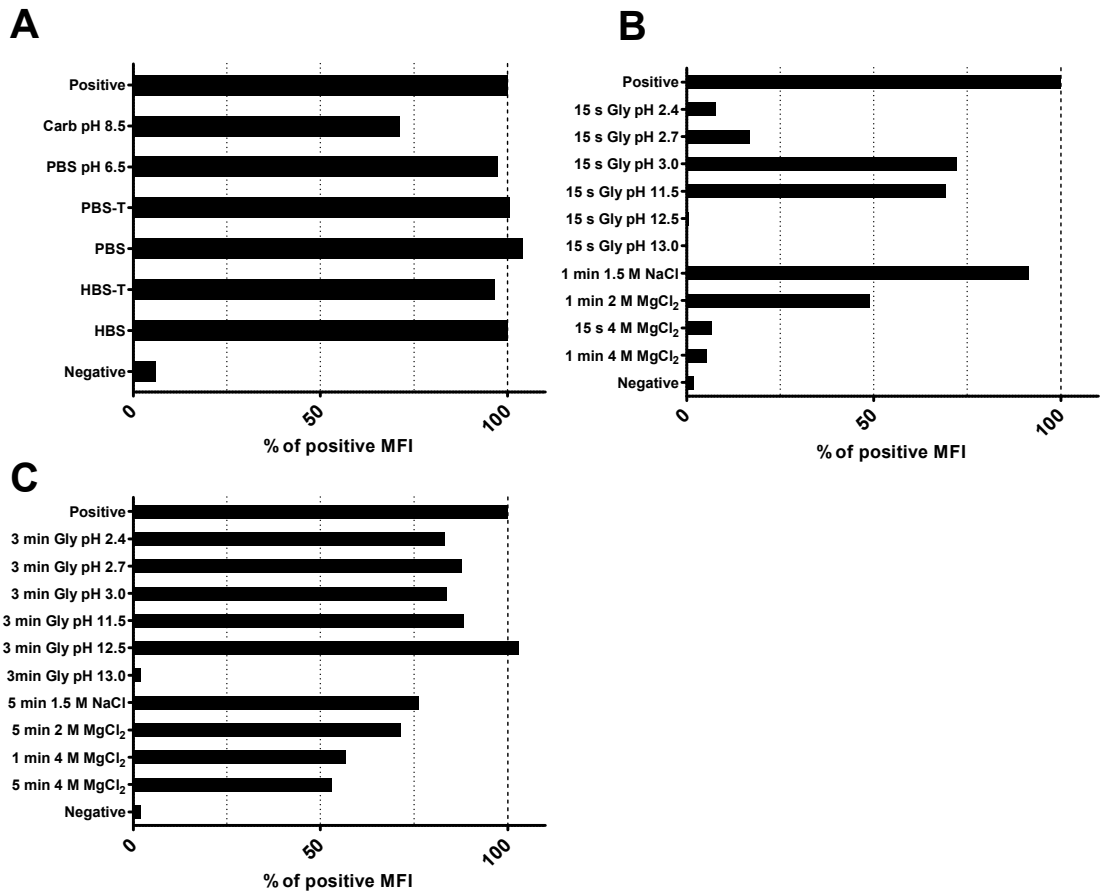


Figure 1 Confirmation of antibody binding to HeLa-Dec1 cells

A Quantification of Dec1 expression of three HeLa cell clones after transfection using flow cytometry and the PE-labeled anti-Dec1 antibody clone 15E2. Transfected clone 217 was selected as positive (HeLa-Dec1) and 220 as negative (HeLa). **B** Comparison of the maximal mean fluorescence intensity (MAX MFI) reached with different antibody binding times for the PE-labeled anti-Dec1 antibody clone 15E2. Both fixed and non-fixed cells reached MAX MFI within 86 seconds and showed comparable binding kinetic. **C** Binding of anti-Dec1 antibodies to HeLa-Dec1: as viewed by Leica TCS SPE confocal microscope. Both antibody clones resulted in similar fluorescence intensities for HeLa-Dec1. Unspecific binding to the negative HeLa clone 220 was negligible.

Figure 2 Procedure optimization for kinetic measurements on the Attana Cell™ 200 by flow cytometry

A HeLa-Dec1 stained with the anti-Dec1 antibody clone 15E2 under different buffer conditions. **B** Efficacy of different regeneration conditions (15 s or 1 min) for the removal of anti-Dec1 antibodies from HeLa-Dec1. **C** Epitope stability, measured by determining the staining intensity that can still be achieved after prolonged incubation under regeneration conditions (1 min to 5 min). In all the plots (A-C) the fluorescence intensity obtained by using MACS buffer (PBS, 2mM EDTA, 0.5% BSA) was defined as positive and set to 100%. Carb, 50 mM sodium carbonate/bicarbonate buffer; Gly, 100 mM glycine; PBS, Phosphate Buffered Saline pH 7.4; HBS, Hepes Buffered Saline (10 mM HEPES, 150 mM NaCl, pH 7.4); T, addition of 0.05% Tween20; Negative, staining of the Dec1-negative HeLa cell clone. **D** Fluorescence microscope pictures of HeLa and HeLa-Dec1 transferred to COP-1 sensor chips by cytospin and fixed with 4% PFA. Nuclei were stained with Höchst 33342. Cell coverage was around 60-80%, considered to be ideal for affinity experiments and kinetic measurements


A
Positive
Carb pH 8.5
PBS pH 6.5
PBS-T
PBS
HBS-T
HBS
Negative
0
50
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% of positive MFI
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Positive
15 s Gly pH 2.4
15 s Gly pH 2.7
15 s Gly pH 3.0
15 s Gly pH 11.5
15 s Gly pH 12.5
15 s Gly pH 13.0
1 min 1.5 M NaCl
1 min 2 M MgCl2
15 s 4 M MgCl2
1 min 4 M MgCl2
Negative
0
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Positive
3 min Gly pH 2.4
3 min Gly pH 2.7
3 min Gly pH 3.0
3 min Gly pH 11.5
3 min Gly pH 12.5
3min Gly pH 13.0
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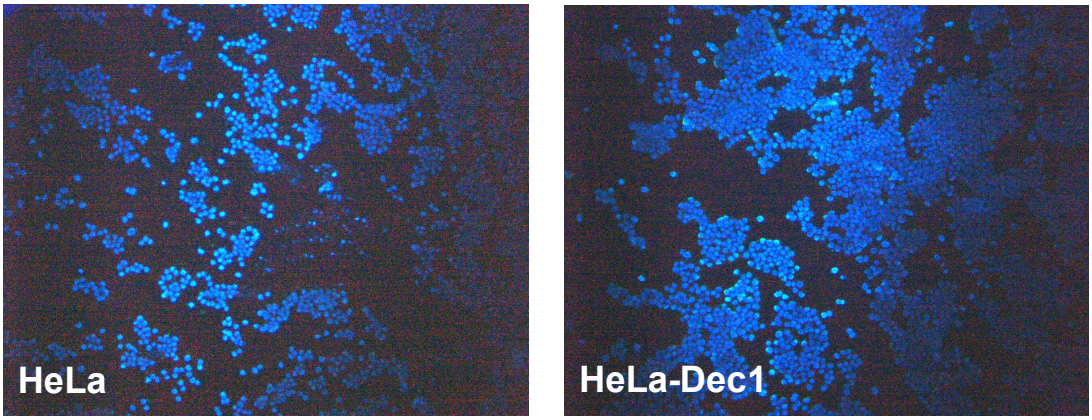
HeLa
HeLa-Dec1

cell fixation, because the running and regeneration buffers that are commonly used are fatal to the cells, which die and detach from the chip. To obtain comparative assays with flow cytometry and extend the suitability of the methodology to suspension and immune cells we developed a general immobilization strategy based on cytospin (described in the "Material and methods" section). We were satisfied with the results that were obtained: the applied suspension of HeLa cells was evenly distributed and we were able to easily control chip coverage. Moreover, no significant loss of cells was observed after fixation (Fig. 2D).

Detection of epitope binding is enhanced by sorting of highly positive HeLa-Dec1 cells

To our initial surprise the first trials to measure the affinity of anti-Dec1 antibodies to primarily transfected HeLa-Dec1 cells with the Attana QCM biosensor revealed no interaction. Despite the clear positivity in flow cytometry, no binding could be detected for antibody concentrations ranging from 13.9 to 111.2 nM (Suppl. Fig. 1). This negative result can be explained by two phenomena; the low accessibility of the targeted epitopes and/or the number of Dec1 molecules on the surface of every cell. In fact, the number of Dec1 molecules was estimated to 48'960 Dec1/cell using PE-fluorescence intensity and the PE-molecules standard curve calculated by QuantiBrite. This suggests a maximum achievable shift (ΔHz_{max}) of 0.8 Hz in the Attana Cell™ 200: in the detectable area but close to detection limit.

To overcome this issue we decided to sort HeLa-Dec1 cells and strictly select for highly positive expression of the Dec1 receptor (Suppl. Fig. 2). This allowed an increase of the number of Dec1 epitopes by almost nine times to an estimated average of 428'563 Dec1/cell (Suppl. Fig. 2), corresponding to a theoretical ΔHz_{max} of 6 Hz. Kinetics and affinity measurements performed on these cells after 3 days of culture were successful and on-rates (k_{on}), off-rates (k_{off}) and the affinity constant (K_D) describing the binding properties of both tested antibodies for fixed cells could be calculated (Fig. 3, Table 1). After successful kinetic measurements the cell coverage was visualized by Höchst 33342 staining, which confirmed an optimal coverage in the range of 60-80% (Fig. 2D).

Comparison of traditional and cell-based biosensor assays

The interaction between anti-Dec1 antibodies and Dec1 on the surface of HeLa cells was compared with their interaction with recombinant Dec1 (rDec1) protein immobilized on a LNB-Carboxyl sensor chip by amine coupling (the conventional approach for antibody affinity measurements on SPR and QCM devices). This enabled a direct comparison between the binding to the intact cell surface and the binding to recombinant protein, which currently represents the golden standard.

Anti-Dec1 antibodies bound to rDec1 in a concentration dependent manner, but not to recombinant ovalbumin (rOVA) used as negative control. As expected no positive signal could be detected for anti-CD8 and anti-CD4 antibodies binding to rDec1 (Fig. 4A). The calculated constants showed comparable k_{on} for both antibodies but

different k_{off} rates, which resulted in affinity constants (k_D) of 3.83 ± 0.64 x 10^{-9} M (clone 15E2) and 1.36 ± 0.28 10^{-9} M (clone 259931, Fig. 4B). The difference between the two antibody clones appears more pronounced by looking at the affinity constants describing their binding to cell-bound Dec1. The K_D for clone 15E2 only observed a modest drop to 2.14 ± 0.24 x 10^{-9} M, while the affinity of clone 259931 almost dropped by a factor six in comparison to the recombinant protein, to 0.24 ± 0.001 x 10^{-9} M. Interestingly, this decrease is explained by a pronounced increase of K_{off}, indicating that this clone is easily washed away from the membrane-bound Dec1.

Table 1 Binding kinetic and dissociation constants

Calculated binding kinetic on-rate and off-rate constants (K_{on}, K_{off}) and the dissociation constant (K_D). Sensograms were fitted with TraceDrawer software using a 1:1 kinetic model compensated for mass-transport limitation.

	K_{on} (10^5 $M^{-1}min^{-1}$)		K_{off} (10^{-4} min^{-1})		K_D (10^{-9} M)	
	on-cell	**Rec. Prot.**	**on-cell**	**Rec. Prot**	**on-cell**	**Rec. Prot.**
cl. 15E2	2.03 ± 0.0026	2.39 ± 0.0001	4.36 ± 0.479	9.15 ± 1.520	2.14 ± 0.238	3.83 ± 0.638
cl. 259931	5.25 ± 0.0015	4.82 ± 0.0001	125 ± 0.035	6.55 ± 1.340	0.24 ± 0.001	1.36 ± 0.279

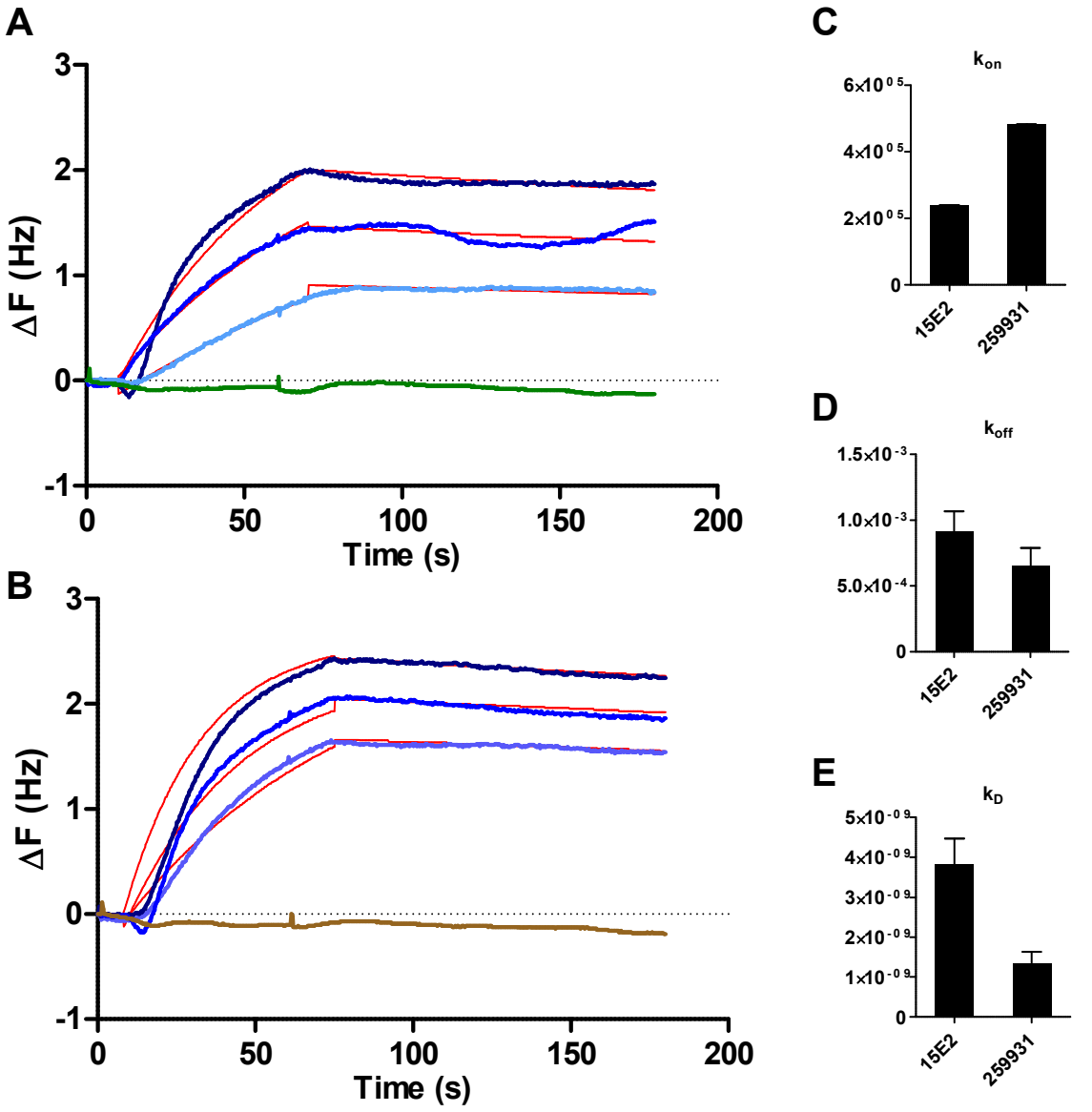

Figure 3 On-cell affinity measurements of anti-Dec1 binding to its membrane-bound receptor

A-B Representative sensograms (blue) illustrating the real time binding of anti-Dec1 antibody onto HeLa-Dec1 cells, monitored using Attana Cell™ 200 equipment. The association and dissociation phases (60 s and 120 s, respectively) were recorded at a concentration of 83.4 nM, 55.6 nM and 27.8 nM for the anti-Dec1 antibody clone 15E2 (**A**), and 41.7 nM, 27.8 nM and 13,9 nM for the clone 259931 (**B**). Darker blue corresponds to higher concentration. As control the binding of an anti-CD4 or anti-CD8 antibody is shown (green and brown, respectively). Red curves represent theoretical curve fitting determined using TraceDrawer software and a 1:1 kinetic model compensated for mass-transport limitation. **C-E** Comparison of on-rate (K_{on}, $M^{-1}min^{-1}$), off-rate (K_{off}, min^{-1}) and affinity constant (K_D, M) calculated for both tested antibodies on cell-bound Dec1. Bars represent the standard error of the mean (s.e.m).

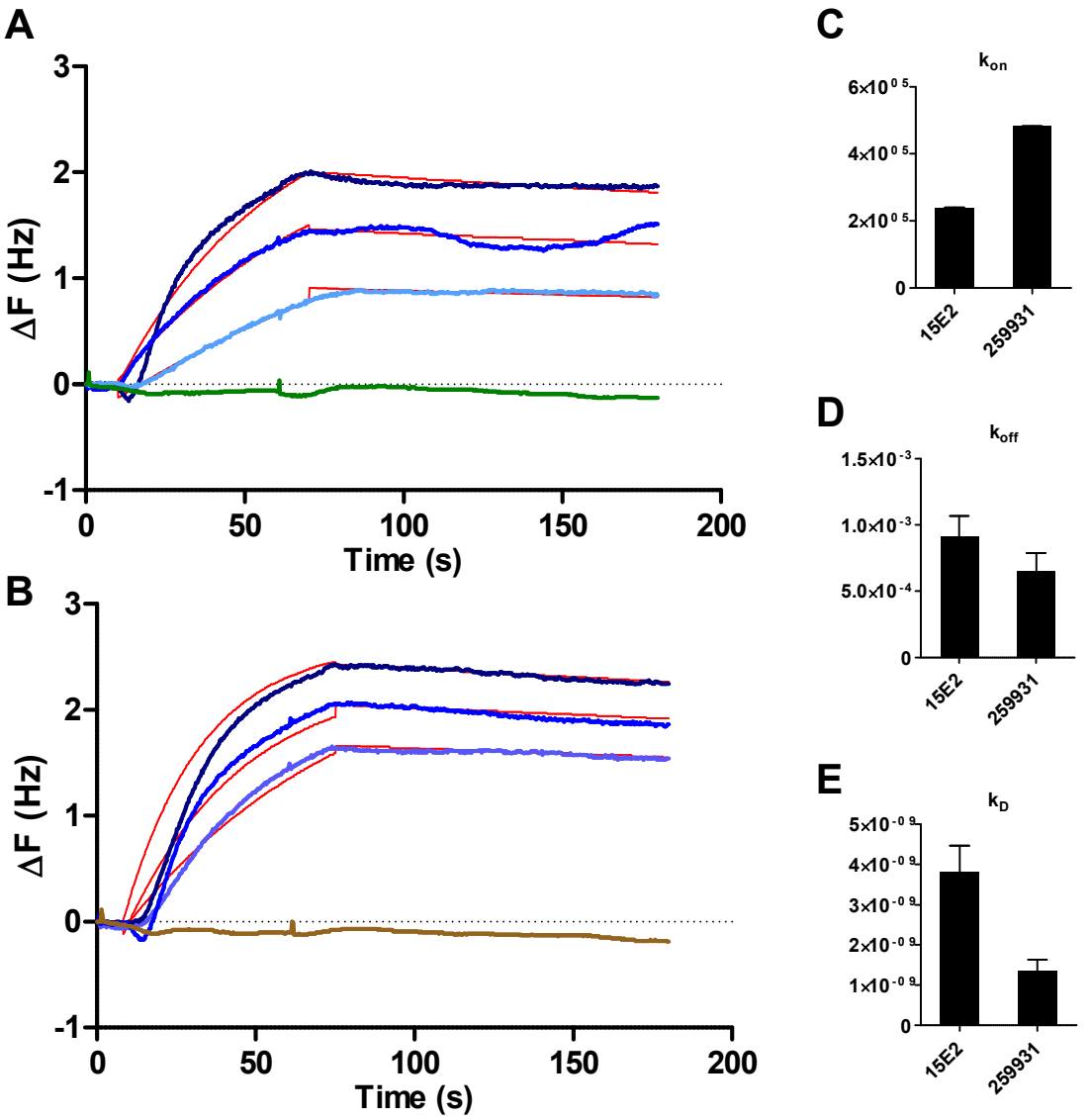

Figure 4 Antibody affinity measurements of anti-Dec1 to covalently bound recombinant protein

A-B Representative sensograms (blue) illustrating real time binding of anti-Dec1 antibody onto recombinant Dec-1 protein immobilized on sensor chip surface. Binding was monitored using Attana Cell™ 200 equipment. The association and dissociation phases (60 s and 120 s, respectively) were monitored at concentrations ranging from 83.4 nM to 27.8 nM for the anti-Dec1 antibody clones 15E2 (**A**) and 259931 (**B**). Red curves represent theoretical curve fitting determined using TraceDrawer software and a 1:1 kinetic model compensated for mass-transport limitation. As control the binding of an anti-CD4 or anti-CD8 antibody is shown (green and brown, respectively). For clarity only one of the recorded three curves for every concentration tested is shown (83.4 nM, 55.6 nM, 27.8 nM). Darker blue corresponds to higher concentration. **C-E** Comparison of on-rate (K_{on}, $M^{-1}min^{-1}$), off-rate (K_{off}, min^{-1}) and affinity constant (K_D, M) calculated for both tested antibodies on cell-bound Dec1. Bars represent the s.e.m.

Discussion

This study reports for the first time the suitability of Quartz Crystal Microbalance devices (Attana Cell™ 200) for the determination of antibody affinity on cells. The methodology developed is suitable for both adherent and non-adherent cells, and hence opens the possibility for cell-based measurements on immunologically interesting cells such as B- and T-cells, or other cells of the immune system. Moreover, no culturing step on the biosensor chip is needed, resulting in the opportunity for measurements on primary cells that cannot be cultured *in vitro*.

As a model system we compared the interaction of two different anti-Dec1 antibodies with cell surface-displayed and recombinant Dec1. Both anti-Dec1 clones showed similar k_{on} values for the on-cell and the on-protein measurement, but clone 259931 had a much higher on-cell k_{off} value, indicating an equally fast, but less-stable binding leading to faster dissociation and decreased overall affinity constant K_D. These differences that we observed for antibody-epitope interactions between cell surface-integrated and immobilized proteins illustrate the importance of studying the binding properties of biomolecules using intact cells.

There are two important considerations that need to be made about QCM-based measurements of on-cell antibody binding. First, the influence of the fixation procedures necessary to keep the cells firmly attached to the QCM crystal. We believe that the use of PFA fixation allows measurements that are closest to *in vivo* situations. For instance, and contrarily to the common vision, this type of fixation maintains the mobility of the membrane receptors [21]. Flow cytometry measurements performed in this work confirmed this view and showed that, in our model system, the binding kinetic is not influenced by cell fixation. Moreover, it has to be kept in mind that the since long accepted procedure where the receptor is purified and immobilized on the sensor surface also includes manipulations with unknown effects on its morphology [22].

Second, the signal to noise ratio; the number and the accessibility of epitopes on the cell surface strongly influence the interaction signal. Our data suggest that measuring shifts smaller than 0.5 Hz is hardly feasible because of the noise caused by other variables such as temperature and small differences in the buffer composition. Even relatively abundant immunologically relevant epitopes such as CD4, CD3 and CD8 that are estimated to occur about 100'000 times per cell [23], would give a maximal theoretical shift of only 1.3 Hz (Suppl. Fig. 2). Moreover, the determination of reliable affinity constants requires the use of different analyte concentrations, and it is unlikely to achieve reliable results within a range of just a few Hz.

Here we suggest to use stably transfected cell lines with strong promoters to increase the number of epitopes displayed on the cell surface; if necessary this can be combined with sorting for cells highly expressing the molecule of interest. In this study the maximal shift obtained after transfection and sorting was about 4 Hz,

which allowed robust measurements. The necessity of transfection in some experimental settings was also recently mentioned by Wright and co-workers, who used transfection to measure the interaction of HEK293 cells with a 15 kDa peptide [24].

Affinity determination on cell surfaces appears more easily achievable for lectins than for antibodies [6-8]. Beside the increased avidity, resulting from multiple sugar binding sites (e.g. Wheat Germ Agglutinin) or multimerization (e.g. Concanavalin A), is the number of easily accessible target epitopes on the cell surface [25] that presumably allowed the strong signals (20-30 Hz) observed in other studies. To the best of our knowledge only Tan et al. [26] investigated the binding of an antibody to its ligand on the cell surface. The approach, however, differs significantly to the one used in this study, because it lacks a microfluidic system and hence cannot measure off-rates. Additionally the cells were bound to the crystal by means of the integrin-binding RGD peptide, which has the advantage of maintaining cell integrity (no fixation step needed), but also introduces a new variable (the binding dynamics of cell-binding to the RGD peptide) and cannot withstand regeneration steps.

An alternative to the use of whole cells for the analysis of membrane receptors is constituted by their integration into an artificial lipid bilayer [27]. Although a bit simplistic, this approach was proven valuable to show that binding of Fc regions of IgG to lipid-bilayer-immobilized protein A from *Staphylococcus aureus* was largely restricted by steric hindrance on the lipid surface [28].

Conclusions

The methodology developed in this study will be of interest to the researchers working with cells in suspension. It sets the basis for future on-cell measurements of antibody affinity using QCM devices and provides some tools that allow from one side to extend the range of application to non-cultivable and suspension cells and on the other side to optimize the assay in a faster way by flow cytometry. Moreover, it identifies boundaries of the technology due to the low number of target epitopes normally expressed by the cells, and evaluates cell transfection and sorting as valuable tools to overcome this limitation.

Future studies will focus on the implementation of QCM technology for additional membrane proteins such as the T- and the B-cell receptor, and the Major Histocompatibility Complexes, which are immunologically important.

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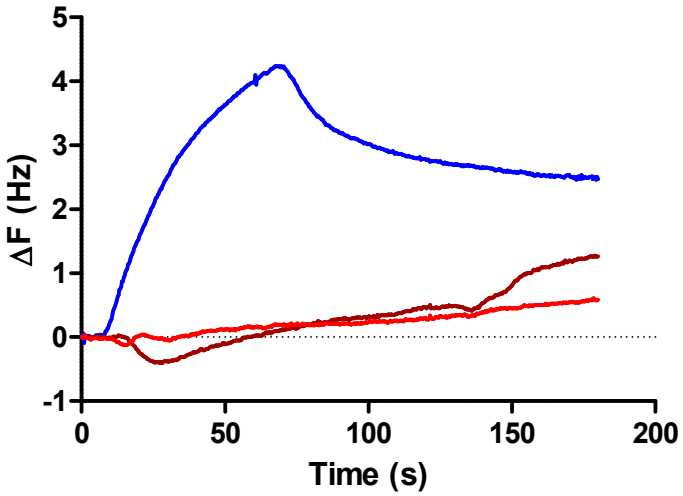
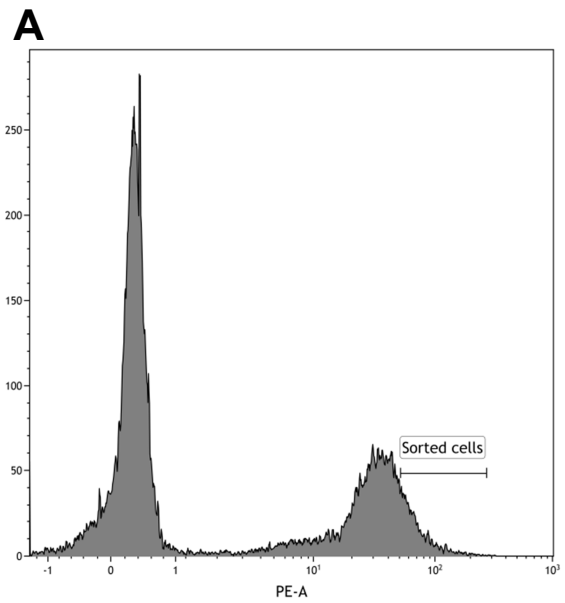
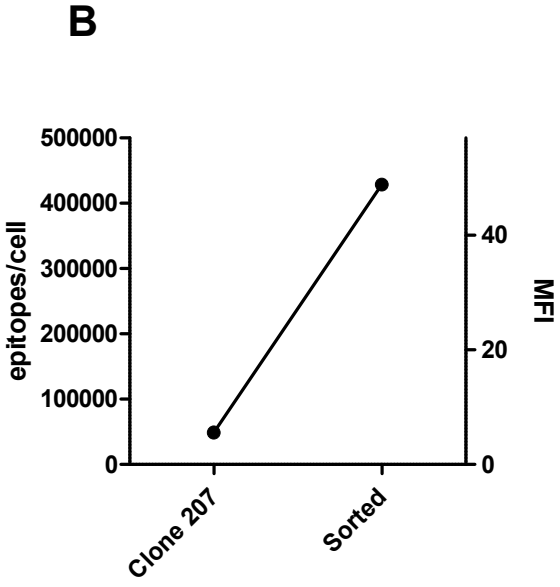


Supplementary Figure 1 Absence of binding detection in unsorted cells

By using unsorted HeLa-Dec1 (clone 207, see figure 1A) no binding could be detected for antibody concentrations of 13.9 nM and 111.2 nM (bright and dark red line, respectively). As comparison a binding curve from a successful experiment with sorted HeLa-Dec1 is shown (blue line).





C

$$\Delta Hz_{max}\ (Clone\ 207) = n_c \cdot \left(\frac{n_e}{2N_A}\right) \cdot \frac{M(Ab)}{k} = 80'000 \cdot \left(\frac{48'960}{2 \cdot 6.022 \cdot 10^{23}}\right) \cdot \frac{180'000}{7 \cdot 10^{-10}} = 0.8\ Hz$$

$$\Delta Hz_{max}\ (Sorted) = n_c \cdot \left(\frac{n_e}{2N_A}\right) \cdot \frac{M(Ab)}{k} = 80'000 \cdot \left(\frac{428'562}{2 \cdot 6.022 \cdot 10^{23}}\right) \cdot \frac{180'000}{7 \cdot 10^{-10}} = 7.3\ Hz$$

Supplementary Figure 2 Cell sorting for highly positive HeLa-Dec1 cells

A Four highly positive HeLa-Dec1 clones were pooled after transfection as soon as the total cell number reached 20 mio and stained with PE-anti-Dec1 (clone 15E2). Cell sorting strictly target highly positive cells **B** Sorting for highly positive HeLa-Dec1 cells resulted in a significant increase in the number of epitopes per cell, as determined using PE-labeled anti-Dec1 and a PE molecules per bead standard curve (QuantiBrite). MFI, Mean Fluorescence Intensity **C** Calculation of ΔHz_{max} for clone 207 and for the sorted cells. Cell sorting brought the maximum achievable shift to 7.3 Hz. n_c, maximum number of cells on the chip (80'000); n_e, number of estimated epitopes per cell; N_A, Avogadro constant ($6.022 \cdot 10^{23}$); M(Ab), molar mass of the antibody (180'000 Da); k, the Sauerbrey constant defining the correlation between frequency shift and mass shift for a 10 MHz quartz crystal ($7 \cdot 10^{-10}$ g/Hz).

General discussion

The increasing prevalence of allergic diseases in industrialized and developing nations [4] and the availability of significant financial means in the greatly affected countries prospect a bright future for allergy research. It is, however, hard to tell which directions will be taken by academic investigators and especially by the pharmaceutical industry. In fact, during the last decade a huge variety of new immunotherapeutic approaches have been proposed, including the development of modified allergens [63, 67, 71, 73], the use of DNA technology [79], the investigation of novel routes for allergen administration [44, 53, 56], and the developments of innovative carrier materials and adjuvants [83, 88]. Most of these strategies are still poorly investigated, especially in clinical trials. The next decade will therefore be decisive and will disclose which approaches will overcome the high hurdles to reach the market.

Fact is that there is still a lot to learn in the field of allergen-specific immunotherapy (SIT). It has now become clear that the mechanisms underlying SIT are complex, and that a benefit can be reached by manipulating different and sometimes independent pathways. This becomes obvious by looking at the outcomes of successful immunotherapies in mice and human. For example the benefits of SLIT mostly rely on the induction of tolerogenic responses dominated by T_{regs} but not on the alteration of antibody responses [49, 50], which on the contrary is typical for SCIT [39].

Even if completely different goals are pursued, many approaches developed for cancer immunotherapy can potentially be implemented to treat allergies. In fact, the alteration of T_{reg} cell responses as well as of the balance between T_H1 and T_H2 responses is common to many interventions against tumors. This includes the development of innovative DC targeting approaches and of immunomodulatory adjuvants triggering T_H1 responses. Much research is also done on the autologous transplantation of DCs, where DCs are loaded and activated *in vitro* and re-injected in the patient for a potent and targeted immune response [226, 227]. In this context the most important hurdle is the relative mildness of allergic diseases in comparison to cancer. Relatively safe treatments that are becoming customary for cancer patients and hence possess a favorable risk/benefit ratio are still too dangerous to be adapted for allergy patients. The achievable benefit (cure from the atopic condition) still does not justify the risks associated with the therapy (mostly auto-immune diseases [228] and cancer itself [uninvestigated]). These elegant approaches will therefore not be at our disposal until the safety of immunomodulatory interventions will be extensively demonstrated and accepted by the scientific community and in a later stage by the public awareness.

In this work we have evaluated two possible methods to improve specific immunotherapies: DC targeting and the use of particulate adjuvants.

Hydroxyapatite microbeads for allergy immunotherapy

The scientific community is becoming aware of the importance of adjuvants and secondary stimuli able to shape the immune response [83], but it is surprising how the information on this crucial subject remains fragmentary. We identify a great research potential on this subject, especially for an increase in the efficacy of immunotherapies.

Collaboration with Prof. Håkan Engqvist and Dr. Wei Xia at the University of Uppsala allowed us to test very homogeneous porous microparticles developed in their labs. Our reasoning was that the theoretically inert hydroxyapatite particles, loaded with a model antigen, would be able to create a subcutaneous depot slowly releasing the antigen but not providing adjuvation, thereby triggering a state of immunological tolerance that can be exploited for immunotherapeutic purposes.

Our data confirmed the immunomodulating properties of SHAS. Particles-coupling could prolong the permanence of the antigen in the LN to one week and showed long-lasting stimulation of $CD8^+$ and $CD4^+$ T-cells in mice. The antibody response *in vivo*, as well as investigations *in vitro* suggested poor inflammatory potential and *in vivo,* SHAS-bound OVA behave analogously to OVA in solution. Unfortunately our experimental setting could neither demonstrate nor exclude a benefit of SHAS in immunotherapy and deeper investigations considering a wider range of therapeutic concentrations and protocols are needed for a conclusive evaluation. Nevertheless, our results show that binding an allergen to SHAS results in reduced side effects in allergic mice, suggesting reduced accessibility. This may reduce the amount of immunotherapeutic injections by allowing the application of larger amounts of allergen, released over a longer time range.

One of the issues awaiting response is the fate of HA-particles, both in mice and humans. Only one week after injection the particles could not be localized in the neck subcutaneous tissue of the mouse (not shown). The absence of the necessary technical means, however, doesn't allow us to undoubtly state that the particles have been absorbed. Moreover, a different site of subcutaneous application in mice or a possible application in humans may have completely different outcomes.

DCpep mediated Dendritic Cell targeting for immunotherapy

Dendritic cell targeting can be achieved in multiple ways, the most common without doubt being the use of antibodies against DC-specific receptors (mostly C-type lectin receptors). The usefulness of this approach for the development of efficient allergy immunotherapies still has to be clearly demonstrated. The DC-receptor, which has the highest potential up to now, is probably DEC-205, because of its tolerogenic potential [191], but it is still not clear what the outcome of antibody-mediated targeting of allergens to DCs via other receptors could be, since the only published approaches rely on bead-mediated passive-targeting or carbohydrate-decorated allergens for enhanced binding to C-type lectin receptors [229]. In this work we decided to use short peptides as targeting, mainly for technical reasons. In fact, we

could demonstrate that the production of targeted antigen constructs bearing the 12-mer peptide DCpep is simple and can be done by using well-established *E. coli*-based techniques. This represents a great advantage in comparison with the mammalian system required for the production of antibodies and the use of complex conjugation techniques. The main open-question regarding DCpep is the unknown binding partner. Our collaborators at the University of Madrid (O. Palomares and co-workers) are currently addressing this important question that will allow us to predict the outcomes of DCpep-mediated targeting and suggest different uses of its potential.

One of the most interesting findings of this investigation is the observation that DCpep possesses a low specificity. Specificity is a key issue for all targeted approaches, because targeting a specific cell subset (and not another) may have relevant influence on the immunological outcomes. In the specific case of DCs it became clear that the organs mostly challenged with environmental threats are populated by a variety of subsets that have been characterized by considering a defined constellation of surface markers [109, 230]. The functional differences among these subsets as well as their plasticity are poorly investigated. However, several indications, point towards the obvious overlap of functional specialization and differential expression of surface markers [104, 106, 107, 231]. For this reason it is important to investigate carefully which cells are targeted and which ones are not.

To our disappointment we had to assert that DCpep has no preference for a particular subset of skin or LN murine DC subset *in vitro*, but the absence of specificity of DCpep goes further. In fact monocytes and macrophages appeared greedy for DCpep bearing constructs as well, and we speculate that clearance through these poorly presenting cells could have abolished the advantages that may results from the specific targeting, leading to the negative results obtained *in vivo*. This is a serious issue affecting DCpep, but potentially also all DC-targeting approaches. Despite the variety of markers “preferentially expressed” on DCs, no single DC-specific marker has yet been identified [184]. At the moment cross-targeting of unwanted and mostly related cell subsets cannot be avoided and could significantly mark the future of targeted therapies.

The observations discussed above rise some questions on the usability of DCpep for immunotherapy and lead us to speculate that DCpep may act as a phagocytosis-booster via interaction with the plasma membrane, rather than by binding to a specific protein receptor. This hypothesis is corroborated by the recent observation that up to now DCpep enhances the binding to the DCs of every tested mammalian species, including human, murine, feline, avian, canine and equine DCs (Owen 2013, A. Ziegler unpublished results).

Undisclosed preliminary results also suggest that the position of DCpep within the construct, together with the nature of the coupled protein, are crucial for its functionality. In 3 different constructs using the allergens Bet v 1, Fel d 1 and OVA positioning DCpep C-terminally, between the allergen and the his-tag (as done in the

original publication [203]) resulted in the abolishment of the binding enhancement. N-terminally located DCpep was always functional, but at different extents.

We also tried to get some insight about the binding of DCpep to mdDCs by looking at binding kinetic on-cell. Unfortunately the experiment failed and we were not able to detect any difference in the binding of DCpep bearing and non-bearing constructs using the Quartz Crystal Microbalance (QCM) device Attana Cell™ 200. We can only speculate on the reasons behind it: the insufficient number of epitopes, the reduced size of the constructs used (about 20 kDa), a negative influence of the fixation procedure or too weak on-rates are only a few of the possible explanations that need to be ruled out in future experiments.

Detection of antibody affinity for cell-bound epitopes

Within the troublesome framework of device set-up and proof-of concept investigations we could, however, achieve a success. We developed a method that for the first time allowed measuring the affinity of antibodies for cell-bound epitopes. This achievement resulted from the difficulties that we had in the obtainment of a detectable signal from the Attana Cell™ 200 for cell-protein interactions that were evident in flow cytometry and confocal microscopy. Briefly, the developed method consists in the stable transfection of HeLa cells with the membrane epitope of interest, followed by the selection of highly positive clones by fluorescence-activated cell sorting and the transfer of the cells onto the quartz crystal of the device by cytospin and fixation with paraformaldehyde. This method reflects some limitations that, to our opinion, are not going to be easy to overcome. First, the investigation of most cell-antibody interactions will require transfection, for the simple reason that a significant number of epitopes (roughly 100'000-200'000 per cell) is needed to obtain a robust signal. Such receptor densities are rarely observed in primary cells [232]. Second, cells need to be fixed on the sensor surface to be kept in place, and fixation procedures may influence the binding kinetic. Third, cell-bound epitopes appear to be more sensitive to the regeneration conditions applied for the removal of bound-antibodies, which is necessary for multiple measurements using the same surface. In our case this resulted in significant binding losses, which limited the number of curves that could be generated.

All these drawbacks are at least partially compensated by the great advantage of measuring antibody affinities on whole cells. The cells can either be cultivated directly on the sensor chip or transferred by cytospin by means of straight-forward procedures without purification steps. This is particularly convenient for membrane-receptors, where a laborious and sometimes impossible purification can be avoided. In addition, maintaining the integrity of the cell allows affinity measurements in more authentic settings that reflect the *in vivo* situation more closely.

Conclusions and outlook

The contribution of this work to the scientific progress is dual. From one side it provides a new technical tool that allows the measurement of antibody affinity for cell-bound epitopes. After the necessary optimization this approach may give a considerable input to the way new binders and antibodies are identified and investigated.

From the other, it shed some more light on two of the main strategies that could radically change the way how allergy (but also cancer) immunotherapy is performed: first, it reveals some limitations of Dendritic Cell targeting therapies, with a special focus on the use of the Dendritic Cell targeting peptide DCpep *in vivo*. Second, it identifies hydroxyapatite microspheres as a potential low-inflammatory adjuvant that can be employed for allergy immunotherapy.

To get a better and conclusive picture further investigation is needed. We would consider the following analyses as a priority: First, the identification of the binding partner for DCpep and a deeper investigation of the fate of DCpep targeted constructs *in vivo*. Second, a deeper evaluation of the immunotherapeutic potential of hydroxyapatite microparticles applied subcutaneously *in vivo*, considering a wider range of therapeutic protocols and dosages.

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Courses and contributions

Credit points

Credits	Lecture / Course	Location, Year
2 CP	MIM Introductory Course	Zurich, 2010
2 CP	Immunology Lectures (SIAF, Prof. Mübeccel Akdis)	Davos, 2011
2 CP	LTK Module 1: Introductory Course in Laboratory Animal Science	Zurich, 2012
1 CP	European Academy of Allergy and Clinical Immunology Congress (Poster)	Geneva, 2012
4 CP	Glycobiology (ETH, Prof. Markus Aebi)	Zurich, 2012
1 CP	World Immune Regulation Meeting VIII (Oral Presentation)	Davos, 2014

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Kunzler, M., et al. (2010). "Biotoxicity assays for fruiting body lectins and other cytoplasmic proteins." Methods Enzymol 480: 141-150

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Garbani, M. and R. Crameri. "Novel vaccines for allergy immunotherapy." MIM Introductory Course, Zurich, Switzerland (16.9.2010).

Garbani, M., et al. "DC-targeting and improved cell penetration in immunotherapy." Wolfsberg Meeting of Swiss PhD Students in Immunology, Ermatingen, Switzerland (2.4.2012).

Garbani, M., et al. "Dendritic cells targeting and improved cell penetration in allergen-specific immunotherapy." MIM Retreat, Davos, Switzerland (4.9.2012).

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Garbani, M. "PhD Committee Meeting 1: Direct Targeting of Dendritic Cells as a novel concept for allergen-specific immunotherapy." (6.4.2011)

Garbani, M. (18.3.2013). "PhD Committee Meeting 2: Dendritic cell targeting in immunotherapy."

Garbani, M. (29.7.2014). "PhD Committee Meeting 3: Mineral and natural adjuvants for immunotherapy."

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Garbani, M. "Targeting (Dendritic) Cells." (15.12.2010)

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