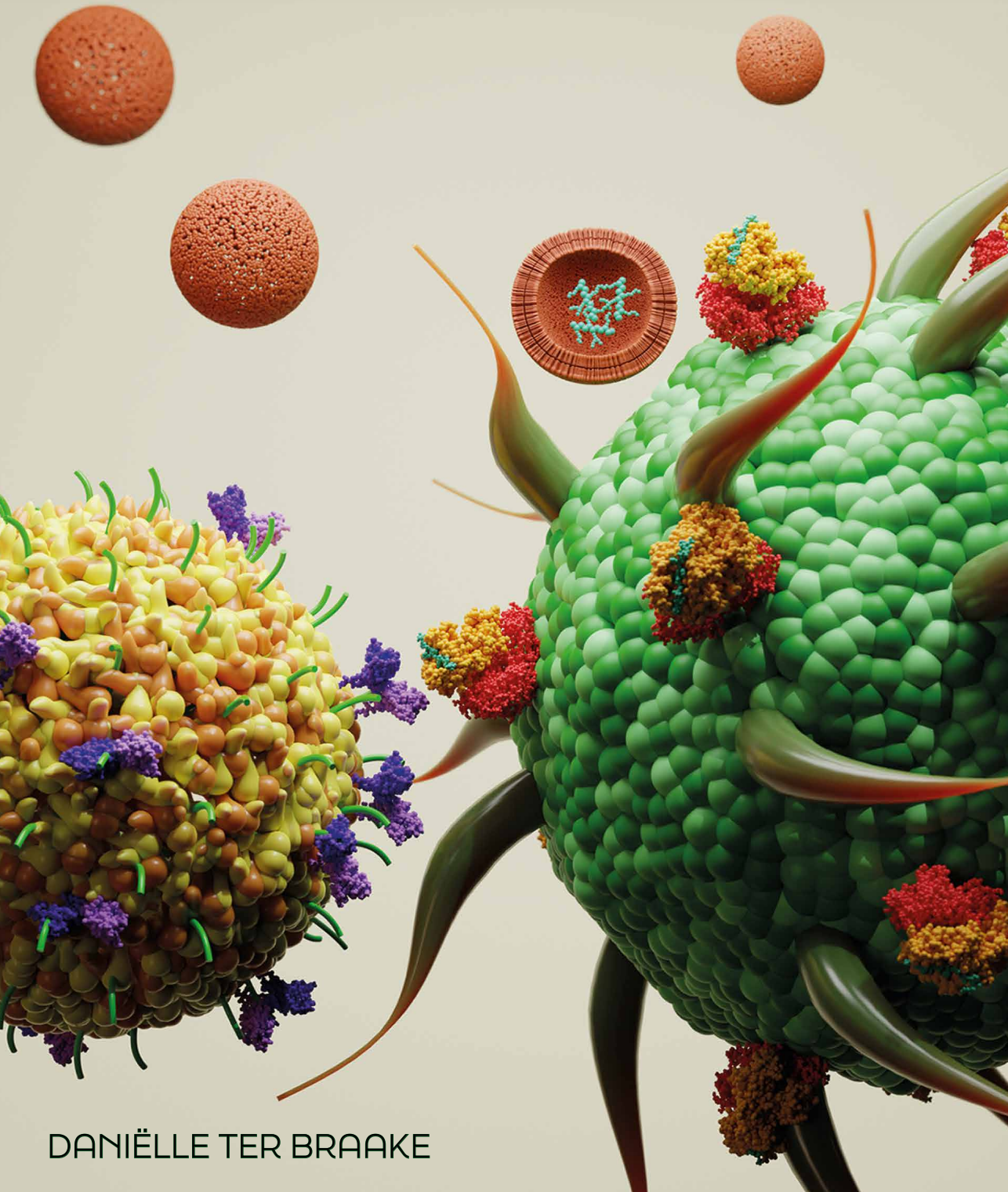
ANTIGEN-SPECIFIC IMMUNE TOLERANCE THROUGH DENDRITIC CELL MODULATION



DANIËLLE TER BRAAKE

Antigen-Specific Immune Tolerance through Dendritic Cell Modulation

Daniëlle ter Braake

Colofon

ISBN: 9789039376744
DOI: 10.33540/2255

The research described in this thesis was conducted at the Division of Immunology, Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

Cover Design: Daniëlle ter Braake
Thesis Layout: André Tuller
Printing: Herman Media Almelo

Printing of this thesis was financially supported by the Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University.

Antigen-Specific Immune Tolerance through Dendritic Cell Modulation

Antigeen-specifieke immuun tolerantie via dendritische cel modulatie
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof. dr. H.R.B.M. Kummeling
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

donderdag 21 mei 2024 des middags te 12.15 uur

door

Daniëlle ter Braake

geboren op 5 maart 1995
te Almelo

Promotoren
Prof. dr. F. Broere
Prof. dr. W. van Eden

Co-promotor
Dr. N. Benne

Beoordelingscommissie
Prof. dr. J. Garssen
Prof. dr. C. Hilkens
Prof. dr. J.M. van Laar
Prof. dr. M.A. Tryfonidou
Prof. dr. F. van Wijk

Table of Contents

General Introduction

General Introduction

The immune system is incredibly fine-tuned and impeccably designed to protect the body from foreign and dangerous invaders while ensuring that it doesn't respond to self-antigens[1,2]. This is called immune tolerance. When self-tolerance mechanisms fail, your immune system will respond to self-antigens, possibly resulting in the development of a plethora of autoimmune diseases, such as rheumatoid arthritis.

The Immune System

The immune system protects the body against a myriad of invading pathogens. It consists of the innate and adaptive immune systems that work collaboratively to detect, neutralize, and generate immunological memory against exogenous and endogenous pathogens[3]. Furthermore, the immune system is very adept at recognizing our own antigens, called autoantigens, thereby avoiding unwarranted immune responses[1,2]. The two parts of the immune system are very distinct from each other, yet serve the same goal of maintaining immune homeostasis and fighting off pathogens. The innate immune system is non-specific, acts rapidly, and does not require previous exposure to the pathogen to become activated[4], whilst the adaptive immune system provides a specific response to pathogens[5]. This immune response is more deliberate and requires the recognition of pathogenic antigens, either due to prior exposure or vaccination. Immunological memory is another remarkable feat of the adaptive immune system, allowing for a rapid immune response upon subsequent encounters with a pathogen.

The first physical barriers pathogens encounter are the skin and mucosal membranes that in most cases prevent them from entering the body[6,7]. The protective functions of these barriers are supported by various chemical substances that have antimicrobial properties, such as saliva[8] and tears[9]. These chemical barriers contain, for example, lysozyme[10], defensin[11], complement proteins[12,13], and antimicrobial peptides (AMPs)[14,15]. However, certain pathogens are extremely adept at evading these first barriers and can invade the host. In the body, the pathogen encounters the innate immune system's cellular defense, including natural killer (NK) cells[16–18] and phagocytes. The main cells that can phagocytose are neutrophils[19–21] and antigen-presenting cells (APCs), such as macrophages and dendritic cells (DCs)[22,23]. The recognition of a pathogen by phagocytes happens through the binding of the pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) with pattern recognition receptors (PRRs) on the cell surface of APCs. PAMPs and DAMPs are conserved molecular patterns that are present in pathogens but absent in the host[24,25]. Toll-like receptors (TLRs) are a class of PRRs that can be found on the surface of phagocytes, or, in the case of TLR3, TLR7, and TLR9, within endosomes of these cells[26–29]. TLRs can recognize a wide variety of PAMPs, including bacterial lipopolysaccharides (LPS)[30] or viral ribonucleic acid (RNA)[31]. TLR activation triggers intracellular signaling pathways, resulting in the production of pro-inflammatory signaling molecules called cytokines and interferons, which in turn promote immune cell activation and recruitment.

The presence of PRRs on the surface of DCs not only allows these cells to detect invading pathogens or cellular stress, thereby rapidly initiating an immune response[26], but it also enhances the DC's ability to capture and process foreign antigens, something they are extremely adept at[32]. For the presentation of antigens, DCs make use of major histocompatibility complex (MHC) molecules. MHC molecules are glycoproteins and in humans are also known as human leukocyte antigens (HLA)[33]. There are two classes of MHC molecules, MHCI and MHCII. MHC class I molecules are found on the surface of most nucleated cells in the body. The peptide-binding groove of MHCI molecules is typically 8-10 amino acids in length, accommodating peptides derived from endogenous proteins.

Antigens presented on MHCI can activate $CD8^+$ cytotoxic T cells. MHC class II molecules are predominantly expressed on professional APCs. Unlike MHCI, MHCII molecules have both an α and β transmembrane chain, making their peptide-binding groove more extensive. This allows them to present longer peptides, typically 13-25 amino acids in length, that are generally derived from exogenous proteins. Antigens presented on MHCII can activate $CD4^+$ T cells[34].

Specialized subsets of DCs can present exogenous antigens on MHCI molecules through a process called cross-presentation[35–37]. This leads to the activation of $CD8^+$ cytotoxic T cells by DCs that are not infected themselves. $CD8^+$ T cells, also known as cytotoxic T cells, can directly kill infected or abnormal cells by releasing vesicles containing perforin and granzyme, inducing cell death, also known as apoptosis in target cells[38]. $CD8^+$ T cells are involved in cellular immune responses against intracellular pathogens but also cancer cells. Cross-presentation of autoantigens that are naturally occurring in the host is essential for the depletion of auto-reactive cytotoxic $CD8^+$ T cells that have escaped central tolerance mechanisms[39–41].

$CD4^+$ T cells, also known as helper T cells, also play an important role in coordinating the adaptive immune response. They can regulate and support both cellular and humoral immune responses[42]. Upon activation by an APC, naïve $CD4^+$ T cells can differentiate into different types of T helper cells, depending on the cytokines, such as interferons (IFN) and interleukins (IL), secreted by the APC in response to antigen recognition[42–45]. The main subsets are T helper 1 (Th1), Th2, Th17, and regulatory T cells (Tregs). Th1 cells are induced through the release of IL-12 and support cellular immunity by activating macrophages (through IFNγ) and $CD8^+$ T cells, enhancing their ability to clear intracellular pathogens. Th2 cells are induced through the release of IL-4 and promote humoral immunity by boosting antibody production by plasma cells, which in turn can target extracellular pathogens. Th17 cells are induced through the release of IL-17 and play a role in inflammation and protection against extracellular bacteria and fungi[46,47].

Tregs are a specialized subset of $CD4^+$ T helper cells. These are essential for the inhibition of (exaggerated) immune responses and for promoting and maintaining immune tolerance towards self-antigens, thereby preventing autoimmune diseases[48,49]. They can achieve immunosuppression through various mechanisms, including the secretion of anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGFβ), cell-to-cell-contact through surface receptors, and metabolic disruption of effector T cells, for example through indoleamine 2,3-dioxygenase (IDO)-mediated depletion of tryptophan. Tregs can be broadly divided into two main subsets: natural Tregs (nTregs) and induced Tregs (iTregs). nTregs are commonly $CD4^+$ and are generated in the thymus during T cell development. They arise from the positive selection of thymocytes with high-affinity T cell receptors (TCRs) for self-antigens that are presented in the thymus. nTregs express high levels of CD25 (IL-2Rα), L-Selectin (CD62L), glucocorticoid-induced TNFR-related protein (GITR), and low levels of CD127. iTregs exert the same functions as nTregs, with the main difference between both subsets being that iTregs differentiate from naïve $CD4^+$ T cells in secondary lymphoid tissues, such as the spleen and lymph nodes. The activation of iTregs can occur in a variety of ways such as 1) by encountering an antigen in the presence of immunomodulators or the absence of co-stimulation[50,51], 2) via signals secreted by activated DCs and naturally occurring tolerogenic DCs localized in the lamina propria of the small intestine or mesenteric lymph nodes, and 3) during chronic inflammation. The transcription factor forkhead box protein P3 (FoxP3) is the master regulator of conventional nTreg and iTreg development and function and therefore is a good discriminatory protein to identify the abovementioned Tregs[52–54]. However, there is another subset of iTregs that does not require FoxP3 for their activation or function,

known as Type 1 regulatory T (Tr1) cells. Tr1 cells are a subset of iTregs that transiently express FoxP3 and produce high levels of the immunosuppressive cytokine IL-10[55]. IL-10 is vital for the regulation of a variety of immune processes, including the downregulation of co-stimulatory molecules and MHCII, modulation of APCs, prevention of the secretion of inflammatory mediators, inhibition of cytokine secretion by effector T cells, inducing T cell anergy, and promoting B cell differentiation and survival[56]. Furthermore, these cells secrete high levels of TGFβ, an intermediate amount of IL-5, and a low amount of IL-2 and IFNγ[57]. Upon Tr1 cell activation through their TCRs, these cells are able to express CD28, CD69, CTLA4, CD25, IL-2Rβγ, and CD40L in normal amounts[58]. Tr1 cells can also express chemokine receptors that are normally associated with Th1 cells (CXCR3 and CCR5) and Th2 cells (CCR3, CCR4, and CCR8), allowing them to migrate to the same tissue sites as effector T cells, resulting in a localized suppressive immune response[59,60]. The polarization of T cells upon activation by dendritic cells is visualized in *figure 1*.

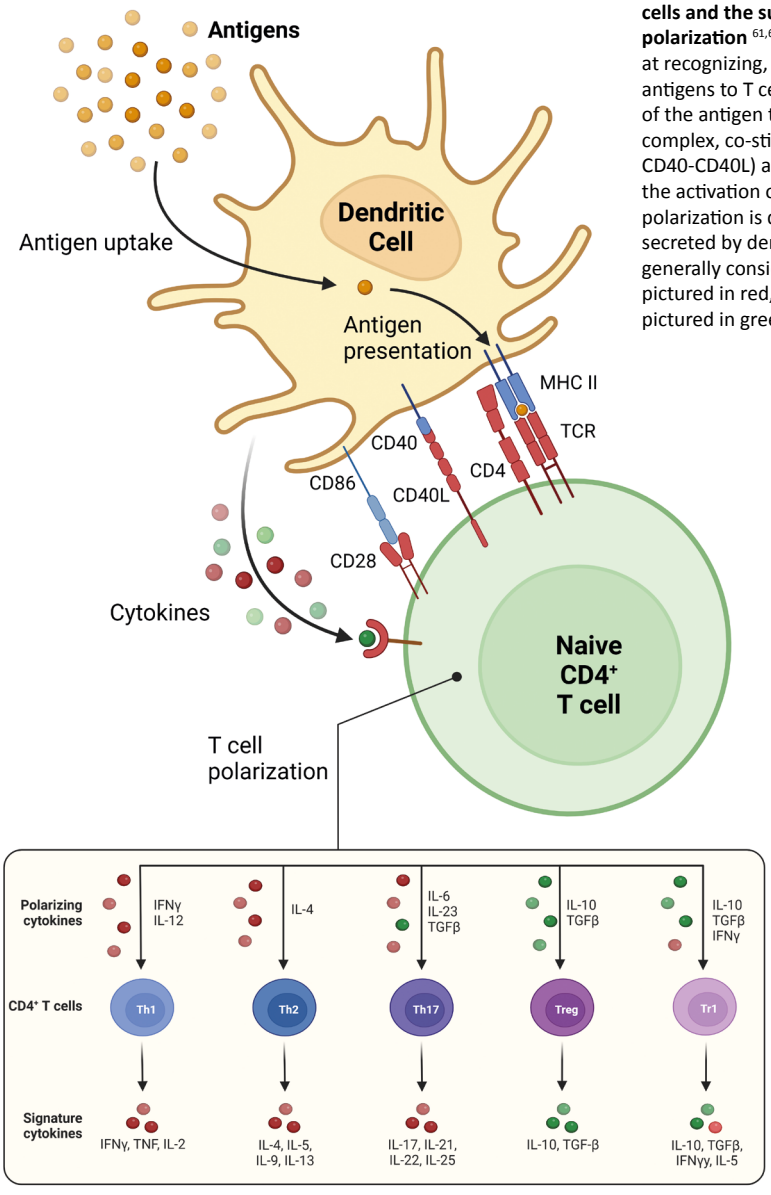


Figure 1. Antigen-presentation by dendritic cells and the subsequent T cell polarization [61,62]. Dendritic cells are very adept at recognizing, processing, and presenting antigens to T cells. Besides the presentation of the antigen through the MHCII-antigen-TCR complex, co-stimulatory signals (CD86-CD28 & CD40-CD40L) and cytokines are necessary for the activation of naïve CD4⁺ T cells. T-cell polarization is determined by cytokines secreted by dendritic cells. Cytokines that are generally considered pro-inflammatory are pictured in red, anti-inflammatory cytokines are pictured in green.

The immune system goes rogue

The immune system maintains a delicate balance between recognizing and attacking foreign antigens whilst ignoring the host's self-antigens. However, when this intricate protection system malfunctions, the immune system will start to treat self-tissues as pathogens and will respond to them accordingly, resulting in autoimmune and chronic inflammatory diseases, such as multiple sclerosis (MS), Type 1 Diabetes (T1D) and rheumatoid arthritis. The precise causes of autoimmune diseases are not fully understood, but it is believed to result from a combination of genetic[63,64], environmental[65–67], and hormonal factors[68–70].

The HLA system plays a fundamental role in the immune system's regulation and identification of self and non-self antigens. This system is controlled by genes located on chromosome 6 and encodes cell surface molecules that are specialized in presenting antigens to TCRs on T cells[71]. HLA types have significant implications for the immune system's ability to recognize and respond to foreign antigens. Previous studies have shown that variations in HLA genes are linked to increased susceptibility to autoimmune diseases[72,73]. Genome-wide association studies (GWAS) have emerged as a powerful tool to better understand the genetic basis of many autoimmune diseases, allowing researchers to look further than HLA genotypes. GWAS is a high-throughput genotyping method focusing on single-nucleotide polymorphisms (SNPs) scattered throughout the human genome. In these studies, the genetic profiles of healthy individuals are compared to those of patients with autoimmune diseases, allowing for the identification of genetic variants that are more prevalent in patients suffering from autoimmune diseases[74,75].

Whilst genetics predispose to the development of autoimmune diseases, environmental triggers often result in the exhibition of the disease. Environmental factors vary and include diet, stress, pollution, smoking, medications, and infections[65–67]. Infections, and in particular viral infections, have been linked to the development of autoimmune diseases through a process called molecular mimicry[76–79]. Molecular mimicry is the phenomenon in which similar structures are shared between self and non-self antigens, resulting in the immune system's inability to distinguish between the two. Together, these factors contribute to increased susceptibility to the development of autoimmune diseases.

Rheumatoid Arthritis

One of the most common autoimmune diseases is rheumatoid arthritis, affecting approximately 0.5-1% of the global adult population. Rheumatoid arthritis is a chronic autoimmune disease characterized by inflammation of the synovial lining of the joints, leading to stiffness, swelling, musculoskeletal pain, and joint deformity. The exact cause of rheumatoid arthritis is not yet fully understood[80–82].

When looking at genetic factors, it has been shown that HLA-DRB1, HLA-DRB1*04 in particular, is strongly associated with increased susceptibility to rheumatoid arthritis. HLA-DR is equivalent to the mouse MHC class II[83–86]. As with most autoimmune diseases, environmental factors play a major role in the development of rheumatoid arthritis[87]. Several environmental stressors, such as smoking and infections, can affect the activity of peptidyl arginine deiminases (PAD) enzymes. These enzymes are responsible for a post-translational modification called citrullination, in which the positively charged amine groups from arginine are removed, forming citrulline[88,89]. In rheumatoid arthritis, the immune system can mistakenly recognize some citrullinated proteins as non-self, leading to the activation of autoreactive Th1 and Th17 cells and the production of autoantibodies such as anti-citrullinated protein antibodies (ACPAs) by B cells. When autoantibodies bind to citrullinated proteins they form immune complexes, which perpetuate the inflammatory response and contribute to the joint destruction observed in rheumatoid arthritis[90,91].

Treatment for Rheumatoid Arthritis

Current treatments for rheumatoid arthritis focus on symptom management, prevention of joint damage, and improving the patient's quality of life. Treatment strategies often involve a combination of medication, lifestyle changes, and, in some cases, surgical interventions[92,93]. The most commonly used treatment modalities are nonsteroidal anti-inflammatory drugs (NSAIDs)[94,95], disease-modifying anti-rheumatic drugs (DMARDs)[96–102], and glucocorticoids[103–106]. Glucocorticoids, including dexamethasone, exhibit rapid and potent immunomodulatory effects upon administration. This makes glucocorticoids particularly interesting for acute rheumatoid arthritis flare-ups, where quick pain relief is required. They also benefit patients who are waiting for the prescribed DMARDs to reach their maximum efficiency, a process that might take weeks or months. Hereafter, the concentration of glucocorticoids is lowered or glucocorticoid treatment is discontinued, to prevent unwanted side effects[107], such as skeletal muscle atrophy[108].

Contemporary treatment strategies for rheumatoid arthritis, such as those mentioned above, provide much-needed relief for patients, however, they do come with some disadvantages. Due to the heterogeneous nature of rheumatoid arthritis, patients respond differently to the available treatment options[109–112], highlighting the need for personalized approaches. Even when an effective treatment strategy has been found, some patients might develop resistance to certain medications over time, rendering them ineffective[113–116]. This leads to a constant search for new drugs. However, the major disadvantage of current treatment modalities is the non-specific immunosuppression, leaving treated patients with an impaired immune response and therefore a higher susceptibility to infections[106].

Taking this into account, there is a clear need for antigen-specific immune interventions. This not only increases the efficacy of rheumatoid arthritis treatment but also reduces systemic side effects. Antigen-specific therapies can be customized to fit each patient, maximizing the chance of successful treatment. For the generation of antigen-specific therapies, interest is being shown in DCs. DCs are fundamental to the activation of (autoreactive) T and B cells, as their antigen-presentation abilities allow for antigen-specific autoimmune responses to occur[117]. Simultaneously, DCs can influence T cell differentiation based on the environmental cues they have received, which makes them key players in the induction of either a pro-inflammatory or an anti-inflammatory immune response[45].

The exact triggers for DC activation in rheumatoid arthritis are not yet fully understood, however, several key causes have been identified. First is the inflammatory nature of rheumatoid arthritis, in which DCs will become activated by inflammatory environmental triggers in the joints. This creates a positive feedback loop, where activated DCs will produce and secrete pro-inflammatory cytokines, including IL-1, TNFα, and IL-6, that further fuel the inflammatory environment. Second, DCs can present self-antigens and citrullinated peptides to T cells, resulting in a pool of autoreactive T cells[81,117–119].

Tolerogenic Dendritic Cells

Given the fundamental role of DCs in the regulation of the immune response, they have emerged as promising targets for novel therapeutic interventions. Various strategies focus on the modulation of DCs, with the ultimate aim of inducing immune tolerance to self-antigens. A specialized subset of DCs, known as tolerogenic DCs (tolDCs) are of particular interest in this field. These cells possess immunosuppressive properties and can actively induce immune tolerance. They are characterized by the expression of low levels of co-stimulatory molecules, reduced production of pro-inflammatory cytokines and an increase in the secretion of anti-inflammatory cytokines, resulting in the induction of Tregs and activation of immune regulatory pathways[120–123]. TolDCs are generated through various

methods, such as pharmacological modulation[124–134], genetic engineering[135], or *ex vivo* culture protocols[136,137]. These interventions collectively aim to boost the ability of tolDCs to dampen immune responses and promote tolerance. The immunomodulators that are most discussed in the context of this thesis are retinoic acid, dexamethasone, and vitamin D3.

Retinoic Acid

Retinoic acid (RA) is a naturally occurring metabolite of Vitamin A (retinol). RA plays an important role in regulating a wide variety of biological processes, including cell growth, differentiation, and homeostasis. Within a cell, RA is derived from retinol via a two-step enzymatic pathway. In the first step, retinol is oxidized to retinaldehyde (retinal). This requires alcohol dehydrogenases (ADH-1,-4,-5). Subsequently, retinal is oxidized to RA by retinal dehydrogenases (RALDH). In the cytoplasm, synthesized RA is catabolized by the CYP26 class of P450 enzymes[138–140].

The presence of RA promotes a tolerogenic phenotype in DCs, as identified by a reduced expression of co-stimulatory molecules (e.g., CD40, CD86) and increased expression of inhibitory molecules (e.g., programmed death-ligand 1 (PD-L1)) that promote immunosuppression. RA-induced tolDCs tend to produce anti-inflammatory cytokines IL-10 and TGF-β, while producing lower levels of pro-inflammatory cytokines, such as IL-12 and IL-6[139,141]. Together these changes help to suppress effector T cell responses and promote the differentiation and expansion of Tregs[141,142], which are essential for maintaining immune homeostasis and preventing autoimmune diseases such as rheumatoid arthritis. RA-treated tolDCs also upregulate metabolic molecules that contribute to their tolerogenic function, including indoleamine 2,3-dioxygenase (IDO)[143]. IDO activity results in the depletion of tryptophan and the subsequent accumulation of tryptophan metabolites, such as kynurenine. This tryptophan depletion directly affects the survival of effector T cells, which are dependent on tryptophan for survival and function, whilst leaving Tregs unaffected, as these cells are less dependent on tryptophan for their function. This helps create an immunosuppressive environment that favors Treg expansion[144,145]. Additionally, the tryptophan metabolite kynurenine has immuno-suppressive functions in itself[146]. It can act as an endogenous ligand for the aryl hydrocarbon receptor (AHR), a transcription factor that can regulate immune responses. When kynurenine binds to AHR, it forms an active complex that can translocate to the nucleus and induce the expression of target genes, including genes that encode pro-apoptotic factors, resulting in T cell apoptosis[147,148]. RA-induced tolDCs also produce retinaldehyde dehydrogenases (RALDHs), enzymes responsible for the synthesis of reti-noic acid, which further reinforces the generation of tolDCs[139].

Dexamethasone

Dexamethasone (dex) is a synthetic glucocorticoid, that has been widely used for its potent anti-inflammatory and immunosuppressive effects. To assert its effect, dex binds to glucocorticoid receptors (GRs), which are a type of nuclear hormone receptor, consisting of several domains, including the N-terminal domain, the DNA-binding domain, the hinge region, and the ligand-binding domain. GRs are intracellular proteins that are located in the cell cytoplasm in an inactive state[149]. In this state, they're bound to chaperone proteins, including heat shock proteins (HSPs) such as HSP90, forming an inactive complex[150,151]. When dex enters the cytoplasm and encounters this complex, it will bind to the ligand-binding domain with high affinity, which induces a conformational change in the GR, leading to dissociation from its chaperone proteins and thereby activating the GR allowing the GR to translocate to the nucleus. Here, the activated GR homodimerizes, resulting in the formation of a complex that binds to specific DNA sequences, known as glucocorticoid response elements (GREs) in the promoter regions of target genes. This interaction facilitates the recruitment of transcriptional

co-activators or co-repressors, which help determine whether a target gene is activated or suppressed[152]. These dex-induced transcriptional effects result in the suppression of pro-inflammatory genes, such as those encoding cytokines (like IFNγ and TNFα), chemokines, and adhesion molecules[153–155]. At the same time, dex can induce the upregulation of anti-inflammatory molecules and proteins that mediate immunosuppression, such as IL-10[156].

Dex-induced tolDCs show a reduced expression of MHCII molecules on their cell surface, impairing the capacity to present antigens to T cells[157], which is beneficial when DCs are presenting auto-antigens in the context of rheumatoid arthritis. Additionally, co-stimulatory molecules are down-regulated in dex-induced tolDCs, limiting their ability to fully activate T cells and resulting in effector T cell anergy[124,158]. Dex also suppresses the production of pro-inflammatory cytokines by DCs, further dampening the immune response. Furthermore, dex-induced tolDCs can efficiently induce regulatory T cells through the production of anti-inflammatory cytokines, such as IL-10 and TGFβ. Together with the elevated expression of PD-L1 and IDO seen in dex-induced tolDCs, this contributes to the differentiation and expansion of Tregs[159–162]. Furthermore, dex can interact with the AHR pathway. As both AHR and GR are ligand-activated transcription factors, their binding to specific co-activators determines the transcriptional outcomes. GRs that have bound dex could outcompete AHR for shared co-activators, reducing AHR-mediated gene transcription and activation. Additionally, dex may promote the degradation of AHR protein through AHR-GR crosstalk, facilitating dex's ability to modulate immune responses and mitigate inflammatory processes[163,164]. This crosstalk between AHR and GRs highlights the intricate interplay between various signaling pathways.

Vitamin D3

Vitamin D3 is a well-known fat-soluble vitamin that can be supplemented through diet or synthesized by the skin through exposure to sunlight. When it arrives in the liver, it will be hydroxylated into 25-hydroxyvitamin D3 [25(OH)D3], which is the major circulating form of vitamin D3 in the body. Subsequently, 25(OH)D3 undergoes another hydroxylation step, mainly occurring in the kidneys, to form the biologically active 1,25-dihydroxy vitamin D3 (1,25(OH)2D3; VitD3)[165].

VitD3-induced tolDCs display a similar tolerogenic phenotype[158,166–170] as dex-induced or RA-induced tolDCs. This phenotype is characterized by low levels of co-stimulatory molecules and an increased expression of PD-L1 compared to activated DCs. Moreover, vitD3-induced tolDCs display higher levels of IDO and secrete more anti-inflammatory IL-10 and TGFβ, contributing to Treg differentiation and expansion. In not yet fully differentiated DCs, VitD3 has been shown to initiate an early transcriptional reprogramming of metabolic pathways that favors oxidative phosphorylation (OXPHOS)[171]. This is accompanied by increased aerobic glycolysis. Furthermore, glucose availability and glycolysis, which is controlled by the PI3K/Akt/mTOR pathway, can dictate the induction and maintenance of VitD3-tolDC phenotype and function[172].

Clinical trials using tolerogenic dendritic cells

The interest in tolDCs as therapeutic agents has been demonstrated by their worldwide use in clinical trials for various autoimmune diseases, including Type 1 Diabetes (NCT00445913, NCT02354911173), Multiple Sclerosis (MS; NCT02618902, NCT02903537174) and rheumatoid arthritis (NCT01352858175, NCT05251870). These trials aim to assess the feasibility of tolDC therapy for the treatment of auto-immune diseases, identify appropriate patient populations, and determine the optimal cell dose, route of administration, and treatment duration. The first-ever clinical trial that made use of tolDCs for restoring immune tolerance in rheumatoid arthritis was performed in 2015 by Benham et al.

(ACTRN12610000373077[176]). In this exploratory phase I trial, tolDCs were generated using NF-κB suppression with the irreversible NF-κB inhibitor Bay11-7082 and exposed to citrullinated peptides for 48 hours and injected intradermally in patients that were ACPA positive. Within one month of treatment, a single injection of these autologous tolDCs was proven to be safe and results indicated a decrease in effector T cells (defined in the study as $CD4^+$ $CD25^+$ $CD127^+$) and an increase in regulatory T cells (defined in the study as $CD4^+$ $CD25^+$ $CD127^+$). When looking at the disease activity score 28(DAS28), which is a common metric for the evaluation of the severity of rheumatoid arthritis, it was found that these scores were decreased in some patients.

One of the most recent ongoing clinical trials in rheumatoid arthritis patients using autologous tolDCs is the TOLERANT trial (NCT05251870) at University Medical Center Utrecht (UMCU), Utrecht, The Netherlands. In this Phase I/II clinical trial, autologous mature Dex-VitD3 tolerogenic monocyte-derived DCs are exposed to the B29 peptide of heat shock protein 70 (HSP70). HSPs are immunogenic, ubiquitously expressed, and are shown to be upregulated in rheumatoid arthritis[177]. The characteristic that makes HSP-derived peptides so interesting in the context of rheumatoid arthritis treatment is that these peptides can be recognized by Tregs, and, as rheumatoid arthritis is a disease where the autoantigens are largely unknown, this offers promising new possibilities for antigen-specific immunotherapy. In a 2012 study by van Herwijnen et al., it was shown that B29-induced Tregs were able to suppress established proteoglycan-induced arthritis (PGIA) in mice and that B29 can be presented by MHCII on DCs [178], highlighting the therapeutic abilities of this peptide. At the moment of writing, the TOLERANT trial is recruiting, and its results have the potential to significantly contribute to the research field.

TolDC therapy holds significant promise for the future of immunotherapy for rheumatoid arthritis. Nevertheless, several challenges need to be addressed for successful clinical implementation. The generation of autologous tolDCs has been proven to be difficult due to the inherent heterogeneity of the tolDC population and the lack of standardized protocols for its generation[179,180]. This results in a time-consuming and costly process, in which manufacturers need to meet the requirements for good manufacturing processes (GMP), whilst the variability in the quality and potency of tolDCs can impact the reproducibility and consistency of therapeutic outcomes. To circumvent the challenges associated with tolDC immunotherapy, the use of nanoparticles has emerged as a promising treatment strategy[181]. Nanoparticles can work as drug delivery vehicles, with the ability to deliver antigens to APCs, which in turn present these antigens on their cell surface and thereby facilitate the activation of antigen-specific Tregs. Nanoparticles are easily modifiable, allowing for the co-encapsulation of immunomodulators and antigens. This holds interesting new possibilities for *in vivo* immune tolerance induction.

Nanoparticles for the restoration of immune tolerance

Nanoparticles can be made of a variety of materials, including polymers[182], metals[183], lipids[184], proteins[185], or a combination of these. For the use of nanoparticles in the treatment of autoimmune diseases, liposomes have gained interest. Liposomes consist of one or more lipid bilayers that encapsulate an aqueous core[186]. This allows them to encapsulate a wide range of both hydrophobic and hydrophilic compounds. Their physiochemical properties are determined by the liposomal composition and formulation process, allowing them to be modified in a way that facilitates immune tolerance induction. For tolerance induction, a favorable approach would be the mimicking of apoptotic cells by using phosphatidylserine in liposomes[187,188], as these can be efficiently taken up and processed via tolerance-promoting effector mechanisms in the spleen and liver. The negatively charged phospholipid phosphatidylserine (PS) is expressed on the cell surface of apoptotic cells[189], leading

researchers to study this phospholipid for use in liposomes. In a 2018 study by Benne et al., the efficiency of negatively charged PS- and phosphatidylglycerol (PG)-containing liposomes was assessed in a mouse model for atherosclerosis[190]. Here, it was found that anionic PG-containing liposomes are able to facilitate greater antigen-specific Treg responses than PS-containing liposomes. Whilst it had been reported that empty PS-containing liposomes do elicit non-specific immune responses[188,191], empty PG-containing liposomes in the atherosclerosis study showed no effect on the disease, suggesting that the effect of these liposomes is antigen-specific.

In a 2021 study, Krienke et al. showed that the encapsulation of MS-relevant antigen mRNA in lipid nanoparticles resulted in the uptake of these particles by splenic $CD11c^+$ DCs *in vivo* after intravenous injection[192]. In the absence of co-stimulatory molecules, these DCs were able to present antigens to T cells, leading to antigen-specific $FoxP3^+$ Treg expansion and the prevention and treatment of experimental autoimmune encephalomyelitis (EAE) in mice. Furthermore, liposomes are ideally suited for the encapsulation of immunomodulators, such as the hydrophobic dex or the hydrophilic RA. Previous research using nanoparticles that encapsulated both an EAE-relevant antigen and dex showed significant improvements in EAE treatment compared to nanoparticles containing either the antigen or dex[193]. In a model for diabetes, it was shown that nanoparticles that co-encapsulated RA and disease-relevant insulin-peptide could prevent diabetes development in non-obese diabetic (NOD) mice[194]. Combining these studies highlights the potential for the use of liposomes for efficient antigen-specific immunotherapy for the treatment of autoimmune diseases.

Tolerogenic nanoparticles for the induction of antigen-specific immune tolerance to Cas9

Given the therapeutic potential of tolerogenic nanoparticles for the treatment of autoimmune diseases, interest has also emerged from the genetic engineering field. The discovery and development of CRISPR-Cas9 technology has revolutionized gene-editing research, allowing researchers to modify DNA with high levels of precision[195,196]. This opens up many new possibilities for the treatment of genetic diseases, whereby specific disease-causing genetic mutations can be corrected. Successful application of *in vivo* gene editing is facing significant challenges, including the efficient delivery of gene-editing components to the target cells and minimizing off-target side effects. One of the main components of gene editing is the Cas9 protein. This is a bacterial protein that was first identified in *Streptococcus pyogenes*, where it has been known to function as an RNA-guided endonuclease[197]. This allows it to cut DNA at specific locations based on the guidance of a single-guide RNA (sgRNA). The Cas9 protein and the sgRNA form a complex that recognizes and binds complementary DNA sequences, allowing for cleavage and modification of target DNA. However, its bacterial origin prompts the immune system to clear it as soon as it is detected[198,199]. For future research into *in vivo* gene editing, immune evasion by inducing Cas9-specific immune tolerance is a promising strategy, as this could help the Cas9 protein escape immune detection. Liposomal delivery of CRISPR-Cas9 is a relatively novel research field[200], with one study showing *in vivo* efficacy of liposomal delivery of Cas9 mRNA and sgRNAs[201]. Here, researchers show high levels of durable *in vivo* CRISPR-Cas9-mediated gene editing after a single administration. In a recent clinical trial (NCT04601051) for the treatment of transthyretin amyloidosis (ATTR amyloidosis) liposomes were used to encapsulate Cas9 mRNA and sgRNA. These liposomes were intravenously injected to target the misfolded transthyretin (TTR) proteins in tissues, that accumulate in this disease[202]. Researchers found that in a small group of patients with hereditary ATTR amyloidosis, administration of these liposomes was associated with a decrease in serum TTR protein concentrations through the targeted knockout of TTR, highlighting the therapeutic potential of Cas9 mRNA-encapsulating liposomes. There is a long way to go in optimizing *in vivo* delivery of the

necessary components of the CRISPR-Cas9 gene editing system[203,204]. Nevertheless, tolerogenic nanoparticles encapsulating Cas9 (peptide, protein, or mRNA) and, potentially, immunomodulators could provide interesting new insights into the advancement of the field.

Conclusion & aim of the thesis

TolDCs are an innovative and promising immunotherapeutic strategy that offers new possibilities for the treatment of a plethora of autoimmune diseases. Harnessing the unique tolerogenic properties of tolDCs allows for selective modulation of immune responses and restoration of tolerance to self-antigens. The emergence of nanoparticles to induce tolDCs *in vivo* holds great potential for *in vivo* antigen-specific immunosuppression in autoimmune and chronic inflammatory diseases. With ongoing advancements in the field of tolerogenic immune therapy, these treatment modalities have the potential to pave the way for a new era of targeted and safe immunotherapies.

Thesis outline

This thesis aims to explore the possibilities for the use of tolDCs and tolerogenic nanoparticles for the restoration and maintenance of immune tolerance in rheumatoid arthritis, and how this knowledge can be used to advance the field of *in vivo* gene editing using CRISPR-Cas9. In **Chapter 2**, promising strategies for immune tolerance induction by nanoparticles are reviewed. Here, cellular targets and nanoparticulate modification, are discussed. In **Chapter 3**, liposomes that co-encapsulate RA as an immunomodulator and human proteoglycan (hPG), a rheumatoid arthritis-relevant antigen, are analyzed for their use in antigen-specific therapy for autoimmune diseases. In **Chapter 4**, a novel nanoparticle treatment strategy for rheumatoid arthritis is explored, where anionic liposomes that encapsulate hPG linked to dexamethasone, are shown to be able to prevent the development and halt the progression of rheumatoid arthritis in PGIA mouse models. In **Chapter 5**, the use of lipid nanoparticles for the *in vivo* delivery of Cas9 protein is explored. Finally, in **Chapter 6** the findings of this thesis are placed in the context of current literature, thoroughly discussed and new insights are offered for the future of immunotherapy for autoimmune diseases and gene editing.

References

1. Chaplin DD. Overview of the immune response. *Journal of Allergy and Clinical Immunology*. 2010;125(2 SUPPL. 2). doi:10.1016/j.jaci.2009.12.980
2. Farhangnia P, Akbarpour M. Immunological Tolerance. In: *Encyclopedia of Infection and Immunity.* Elsevier; 2022. doi:10.1016/B978-0-12-818731-9.00165-8
3. Marshall JS, Warrington R, Watson W, Kim HL. An introduction to immunology and immunopathology. *Allergy, Asthma and Clinical Immunology.* 2018;14. doi:10.1186/s13223-018-0278-1
4. Turvey SE, Broide DH. Innate immunity. Journal of Allergy and Clinical Immunology. 2010;125(2 SUPPL. 2). doi:10.1016/j.jaci.2009.07.016
5. Bonilla FA, Oettgen HC. Adaptive immunity. *Journal of Allergy and Clinical Immunology.* 2010;125(2 SUPPL. 2). doi:10.1016/j.jaci.2009.09.017
6. Nestle FO, Di Meglio P, Qin JZ, Nickoloff BJ. Skin immune sentinels in health and disease. *Nat Rev Immunol.* 2009;9(10):679-691. doi:10.1038/nri2622
7. Chambers ES, Vukmanovic-Stejic M. Skin barrier immunity and aging. *Immunology*. 2020;160(2):116-125. doi:10.1111/imm.13152
8. Lynge Pedersen AM, Belstrøm D. The role of natural salivary defences in maintaining a healthy oral microbiota. *J Dent.* 2019;80:S3-S12. doi:10.1016/j.jdent.2018.08.010
9. McDermott AM. Antimicrobial compounds in tears. *Exp Eye Res.* 2013;117:53-61. doi:10.1016/j exer.2013.07.014
10. Ferraboschi P, Ciceri S, Grisenti P. Applications of lysozyme, an innate immune defense factor, as analternative antibiotic. *Antibiotics.* 2021;10(12). doi:10.3390/antibiotics10121534
11. Xu D, Lu W. Defensins: A Double-Edged Sword in Host Immunity. *Front Immunol.* 2020;11. doi:10.3389/fimmu.2020.00764
12. Merle NS, Church SE, Fremeaux-Bacchi V, Roumenina LT. Complement system part I - molecular mechanisms of activation and regulation. *Front Immunol.* 2015;6(JUN). doi:10.3389/ fimu.2015.00262
13. Dunkelberger JR, Song WC. Complement and its role in innate and adaptive immune responses. *Cell Res.* 2010;20(1):34-50. doi:10.1038/cr.2009.139
14. Bastos P, Trindade F, da Costa J, Ferreira R, Vitorino R. Human Antimicrobial Peptides in Bodily Fluids: Current Knowledge and Therapeutic Perspectives in the Postantibiotic Era. *Med Res Rev.* 2018;38(1):101-146. doi:10.1002/MED.21435
15. Pasupuleti M, Schmidtchen A, Malmsten M. Antimicrobial peptides: key components of the innate immune system. *Crit Rev Biotechnol.* 2012;32(2). doi:10.3109/07388551.2011.594423
16. Kucuksezer UC, Aktas Cetin E, Esen F, et al. The Role of Natural Killer Cells in Autoimmune Diseases. *Front Immunol.* 2021;12. doi:10.3389/fimmu.2021.622306
17. Yokoyama WM. Natural killer cell immune responses. *Immunol Res.* 2005;32(1-3):317-325.
18. Paul S, Lal G. The molecular mechanism of natural killer cells function and its importance in cancer immunotherapy. *Front Immunol.* 2017;8(SEP). doi:10.3389/fimmu.2017.01124
19. Kumar V, Sharma A. Neutrophils: Cinderella of innate immune system. *Int Immunopharmacol.* 2010;10(11):1325-1334. doi:10.1016/j.intimp.2010.08.012
20. Malech HL, Deleo FR, Quinn MT. The role of neutrophils in the immune system: An overview. *Methods in Molecular Biology.* 2014;1124:3-10. doi:10.1007/978-1-62703-845-4_1
21. Rosales C. Neutrophils at the crossroads of innate and adaptive immunity. *J Leukoc Biol.* 2020;108(1):377-396. doi:10.1002/JLB.4MIR0220-574RR
22. Rosales C, Uribe-Querol E. Phagocytosis: A Fundamental Process in Immunity. *Biomed Res Int.* 2017;2017. doi:10.1155/2017/9042851

23. Uribe-Querol E, Rosales C. Phagocytosis: Our Current Understanding of a Universal Biological Process. *Front Immunol.* 2020;11. doi:10.3389/fimmu.2020.01066
24. Roh JS, Sohn DH. Damage-associated molecular patterns in inflammatory diseases. *Immune Netw.* 2018;18(4). doi:10.4110/in.2018.18.e27
25. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol.* 2007;81(1):1-5. doi:10.1189/jlb.0306164
26. Li D, Wu M. Pattern recognition receptors in health and diseases. *Signal Transduct Target Ther.* 2021;6(1). doi:10.1038/s41392-021-00687-0
27. Duan T, Du Y, Xing C, Wang HY, Wang RF. Toll-Like Receptor Signaling and Its Role in Cell-Mediated Immunity. *Front Immunol.* 2022;13. doi:10.3389/fimmu.2022.812774
28. Lind NA, Rael VE, Pestal K, Liu B, Barton GM. Regulation of the nucleic acid-sensing Toll-like receptors. *Nat Rev Immunol.* 2022;22(4):224-235. doi:10.1038/s41577-021-00577-0
29. Matsushima H, Yamada N, Matsue H, Shimada S. *TLR3-, TLR7-, and TLR9-Mediated Production of Proinflammatory Cytokines and Chemokines from Murine Connective Tissue Type Skin-Derived Mast Cells but Not from Bone Marrow-Derived Mast Cells 1.* Vol 173.; 2004. http://journals.aai.org/jimmunol/article-pdf/173/1/531/1187565/531.pdf
30. Ciesielska A, Matyjek M, Kwiatkowska K. TLR4 and CD14 trafficking and its influence on LPS-induced pro-inflammatory signaling. *Cellular and Molecular Life Sciences.* 2021;78(4): 1233-1261. doi:10.1007/s00018-020-03656-y
31. Dalpke AH, Helm M. RNA mediated Toll-like receptor stimulation in health and disease. *RNA Biol.* 2012;9(6):828-842. doi:10.4161/rna.20206
32. Dzopalic T, Rajkovic I, Dragicevic A, Colic M. The response of human dendritic cells to co-ligation of pattern-recognition receptors. *Immunol Res.* 2012;52(1-2):20-33. doi:10.1007/s12026-012-8279-5
33. Patente TA, Pinho MP, Oliveira AA, Evangelista GCM, Bergami-Santos PC, Barbuto JAM. Human dendritic cells: Their heterogeneity and clinical application potential in cancer immunotherapy. *Front Immunol.* 2019;10(JAN). doi:10.3389/fimmu.2018.03176
34. Wieczorek M, Abualrous ET, Sticht J, et al. Major histocompatibility complex (MHC) class I and MHC class II proteins: Conformational plasticity in antigen presentation. *Front Immunol.* 2017;8(MAR). doi:10.3389/fimmu.2017.00292
35. Jung S, Unutmaz D, Wong P, et al. In Vivo Depletion of CD11c Dendritic Cells Abrogates Priming of CD8 T Cells by Exogenous Cell-Associated Antigens. *Immunity.* 2002;17:211-220.
36. Joffre OP, Segura E, Savina A, Amigorena S. Cross-presentation by dendritic cells. *Nat Rev Immunol.* 2012;12(8):557-569. doi:10.1038/nri3254
37. Gutiérrez-Martínez E, Planès R, Anselmi G, et al. Cross-presentation of cell-associated antigens by MHC class I in dendritic cell subsets. *Front Immunol.* 2015;6(JUL). doi:10.3389/fimmu.2015.00363
38. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol.* 2002;2(10):735-747. doi:10.1038/nri911
39. Kurts C, Robinson BWS, Knolle PA. Cross-priming in health and disease. *Nat Rev Immunol.* 2010;10(6):403-414. doi:10.1038/nri2780
40. Kurts C, Cannarile M, Klebba I, Brocker T. Cutting Edge: Dendritic Cells Are Sufficient to Cross-Present Self-Antigens to CD8 T Cells In Vivo. *The Journal of Immunology.* 2001;166(3):1439-1442. doi:10.4049/jimmunol.166.3.1439
41. Kurts C, Kosaka H, Carbone FR, Miller JFAP, Heath WR. *Class I-Restricted Cross-Presentation of Exogenous Self-Antigens Leads to Deletion of Autoreactive CD8 T Cells.* Vol 186.; 1997. http://rupress.org/jem/article-pdf/186/2/239/1685592/97-0372.pdf

42. Chatzileontiadou DSM, Sloane H, Nguyen AT, Gras S, Grant EJ. The Many Faces of CD4 + T Cells: Immunological and Structural Characteristics. *Int J Mol Sci.* Published online 2020. doi:10.3390/ijms
43. Luckheeram RV, Zhou R, Verma AD, Xia B. CD4 +T cells: Differentiation and functions. *Clin Dev Immunol.* 2012;2012. doi:10.1155/2012/925135
44. Swain SL, McKinstry KK, Strutt TM. Expanding roles for CD4 + T cells in immunity to viruses. *Nat Rev Immunol.* 2012;12(2):136-148. doi:10.1038/nri3152
45. Yin X, Chen S, Eisenbarth SC. Dendritic Cell Regulation of T Helper Cells. *Annu Rev Immunol* 2021. 2021;39:759-790. doi:10.1146/annurev-immunol-101819
46. Mousset CM, Hobo W, Woestenenk R, Preijers F, Dolstra H, van der Waart AB. Comprehensive Phenotyping of T Cells Using Flow Cytometry. *Cytometry Part A.* 2019;95(6):647-654. doi:10.1002/cyto.a.23724
47. Zhu X, Zhu J. CD4 T helper cell subsets and related human immunological disorders. *Int J Mol Sci.* 2020;21(21):1-26. doi:10.3390/ijms21218011
48. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: Mechanisms of differentiation and function. *Annu Rev Immunol.* 2012;30:531-564. doi:10.1146/annurev.immunol.25.022106.141623
49. Rocamora-Reverte L, Melzer FL, Würzner R, Weinberger B. The Complex Role of Regulatory T Cells in Immunity and Aging. *Front Immunol.* 2021;11. doi:10.3389/fimmu.2020.616949
50. Thorstenson KM, Khoruts A. *Generation of Anergic and Potentially Immunoregulatory CD25 CD4 T Cells In Vivo After Induction of Peripheral Tolerance with Intravenous or Oral Antigen 1*. Vol 167.; 2001. http://journals.aai.org/jimmunol/article-pdf/167/1/188/1132853/188.pdf
51. Apostolou I, Von Boehmer H. In vivo instruction of suppressor commitment in naive T cells. *Journal of Experimental Medicine.* 2004;199(10):1401-1408. doi:10.1084/jem.20040249
52. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3 + regulatory T cells in the human immune system. *Nat Rev Immunol.* 2010;10(7):490-500. doi:10.1038/nri2785
53. Li Z, Li D, Tsun A, Li B. FOXP3+ regulatory T cells and their functional regulation. *Cell Mol Immunol.* 2015;12(5):558-565. doi:10.1038/cmi.2015.10
54. Rudensky AY. Regulatory T cells and Foxp3. *Immunol Rev.* 2011;241(1):260-268. doi:10.1111/j.1600-065X.2011.01018.x
55. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M, Gagliani N. The Biology of T Regulatory Type 1 Cells and Their Therapeutic Application in Immune-Mediated Diseases. *Immunity*. 2018;49(6):1004-1019. doi:10.1016/j.immuni.2018.12.001
56. Subramanian Iyer S, Cheng G. Role of Interleukin 10 Transcriptional Regulation in Inflammation and Autoimmune Disease. *Crit Rev Immunol.* 2012;32(1):23-63.
57. Chihara N, Madi A, Karwacz K, Awasthi A, Kuchroo VK. Differentiation and characterization of Tr1 cells. *Curr Protoc Immunol.* 2016;2016:3.27.1-3.27.10. doi:10.1002/0471142735.im0327s113
58. Grazia Roncarolo M, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev.* 2006;212(1):28-50. doi:10.1111/j.0105-2896.2006.00420.x
59. Roncarolo MG, Gregori S, Bacchetta R, Battaglia M. Tr1 Cells and the Counter-Regulation of Immunity: Natural Mechanisms and Therapeutic Applications. In: *Interleukin-10 in Health and Disease*. Vol 380. ; 2014:39-68. http://www.springer.com/series/82
60. Yao Y, Vent-Schmidt J, McGeough MD, et al. Tr1 Cells, but Not Foxp3+ Regulatory T Cells, Suppress NLRP3 Inflammasome Activation via an IL-10–Dependent Mechanism. *The Journal of Immunology*. 2015;195(2):488-497. doi:10.4049/jimmunol.1403225

61. Raphael I, Nalawade S, Eagar TN, Forsthuber TG. T cell subsets and their signature cytokines in autoimmune and inflammatory diseases. *Cytokine*. 2015;74(1):5-17. doi:10.1016/j.cyto.2014.09.011
62. Song Y, Wang N, Chen L, Fang L. Tr1 Cells as a Key Regulator for Maintaining Immune Homeostasis in Transplantation. *Front Immunol.* 2021;12. doi:10.3389/fimmu.2021.671579
63. Ceccarelli F, Agmon-Levin N, Perricone C. Genetic Factors of Autoimmune Diseases. *J Immunol Res.* 2016;2016. doi:10.1155/2016/3476023
64. Ramos PS, Shedlock AM, Langefeld CD. Genetics of autoimmune diseases: Insights from population genetics. *J Hum Genet.* 2015;60(11):657-664. doi:10.1038/jhg.2015.94
65. Jörg S, Grohme DA, Erzler M, et al. Environmental factors in autoimmune diseases and their role in multiple sclerosis. *Cellular and Molecular Life Sciences.* 2016;73(24):4611-4622. doi:10.1007/s00018-016-2311-1
66. Khan MF, Wang H. Environmental Exposures and Autoimmune Diseases: Contribution of Gut Microbiome. *Front Immunol.* 2020;10. doi:10.3389/fimmu.2019.03094
67. Vojdani A, Pollard KM, Campbell AW. Environmental triggers and autoimmunity. *Autoimmune Dis.* 2014;2014. doi:10.1155/2014/798029
68. Rubtsov A V., Rubtsova K, Kappler JW, Marrack P. Genetic and hormonal factors in female-biased autoimmunity. *Autoimmun Rev.* 2010;9(7):494-498. doi:10.1016/j.autrev.2010.02.008
69. Moulton VR. Sex hormones in acquired immunity and autoimmune disease. *Front Immunol.* 2018;9(OCT). doi:10.3389/fimmu.2018.02279
70. Lasrado N, Jia T, Massilamany C, Franco R, Illes Z, Reddy J. Mechanisms of sex hormones in autoimmunity: Focus on EAE. *Biol Sex Differ.* 2020;11(1). doi:10.1186/s13293-020-00325-4
71. Breuning MH, Van Den Berg-Loonen EM, Bernini LF, et al. Localization of HLA on the Short Arm of Chromosome 6*. *Hum Genet.* 1977;37:131-139.
72. Gough SCL, Simmonds MJ. *The HLA Region and Autoimmune Disease: Associations and Mechanisms of Action*. Vol 8.; 2007.
73. Cruz-Tapias P, Castiblanco J, Anaya JM. HLA Association with Autoimmune Diseases. In: *Autoimmunity: From Bench to Bedside*. El Rosario University Press; 2013.
74. Uffelmann E, Huang QQ, Munung NS, et al. Genome-wide association studies. *Nature Reviews Methods Primers.* 2021;1(1). doi:10.1038/s43586-021-00056-9
75. Seldin MF. The genetics of human autoimmune disease: A perspective on progress in the field and future directions. *J Autoimmun.* 2015;64:1-12. doi:10.1016/j.jaut.2015.08.015
76. Rojas M, Restrepo-Jiménez P, Monsalve DM, et al. Molecular mimicry and autoimmunity. *J Autoimmun.* 2018;95:100-123. doi:10.1016/j.jaut.2018.10.012
77. Thaper D, Prabha V. Molecular mimicry: An explanation for autoimmune diseases and infertility. *Scand J Immunol.* 2018;88(2). doi:10.1111/SJI.12697
78. Guilherme L, Kalil J, Cunningham M. Molecular mimicry in the autoimmune pathogenesis of rheumatic heart disease. *Autoimmunity.* 2006;39(1):31-39. doi:10.1080/08916930500484674
79. Cusick MF, Libbey JE, Fujinami RS. Molecular mimicry as a mechanism of autoimmune disease. *Clin Rev Allergy Immunol.* 2012;42(1):102-111. doi:10.1007/s12016-011-8294-7
80. Smolen JS, Aletaha D, Barton A, et al. Rheumatoid arthritis. *Nat Rev Dis Primers.* 2018;4. doi:10.1038/nrdp.2018.1
81. Guo Q, Wang Y, Xu D, Nossent J, Pavlos NJ, Xu J. Rheumatoid arthritis: Pathological mechanisms and modern pharmacologic therapies. *Bone Res.* 2018;6(1). doi:10.1038/s41413-018-0016-9
82. Amaya-Amaya J, Rojas-Villarraga A, Mantilla RD. Rheumatoid Arthritis. In: *Autoimmunity: From Bench to Bedside.* El Rosario Universyt Press; 2013.

83. Wysocki T, Olesińska M, Paradowska-Gorycka A. Current Understanding of an Emerging Role of HLA-DRB1 Gene in Rheumatoid Arthritis-From Research to Clinical Practice. *Cells.* 2020;9(5). doi:10.3390/cells9051127
84. Balsa A, Cabezón A, Orozco G, et al. *Influence of HLA DRB1 Alleles in the Susceptibility of Rheumatoid Arthritis and the Regulation of Antibodies against Citrullinated Proteins and Rheumatoid Factor.* Vol 12.; 2010. http://arthritis-research.com/content/12/2/R62
85. Larid G, Pancarte M, Offer G, et al. In Rheumatoid Arthritis Patients, HLA-DRB1*04:01 and Rheumatoid Nodules Are Associated With ACPA to a Particular Fibrin Epitope. *Front Immunol.* 2021;12. doi:10.3389/fimmu.2021.692041
86. van Drongelen V, Holoshitz J. Human Leukocyte Antigen–Disease Associations in Rheumatoid Arthritis. *Rheumatic Disease Clinics of North America.* 2017;43(3):363-376. doi:10.1016/j.rdc.2017.04.003
87. Liao KP, Alfredsson L, Karlson EW. Environmental influences on risk for rheumatoid arthritis. *Curr Opin Rheumatol.* 2009;21(3):279-283. doi:10.1097/BOR.0b013e32832a2e16
88. Deane KD, Demoruelle MK, Kelmenson LB, Kuhn KA, Norris JM, Holers VM. Genetic and environmental risk factors for rheumatoid arthritis. *Best Pract Res Clin Rheumatol.* 2017;31(1):3-18. doi:10.1016/j.berh.2017.08.003
89. Pradeepkiran JA. Insights of rheumatoid arthritis risk factors and associations. *J Transl Autoimmun.* 2019;2. doi:10.1016/j.jtauto.2019.100012
90. Kurowska W, Kuca-Warnawin EH, Radzikowska A, Maslinski W. The role of anti-citrullinated protein antibodies (ACPA) in the pathogenesis of rheumatoid arthritis. *Central European Journal of Immunology.* 2017;42(4):390-398. doi:10.5114/ceji.2017.72807
91. Liu J, Gao J, Wu Z, et al. Anti-citrullinated Protein Antibody Generation, Pathogenesis, Clinical Application, and Prospects. *Front Med (Lausanne).* 2022;8. doi:10.3389/fmed.2021.802934
92. Radu AF, Bungau SG. Management of rheumatoid arthritis: An overview. *Cells*. 2021;10(11). doi:10.3390/cells10112857
93. Bullock J, Rizvi SAA, Saleh AM, et al. Rheumatoid arthritis: A brief overview of the treatment. *Medical Principles and Practice.* 2019;27(6):501-507. doi:10.1159/000493390
94. Del Grossi Paglia M, Silva MT, Lopes LC, et al. Use of corticoids and non-steroidal antiinflammatories in the treatment of rheumatoid arthritis: Systematic review and network me ta-analysis. *PLoS One.* 2021;16(4 April). doi:10.1371/journal.pone.0248866
95. Crofford LJ. Use of NSAIDs in treating patients with arthritis. *Arthritis Res Ther.* 2013;15:undefined.
96. Hazlewood GS, Whittle SL, Kamso MM, et al. Disease-modifying anti-rheumatic drugs for rheumatoid arthritis: a systematic review and network meta-analysis. *Cochrane Database of Systematic Reviews.* 2020;2020(3). doi:10.1002/14651858.CD013562
97. Padjen I, Crnogaj MR, Anić B. Conventional disease-modifying agents in rheumatoid arthritis – a review of their current use and role in treatment algorithms. *Reumatologia.* 2021;58(6):390-400. doi:10.5114/REUM.2020.101400
98. Mysler E, Caubet M, Lizarraga A. Current and emerging DMARDs for the treatment of rheumatoid arthritis. *Open Access Rheumatol.* 2021;13:139-152. doi:10.2147/OARRR.S282627
99. Benjamin O, Goyal A, Lappin SL. *Disease-Modifying Antirheumatic Drugs (DMARD).*; 2023.
100. Salliot C, Van Der Heijde D. Long-term safety of methotrexate monotherapy in patients with rheumatoid arthritis: A systematic literature research. *Ann Rheum Dis.* 2009;68(7): 1100-1104. doi:10.1136/ard.2008.093690
101. Yazici Y. Long-term safety of methotrexate in the treatment of rheumatoid arthritis. *Clin Exp Rheumatol.* 2010;28:S65-S67.

102. Harrington R, Al Nokhatha SA, Conway R. Jak inhibitors in rheumatoid arthritis: An evidence-based review on the emerging clinical data. *J Inflamm Res.* 2020;13:519-531. doi:10.2147/JIR.S219586
103. Hua C, Buttgereit F, Combe B. Glucocorticoids in rheumatoid arthritis: Current status and future studies. *RMD Open.* 2020;6(1). doi:10.1136/rmdopen-2017-000536
104. McKenzie BJ, Whittle SL, Johnston R V., et al. Long-term glucocorticoids for rheumatoid arthritis. *Cochrane Database of Systematic Reviews.* 2021;2021(12). doi:10.1002/14651858.CD014899
105. Verhoef CM, G van Roon JA, Vianen ME, J G Lafeber FP, J Bijlsma JW. *The Immune Suppressive effect of Dexamethasone in Rheumatoid Arthritis Is Accompanied by Upregulation of Interleukin 10 and by DiVerential Changes in Interferon and Interleukin 4 Production.* Vol 58.; 1999.
106. Ding Q, Hu W, Wang R, et al. Signaling pathways in rheumatoid arthritis: implications for targeted therapy. *Signal Transduct Target Ther.* 2023;8(1). doi:10.1038/s41392-023-01331-9
107. Chen F, Hao L, Zhu S, et al. Potential Adverse Effects of Dexamethasone Therapy on COVID-19 Patients: Review and Recommendations. *Infect Dis Ther.* 2021;10(4):1907-1931. doi:10.1007/s40121-021-00500-z
108. Mourkas M, VASSILIOU MP, AMYGDALOU A, MANDRAGOS C, TAKIS F, BEHRAKIS PK. Muscular mass assessed by ultrasonography after administration of low-dose corticosteroids and muscle relaxants in critically ill hemiplegic patients. *Clinical Nutrition.* 2002;21(4):297-302. doi:10.1054/clnu.2001.0532
109. Zhao J, Guo S, Schrodi SJ, He D. Molecular and Cellular Heterogeneity in Rheumatoid Arthritis: Mechanisms and Clinical Implications. *Front Immunol.* 2021;12. doi:10.3389/fimmu.2021.790122
110. Weyand CM, Klimiuk PA, Goronzy J. Heterogeneity of rheumatoid arthritis: from phenotypes to genotypes. *Springer Semin Immunopathol.* 1998;20:5-22.
111. Ouboussad L, Burska AN, Melville A, Buch MH. Synovial Tissue Heterogeneity in Rheumatoid Arthritis and Changes With Biologic and Targeted Synthetic Therapies to Inform Stratified Therapy. *Front Med (Lausanne).* 2019;6. doi:10.3389/fmed.2019.00045
112. Townsend MJ. Molecular and cellular heterogeneity in the Rheumatoid Arthritis synovium: Clinical correlates of synovitis. *Best Pract Res Clin Rheumatol.* 2014;28(4):539-549. doi:10.1016/j.berh.2014.10.024
113. Smolen JS, Aletaha D, Bijlsma JWJ, et al. Treating rheumatoid arthritis to target: Recommendations of an international task force. *Ann Rheum Dis.* 2010;69(4):631-637. doi:10.1136/ard.2009.123919
114. Morgan C, Lunt M, Brightwell H, et al. Contribution of patient related differences to multidrug resistance in rheumatoid arthritis. *Ann Rheum Dis.* 2003;62(1):15-19. doi:10.1136/ard.62.1.15
115. Zhang KX, Ip CK, Chung SK, et al. Drug-resistance in rheumatoid arthritis: the role of p53 gene mutations, ABC family transporters, and personal factors. *Curr Opin Pharmacol.* 2020;54:59-71. doi:10.1016/j.coph.2020.08.002
116. Boyadzieva V V., Stoilov N, Stoilov RM, et al. Quality of life and cost study of rheumatoid arthritis therapy with biological medicines. *Front Pharmacol.* 2018;9(JUL). doi:10.3389/fphar.2018.00794
117. Wehr P, Purvis H, Law SC, Thomas R. Dendritic cells, T cells and their interaction in rheumatoid arthritis. *Clin Exp Immunol.* 2019;196(1):12-27. doi:10.1111/cei.13256
118. Edilova MI, Akram A, Abdul-Sater AA. Innate immunity drives pathogenesis of rheumatoid arthritis. *Biomed J.* 2021;44(2):172-182. doi:10.1016/j.bj.2020.06.010
119. Ding Q, Hu W, Wang R, et al. Signaling pathways in rheumatoid arthritis: implications for targeted therapy. *Signal Transduct Target Ther.* 2023;8(1). doi:10.1038/s41392-023-01331-9
120. Morelli AE, Thomson AW. Tolerogenic dendritic cells and the quest for transplant tolerance. *Nat Rev Immunol.* 2007;7(8):610-621. doi:10.1038/nri2132

121. Maldonado RA, von Andrian UH. How Tolerogenic Dendritic Cells Induce Regulatory T Cells. In: *Advances in Immunology.* Vol 108. Academic Press Inc.; 2010:111-165. doi:10.1016/B978-0-12-380995-7.00004-5
122. Navarro-Barriuso J, Mansilla MJ, Quirant-Sánchez B, Teniente-Serra A, Ramo-Tello C, Martínez-Cáceres EM. Vitamin D3-Induced Tolerogenic Dendritic Cells Modulate the Transcriptomic Profile of T CD4+ Cells Towards a Functional Hyporesponsiveness. *Front Immunol*. 2021;11. doi:10.3389/fimmu.2020.599623
123. Raïch-Regué D, Glancy M, Thomson AW. Regulatory dendritic cell therapy: From rodents to clinical application. *Immunol Lett.* 2014;161(2):216-221. doi:10.1016/j.imlet.2013.11.016
124. Jansen MAA, Spiering R, Ludwig IS, van Eden W, Hilkens CMU, Broere F. Matured tolerogenic dendritic cells effectively inhibit autoantigen specific CD4+ T cells in a murine arthritis model. *Front Immunol.* 2019;10(AUG). doi:10.3389/fimmu.2019.02068
125. Hilkens CMU, Isaacs JD, Thomson AW. Development of Dendritic Cell-Based Immunotherapy for Autoimmunity. *Int Rev Immunol.* 2010;29(2). doi:10.3109/08830180903281193
126. Bhatt S, Qin J, Bennett C, et al. All- trans Retinoic Acid Induces Arginase-1 and Inducible Nitric Oxide Synthase–Producing Dendritic Cells with T Cell Inhibitory Function . *The Journal of Immunology.* 2014;192(11):5098-5108. doi:10.4049/jimmunol.1303073
127. Kim N, Park CS, Im SA, et al. Minocycline promotes the generation of dendritic cells with regulatory properties. *Oncotarget.* 2016;7(33). www.impactjournals.com/oncotarget
128. Gordon JR, Ma Y, Churchman L, Gordon SA, Dawicki W. Regulatory dendritic cells for immunotherapy in immunologic diseases. *Front Immunol.* 2014;5(JAN). doi:10.3389/fimmu.2014.00007
129. Li XL, Liu Y, Cao LL, et al. Atorvastatin-modified dendritic cells in vitro ameliorate experimental autoimmune myasthenia gravis by up-regulated Treg cells and shifted Th1/Th17 to Th2 cytokines. *Molecular and Cellular Neuroscience.* 2013;56:85-95. doi:10.1016/j.mcn.2013.03.005
130. Woltman AM, De Fijter JW, Kamerling SWA, Paul LC, Daha MR, Van Kooten C. The effect of calcineurin inhibitors and corticosteroids on the differentiation of human dendritic cells. *Eur J Immunol.* 2000;30(7):1807-1812. doi:10.1002/1521-4141(200007)30:7<1807::AID-IMU1807>3.0.CO;2-N
131. Suuring M, Moreau A. Regulatory macrophages and tolerogenic dendritic cells in myeloid regulatory cell-based therapies. *Int J Mol Sci.* 2021;22(15). doi:10.3390/ijms22157970
132. Švajger U, Obermajer N, Jeras M. Novel findings in drug-induced dendritic cell tolerogenicity. *Int Rev Immunol.* 2010;29(6):574-607. doi:10.3109/08830185.2010.522280
133. Piemonti L, Monti P, Allavena P, et al. Glucocorticoids Affect Human Dendritic Cell Differentiation and Maturation. *The Journal of Immunology.* 1999;162(11):6473-6481. doi:10.4049/jimmunol.162.11.6473
134. Lagaraine C, Hoarau C, Chabot V, Velge-Roussel F, Lebranchu Y. Mycophenolic acid-treated human dendritic cells have a mature migratory phenotype and inhibit allogeneic responses via direct and indirect pathways. *Int Immunol.* 2005;17(4):351-363. doi:10.1093/intimm/dxh215
135. Passeri L, Andolfi G, Bassi V, et al. Tolerogenic IL-10-engineered dendritic cell-based therapy to restore antigen-specific tolerance in T cell mediated diseases. *J Autoimmun.* 2023;138. doi:10.1016/j.jaut.2023.103051
136. Kim SH, Jung HH, Lee CK. Generation, characteristics and clinical trials of ex vivo generated tolerogenic dendritic cells. *Yonsei Med J.* 2018;59(7):807-815. doi:10.3349/ymj.2018.59.7.807
137. Harry RA, Anderson AE, Isaacs JD, Hilkens CMU. Generation and characterisation of therapeutic tolerogenic dendritic cells for rheumatoid arthritis. *Ann Rheum Dis.* 2010;69(11):2042-2050. doi:10.1136/ard.2009.126383

138. Li E, Tso P. Vitamin A uptake from foods. *Curr Opin Lipidol.* 2003;14(3). doi:10.1097/00041433-200306000-00003
139. Manicassamy S, Pulendran B. Retinoic acid-dependent regulation of immune responses by dendritic cells and macrophages. *Semin Immunol.* 2009;21(1):22-27. doi:10.1016/j.smim.2008.07.007
140. Budhu AS, Noy N. Direct Channeling of Retinoic Acid between Cellular Retinoic Acid-Binding Protein II and Retinoic Acid Receptor Sensitizes Mammary Carcinoma Cells to Retinoic Acid-Induced Growth Arrest. *Mol Cell Biol.* 2002;22(8):2632-2641. doi:10.1128/mcb.22.8.2632-2641.2002
141. Bakdash G, Vogelpoel LTC, Van Capel TMM, Kapsenberg ML, De Jong EC. Retinoic acid primes human dendritic cells to induce gut-homing, IL-10-producing regulatory T cells. *Mucosal Immunol.* 2015;8(2):265-278. doi:10.1038/mi.2014.64
142. Manicassamy S, Ravindran R, Deng J, et al. Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. *Nat Med.* 2009;15(4):401-409. doi:10.1038/nm.1925
143. Ritprajak P, Kaewraemruaen C, Hirankarn N. Current paradigms of tolerogenic dendritic cells and clinical implications for systemic lupus erythematosus. *Cells*. 2019;8(10). doi:10.3390/cells8101291
144. Baban B, Chandler PR, Sharma MD, et al. IDO Activates Regulatory T Cells and Blocks Their Conversion into Th17-Like T Cells. *The Journal of Immunology*. 2009;183(4):2475-2483. doi:10.4049/jimmunol.0900986
145. Yan Y, Zhang GX, Gran B, et al. IDO Upregulates Regulatory T Cells via Tryptophan Catabolite and Suppresses Encephalitogenic T Cell Responses in Experimental Autoimmune Encephalomyelitis. *The Journal of Immunology.* 2010;185(10):5953-5961. doi:10.4049/jimmunol.1001628
146. Stone TW, Williams RO. Modulation of T cells by tryptophan metabolites in the kynurenine pathway. *Trends Pharmacol Sci.* 2023;44(7):442-456. doi:10.1016/j.tips.2023.04.006
147. Kaiser H, Parker E, Hamrick MW. Kynurenine signaling through the aryl hydrocarbon receptor: Implications for aging and healthspan. *Exp Gerontol.* 2020;130. doi:10.1016/j.exger.2019.110797
148. Shadboorestan A, Koual M, Dairou J, Coumoul X. The Role of the Kynurenine/AhR Pathway in Diseases Related to Metabolism and Cancer. *International Journal of Tryptophan Research.* 2023;16. doi:10.1177/11786469231185102
149. Heitzer MD, Wolf IM, Sanchez ER, Witchel SF, DeFranco DB. Glucocorticoid receptor physiology. *Rev Endocr Metab Disord.* 2007;8(4):321-330. doi:10.1007/s11154-007-9059-8
150. Stojceski F, Buetti-Dinh A, Stoddart MJ, Danani A, Della Bella E, Grasso G. Influence of dexamethasone on the interaction between glucocorticoid receptor and SOX9: A molecular dynamics study. *J Mol Graph Model.* 2023;125. doi:10.1016/j.jmgm.2023.108587
151. Picard D, Khursheed B, Garabedian MJ, Fortin MG, Lindquist S, Yamamoto KR. Reduced levels of hsp90 compromise steroid receptor action in vivo. *Nature.* 1990;348.
152. Weikum ER, Knuesel MT, Ortlund EA, Yamamoto KR. Glucocorticoid receptor control of transcription: Precision and plasticity via allostery. *Nat Rev Mol Cell Biol.* 2017;18(3):159-174. doi:10.1038/nrm.2016.152
153. Smits HH, Gru¨nberg K, Gru¨nberg G, Derijk RH, Sterk PJ, Hiemstra PS. Cytokine release and its modulation by dexamethasone in whole blood following exercise. *Clin Exp Immunol.* 1998;111:463-468.
154. Aghai ZH, Kumar S, Farhath S, et al. Dexamethasone suppresses expression of nuclear factor-kappaB in the cells of tracheobronchial lavage fluid in premature neonates with respiratory distress. *Pediatr Res.* 2006;59(6):811-815. doi:10.1203/01.pdr.0000219120.92049.b3

155. Lammer NC, Ashraf HM, Ugay DA, et al. RNA binding by the glucocorticoid receptor attenuates dexamethasone-induced gene activation. *Sci Rep.* 2023;13(1). doi:10.1038/s41598-023-35549-y
156. Mozo L, Suárez A, Gutiérrez C. Glucocorticoids up-regulate constitutive interleukin-10 production by human monocytes. *Clinical & Experimental Allergy.* 2004;34(3):406-412. doi:10.1111/j.1365-2222.2004.01824.x
157. Obregon C, Kumar R, Pascual MA, Vassalli G, Golshayan D. Update on dendritic cell-induced immunological and clinical tolerance. *Front Immunol.* 2017;8(NOV). doi:10.3389/fimmu.2017.01514
158. Navarro-Barriuso J, Mansilla MJ, Naranjo-Gómez M, et al. Comparative transcriptomic profile of tolerogenic dendritic cells differentiated with vitamin D3, dexamethasone and rapamycin. *Sci Rep.* 2018;8(1). doi:10.1038/s41598-018-33248-7
159. García-González PA, Schinnerling K, Sepúlveda-Gutiérrez A, et al. Dexamethasone and monophosphoryl lipid a induce a distinctive profile on monocyte-derived dendritic cells through transcriptional modulation of genes associated with essential processes of the immune response. *Front Immunol.* 2017;8(OCT). doi:10.3389/fimmu.2017.01350
160. Li V, Binder MD, Kilpatrick TJ. The Tolerogenic Influence of Dexamethasone on Dendritic Cells Is Accompanied by the Induction of Efferocytosis, Promoted by MERTK. *Int J Mol Sci.* 2023;24(21). doi:10.3390/ijms242115903
161. Maldonado RA, Von Andrian UH. How Tolerogenic Dendritic Cells Induce Regulatory T Cells. *Adv Immunol.* 2010;108. doi:10.1016/S0065-2776(10)08004-1
162. García-González P, Ubilla-Olguín G, Catalán D, Schinnerling K, Aguillón JC. Tolerogenic dendritic cells for reprogramming of lymphocyte responses in autoimmune diseases. *Autoimmun Rev.* 2016;15(11):1071-1080. doi:10.1016/j.autrev.2016.07.032
163. Dvořák Z, Vrzal R, Pávek P, Ulrichová J, Dvořák Z. An Evidence for Regulatory Cross-Talk between Aryl Hydrocarbon Receptor and Glucocorticoid Receptor in HepG2 Cells. *Physiol Res.* 2008;57:427-435. www.biomed.cas.cz/physiolres
164. Jin HL, Choi Y, Jeong KW. Crosstalk between Aryl Hydrocarbon Receptor and Glucocorticoid Receptor in Human Retinal Pigment Epithelial Cells. *Int J Endocrinol.* 2017;2017. doi:10.1155/2017/5679517
165. Bikle DD. Vitamin D metabolism, mechanism of action, and clinical applications. *Chem Biol.* 2014;21(3):319-329. doi:10.1016/j.chembiol.2013.12.016
166. Mansilla MJ, González-larreategui I, Figa-martín N, et al. Transfection of vitamin D3-induced tolerogenic dendritic cells for the silencing of potential tolerogenic genes. Identification of CSF1R-CSF1 signaling as a glycolytic regulator. *Int J Mol Sci.* 2021;22(14). doi:10.3390/ijms22147363
167. Mansilla MJ, Contreras-Cardone R, Navarro-Barriuso J, et al. Cryopreserved vitamin D3-tolerogenic dendritic cells pulsed with autoantigens as a potential therapy for multiple sclerosis patients. *J Neuroinflammation.* 2016;13(1). doi:10.1186/s12974-016-0584-9
168. Raïch-Regué D, Grau-López L, Naranjo-Gómez M, et al. Stable antigen-specific T-cell hyporesponsiveness induced by tolerogenic dendritic cells from multiple sclerosis patients. *Eur J Immunol.* 2012;42(3):771-782. doi:10.1002/eji.201141835
169. Mansilla MJ, Sellès-Moreno C, Fàbregas-Puig S, et al. Beneficial Effect of Tolerogenic Dendritic Cells Pulsed with MOG Autoantigen in Experimental Autoimmune Encephalomyelitis. *CNS Neurosci Ther.* 2015;21(3):222-230. doi:10.1111/cns.12342
170. Naranjo-Gómez M, Raïch-Regué D, Oñate C, et al. Comparative study of clinical grade human tolerogenic dendritic cells. *J Transl Med.* 2011;9(1). doi:10.1186/1479-5876-9-89

171. Quigley M, Rieger S, Capobianco E, et al. Vitamin D Modulation of Mitochondrial Oxidative Metabolism and mTOR Enforces Stress Adaptations and Anticancer Responses. *JBMR Plus.* 2022;6(1). doi:10.1002/jbm4.10572
172. Ferreira GB, Vanherwegen AS, Eelen G, et al. Vitamin D3 induces tolerance in human dendritic cells by activation of intracellular metabolic pathways. *Cell Rep.* 2015;10(5):711-725. doi:10.1016/j.celrep.2015.01.013
173. Phillips BE, Garciafigueroa Y, Engman C, Trucco M, Giannoukakis N. Tolerogenic dendritic cells and T-regulatory cells at the clinical trials crossroad for the treatment of autoimmune disease; emphasis on type 1 diabetes therapy. *Front Immunol.* 2019;10(FEB). doi:10.3389/fimmu.2019.00148
174. Willekens B, Presas-Rodríguez S, Mansilla MJ, et al. Tolerogenic dendritic cell-based treatment for multiple sclerosis (MS): A harmonised study protocol for two phase i clinical trials comparing intradermal and intranodal cell administration. *BMJ Open.* 2019;9(9). doi:10.1136/bmjopen-2019-030309
175. Bell GM, Anderson AE, Diboll J, et al. Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis. *Ann Rheum Dis.* 2017;76(1):227-234. doi:10.1136/annrheumdis-2015-208456
176. Benham H, Nel HJ, Law SC, et al. *Citrullinated Peptide Dendritic Cell Immunotherapy in HLA Risk Genotype-Positive Rheumatoid Arthritis Patients.* https://www.science.org
177. Tukaj S, Mantej J, Sobala M, Potrykus K, Sitko K. Autologous extracellular Hsp70 exerts a dual role in rheumatoid arthritis. doi:10.1007/s12192-020-01114-z/Published
178. Van Herwijnen MJC, Wieten L, Van Der Zee R, et al. Regulatory T cells that recognize a ubiquitous stress-inducible self-antigen are long-lived suppressors of autoimmune arthritis. *Proc Natl Acad Sci U S A.* 2012;109(35):14134-14139. doi:10.1073/pnas.1206803109
179. Mansilla MJ, Hilkens CMU, Martínez-Cáceres EM. Challenges in tolerogenic dendritic cell therapy for autoimmune diseases: the route of administration. *Immunotherapy Advances.* 2023;3(1). doi:10.1093/immadv/ltad012
180. Passeri L, Marta F, Bassi V, Gregori S. Tolerogenic dendritic cell-based approaches in autoimmunity. *Int J Mol Sci.* 2021;22(16). doi:10.3390/ijms22168415
181. Benne N, ter Braake D, Stoppelenburg AJ, Broere F. Nanoparticles for Inducing Antigen-Specific T Cell Tolerance in Autoimmune Diseases. *Front Immunol.* 2022;13. doi:10.3389/fimmu.2022.864403
182. Rao JP, Geckeler KE. Polymer nanoparticles: Preparation techniques and size-control parameters. *Progress in Polymer Science (Oxford).* 2011;36(7):887-913. doi:10.1016/j.progpolymsci.2011.01.001
183. Kelly KL, Coronado E, Zhao LL, Schatz GC. The optical properties of metal nanoparticles: The influence of size, shape, and dielectric environment. *Journal of Physical Chemistry B.* 2003;107(3):668-677. doi:10.1021/jp026731y
184. Pattni BS, Chupin V V., Torchilin VP. New Developments in Liposomal Drug Delivery. *Chem Rev.* 2015;115(19):10938-10966. doi:10.1021/acs.chemrev.5b00046
185. Hawkins MJ, Soon-Shiong P, Desai N. Protein nanoparticles as drug carriers in clinical medicine. *Adv Drug Deliv Rev.* 2008;60(8):876-885. doi:10.1016/j.addr.2007.08.044
186. Dua JS, Rana AC, Bhandari AK. International Journal of Pharmaceutical Studies and Research LIPOSOME: METHODS OF PREPARATION AND APPLICATIONS.
187. Shi D, Fu M, Fan P, et al. Artificial phosphatidylserine liposome mimics apoptotic cells in inhibiting maturation and immunostimulatory function of murine myeloid dendritic cells in response to 1-chloro-2,4-dinitrobenze in vitro. Arch Dermatol Res. 2007;299(7):327-336.

doi:10.1007/s00403-007-0770-9
188. Hosseini H, Li Y, Kanellakis P, et al. Phosphatidylserine liposomes mimic apoptotic cells to attenuate atherosclerosis by expanding polyreactive IgM producing B1a lymphocytes. *Cardiovasc Res.* 2015;106(3):443-452. doi:10.1093/cvr/cvv037
189. Sordi R, Bet ÂC, Della Justina AM, Ramos GC, Assreuy J. The apoptosis clearance signal phosphati dylserine inhibits leukocyte migration and promotes inflammation resolution in vivo. *Eur J Pharmacol.* 2020;877. doi:10.1016/j.ejphar.2020.173095
190. Benne N, van Duijn J, Lozano Vigario F, et al. Anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) liposomes induce antigen-specific regulatory T cells and prevent atherosclerosis in mice. *Journal of Controlled Release.* 2018;291:135-146. doi:10.1016/j.jconrel.2018.10.028
191. Ramos GC, Fernandes D, Charão CT, Souza DG, Teixeira MM, Assreuy J. Apoptotic mimicry: Phosp hatidylserine liposomes reduce inflammation through activation of peroxisome proliferator-activated receptors (PPARs) in vivo. *Br J Pharmacol.* 2007;151(6):844-850. doi:10.1038/sj.bjp.0707302
192. Krienke C, Kolb L, Diken E, et al. *A Noninflammatory MRNA Vaccine for Treatment of Experimental Autoimmune Encephalomyelitis.* https://www.science.org
193. Peine KJ, Guerau-De-Arellano M, Lee P, et al. Treatment of experimental autoimmune encephalomyelitis by codelivery of disease associated peptide and dexamethasone in acetalated dextran microparticles. *Mol Pharm.* 2014;11(3):828-835. doi:10.1021/mp4005172
194. Phillips BE, Garciafigueroa Y, Engman C, et al. Arrest in the Progression of Type 1 Diabetes at the Mid-Stage of Insulitic Autoimmunity Using an Autoantigen-Decorated All-trans Retinoic Acid and Transforming Growth Factor Beta-1 Single Microparticle Formulation. *Front Immunol.* 2021;12. doi:10.3389/fimmu.2021.586220
195. Tavakoli K, Pour-Aboughadareh A, Kianersi F, Poczai P, Etminan A, Shooshtari L. Applications of CRISPR-Cas9 as an advanced genome editing system in life sciences. *BioTech.* 2021;10(3). doi:10.3390/biotech10030014
196. Zhu Y. Advances in CRISPR/Cas9. Biomed Res Int. 2022;2022. doi:10.1155/2022/9978571
197. Le Rhun A, Escalera-Maurer A, Bratovič M, Charpentier E. CRISPR-Cas in Streptococcus pyogenes. *RNA Biol.* 2019;16(4):380-389. doi:10.1080/15476286.2019.1582974
198. Mehta A, Merkel OM. Immunogenicity of Cas9 Protein. *J Pharm Sci.* 2020;109(1):62-67. doi:10.1016/j.xphs.2019.10.003
199. Gough V, Gersbach CA. Immunity to Cas9 as an Obstacle to Persistent Genome Editing. *Molecular Therapy.* 2020;28(6):1389-1391. doi:10.1016/j.ymthe.2020.05.007
200. Zhen S, Li X. Liposomal delivery of CRISPR/Cas9. *Cancer Gene Ther.* 2020;27(7-8):515-527. doi:10.1038/s41417-019-0141-7
201. Finn JD, Smith AR, Patel MC, et al. A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In Vivo Genome Editing. *Cell Rep.* 2018;22(9):2227-2235. doi:10.1016/j.celrep.2018.02.014
202. Gillmore JD, Gane E, Taubel J, et al. CRISPR-Cas9 In Vivo Gene Editing for Transthyretin Amyloidosis. *New England Journal of Medicine.* 2021;385(6):493-502. doi:10.1056/nejmoa2107454
203. Rasul MF, Hussen BM, Salihi A, et al. Strategies to overcome the main challenges of the use of CRISPR/Cas9 as a replacement for cancer therapy. *Mol Cancer.* 2022;21(1). doi:10.1186/s12943-021-01487-4
204. Taha EA, Lee J, Hotta A. Delivery of CRISPR-Cas tools for in vivo genome editing therapy: Trends and challenges. *Journal of Controlled Release.* 2022;342:345-361. doi:10.1016/j.jconrel.2022.01.013

Nanoparticles for Inducing Antigen-Specific T Cell Tolerance in Autoimmune Diseases

Nanoparticles for Inducing Antigen-Specific T Cell Tolerance in Autoimmune Diseases

Naomi Benne[1], Daniëlle ter Braake[1], Arie Jan Stoppelenburg[1,2], Femke Broere[1,3]

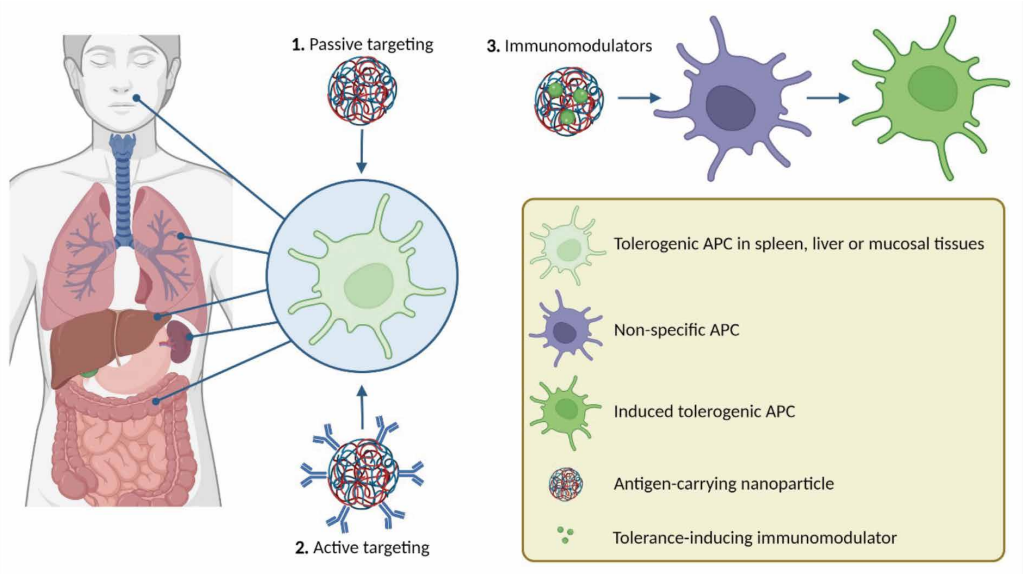
[1] Department of Biomolecular Health Sciences, Faculty of Veterinary Medicine, Utrecht University, 3584 CL Utrecht, The Netherlands
[2] Department of Rheumatology and Clinical Immunology, University Medical Center Utrecht, 3584 CX Utrecht, The Netherlands
[3] Department of Clinical Sciences, Faculty of Veterinary Medicine, Utrecht University, 3584 CL Utrecht, The Netherlands

Frontiers in Immunology (2022), Volume 13
doi: 10.3389/fimmu.2022.864403

Abstract

Autoimmune diseases affect many people worldwide. Current treatment modalities focus on the reduction of disease symptoms using anti-inflammatory drugs which can lead to side effects due to systemic immune suppression. Restoration of immune tolerance by down-regulating auto-reactive cells in an antigen-specific manner is currently the “holy grail” for the treatment of autoimmune diseases. A promising strategy is the use of nanoparticles that can deliver antigens to antigen-presenting cells which in turn can enhance antigen-specific regulatory T cells. In this review, we highlight some promising cell targets (e.g. liver sinusoidal endothelial cells and splenic marginal zone macrophages) for exploiting natural immune tolerance processes, and several strategies by which antigen-carrying nanoparticles can target these cells. We also discuss how nanoparticles carrying immunomodulators may be able to activate tolerance in other antigen-presenting cell types. Finally, we discuss some important aspects that must be taken into account when translating data from animal studies to patients.

Graphical abstract



Introduction

1.1 General

Our immune system has evolved to distinguish non-self from self-antigens to protect us against pathogens while also maintaining tissue homeostasis. Immune cells of both the innate and the adaptive immune systems are involved in this complex protection against potentially harmful intruders. The process of combating exogenous antigens by these immune cells is a powerful effector mechanism. However, when directed toward our own cells, tissues, or commensal microbes, this mechanism could be very destructive[1]. To maintain immune homeostasis and prevent tissue damage, the immune system must be able to distinguish innocuous endogenous antigens from potentially harmful exogenous antigens[2]. This mechanism is known as self-tolerance and is maintained by specialized immune cell subsets such as tolerogenic antigen-presenting cells (APCs) and regulatory T cells (Tregs)[3]. In autoimmunity, Tregs are the most well-studied cell type and the focus of this review. Tregs can be derived from the thymus[4] or peripherally induced from effector T cell populations[5]. Tregs are typically defined as $CD45RO^{+}TCR^{+}CD4^{+}CD25^{hi}FoxP3^{+}CD127^{-}$ cells in humans, and $CD4^{+}CD25^{+}FoxP3^{+}$ in mice[6], but can also express the suppressive receptors CTLA-4, PD-1, TIGIT, and GITR, among others[7,8]. Other suppressive T cell populations that do not express FoxP3 include T regulatory 1 (Tr1) cells, which secrete high levels of IL-10 and express CD49b and LAG-3 in humans and mice[9], and T helper 3 (Th3) cells, which secrete high levels of TGF-β[10]. Apart from T cells, B cells also play an important role in maintaining immune homeostasis, including regulatory B cells (Bregs)[11], of which there are also several subtypes, as reviewed elsewhere[12]. Immune tolerance can be disrupted due to multifactorial causes[13]. This can prompt an overactive inflammatory response towards (auto)antigens[14], resulting in the development of autoimmune diseases. Current treatment modalities focus on the reduction of disease symptoms using anti-inflammatory drugs (e.g. corticosteroids) or biologicals (e.g. tumor necrosis factor (TNF)-α inhibitors)[15]. Long-term use of these drugs coincides with unwanted side effects such as increased susceptibility to opportunistic infections and tumors[16]. Significant research has focused on improving the targeting of these drugs, thereby reducing the dosage, and limiting off-target effects (as reviewed by Fang et al.[17]). Although these therapies have improved patient care over the last decades, they fail to cure patients that suffer from autoimmune diseases, necessitating lifelong therapy.

Restoring the immune balance by down-regulating auto-reactive cells and enhancing Treg function is a promising strategy to treat autoimmune diseases. Importantly, this could result in long-lasting medication-free disease remission[18]. Unfortunately, the injection of free antigen is unlikely to result in sufficient accumulation in the specific cells that can induce tolerance. Besides, many autoantigens are poorly soluble, and due to their size or charge can bring about unwanted immunogenic effects if administered freely[19]. A very promising approach is the use of APC-like nanoparticles[20], as reviewed elsewhere[21]. Another strategy is to develop nanoparticulate delivery systems to facilitate the delivery of antigens to APCs *in vivo*.

Nanoparticles are drug delivery systems ranging from 1 to 1000 nm in size[22]. These particles protect their cargo from enzymatic degradation and can be designed to accommodate for an antigen's size, charge, and solubility, and (passively) target specific immune cells. This allows for higher therapeutic efficacy, reduces the required dose, and minimizes off-target effects or side effects associated with high doses or the intrinsic properties of the free antigen[23]. Nanoparticles can target organs or cells depending on their physicochemical properties (e.g. size, charge, and rigidity)[24-27], or by the incorporation of targeting moieties, such as antibodies[28]. Nanoparticles can be made of different materials, including polymers[29], metals[30], lipids[31], proteins[32], or a combination of the above. Some of

these materials have intrinsic immunomodulatory effects[33], which makes them interesting for use in immunotherapy. Furthermore, nanoparticles can carry both an antigen and an immunomodulator to induce tolerogenic phenotypes in APCs *in vivo*. In this review, we highlight some promising cell targets and several strategies by which antigen-carrying nanoparticles, with or without immunomodulators can target these cells.

1.2 Cellular targets

Immune tolerance to circulating antigens is maintained by the spleen and liver[34,35]. These organs are responsible for filtering the blood and contain many specialized cell types that are involved in the clearance of apoptotic cells. This process is termed efferocytosis and is vital for immune homeostasis; the dysregulation of this process has been implicated in several autoimmune diseases[36,37]. Some APC subsets such as plasmacytoid dendritic cells (pDCs) express CD36 and CD61, which are efferocytic scavenger receptors involved in immune regulation[38]. Cells expressing these receptors often show a decrease in the surface expression of the co-stimulatory molecules CD40 and CD86 and it has been shown that efferocytosis by DC subsets leads to a suppressive phenotype[39,40]. Furthermore, deficiency in scavenger receptor function has been described to be involved in the development of autoimmunity[41], and activation of the efferocytic receptor MER protected mice against the development of arthritis in collagen-induced arthritis and KRN serum transfer mouse models[42]. In a study by Watkins et al., the importance of efferocytosis in immune tolerance was demonstrated by directly targeting antigens towards apoptotic erythrocytes. When these erythrocytes were efferocytosed in the spleen, the antigen was presented in a tolerogenic manner and induced longlasting antigen-specific T cell anergy[43]. In another study, it was shown that human apoptotic cells derived from peripheral blood mononuclear cells have low expression of HLA-DR and CD86, produce the anti-inflammatory cytokines IL-10 and TGF-β, and expressed Fas and caspase-3. In a mixed lymphocyte reaction, the apoptotic cells greatly reduced the proliferation of T cells as compared to non-apoptotic cells and reduced the expression of CD25, CD45RO, and OX40 on proliferated T cells. These apoptotic cells inhibited allogeneic immune responses in humanized non-obese diabetic (NOD)/ severe combined immune deficiency (SCID)/γC mice[44]. Furthermore, i.v. injection of apoptotic cells that expressed a MOG peptide prevented the development of EAE in mice. This was shown to be due to the accumulation of the apoptotic cells in the splenic marginal zone and antigen-specific T cell unresponsiveness, as measured by reduced proliferation of T cells and reduced production of IFNγ and IL-17 by T cells. Unfortunately, injection of the apoptotic cells after MOG immunization did not affect disease progression[45]. Splenectomy abolishes the immune-suppressing effects of apoptotic cell-mimicking liposomes[35]. These studies show that when antigen is taken up in the context of efferocytosis, it leads to immune suppression and that the spleen is vital in this process. The splenic marginal zone contains several cell subtypes such as marginal zone macrophages (MZMs), B cells, and DCs[46]. For instance, MZMs expressing the macrophage receptor with a collagenous structure (MARCO)[47] and macrophages expressing CD169/sialic acid-binding immunoglobulin-type lectin-1 (Siglec-1)[48] can induce tolerance. Depletion of Siglec-1$^+$ macrophages from the spleens in mice susceptible to EAE abrogated the protective effects of MOG-apoptotic cells[45]. Siglec-1 is important for cell-cell contact and binds to CD8α$^+$ DCs, which suggests that the tolerogenic immune effects of Siglec-1$^+$ macrophages may be mediated through marginal zone DCs[49]. CD8α$^+$CD103$^+$ DCs in the marginal zone of the spleen efficiently efferocytose apoptotic cells from the blood, and subsequently migrate to splenic T cells to present antigens in a tolerogenic fashion[50]. Indeed, injected apoptotic cells are filtered by CD8α$^+$ DCs in WT mice and lead to tolerance, while Siglec-1 depletion leads to uptake by CD8α$^-$CD11b$^+$ DCs instead[45].

The liver microenvironment also promotes tolerance[51] and human DCs derived from the liver are more suppressive than blood-derived DCs[52]. The liver contains a multitude of cells that are inherently suppressive[34]. For example, liver sinusoidal epithelial cells (LSECs) have been shown to induce differentiation of T cells to both FoxP3$^+$ Tregs and FoxP3$^-$LAG3$^+$ Tr1 cells[53]. Furthermore, Kupffer cells in the liver express low levels of co-stimulatory molecules and suppress T cell responses[54].

Finally, it is well known that oral and mucosal antigen application favors tolerance induction. In a collagen-induced arthritis mouse model, multiple oral administrations of type II collagen significantly reduced the severity of the disease[55], and in a human trial in rheumatoid arthritis patients, oral administration of type II collagen significantly reduced joint swelling and pain[56]. Oral tolerance induction is independent of apoptotic pathways as splenectomy does not abrogate oral tolerance[57]. The mesenteric lymph nodes are important for oral tolerance induction, as it was found that transplantation of peripheral lymph nodes into the gut mesenteries in mice did not allow for the induction of oral tolerance[58]. This points to the importance of the microenvironment created by stromal cells in these lymphoid structures that facilitates tolerance induction. In mice removal of the superficial cervical and internal jugular lymph nodes which drain the nasal mucosa abrogated nasal tolerance induction[59]. Specifically, it has been shown that CD11b$^+$ DCs are important for oral tolerance in a collagen-induced arthritis model[60].

APCs involved in mucosal tolerance are mainly macrophages and DCs in the lungs[61], along with B cells, DCs, and macrophages in the nasal- and gut-associated lymphoid tissues[62-64]. Other DCs involved in nasal and oral tolerance express the inhibitory Fc receptor for IgG IIB (FcγRIIB)[65,66]. FcγRIIB plays a key role in DC uptake, processing, and presentation of antigens[67] and a loss of this receptor has been shown to induce autoimmunity in mice[68]. Another group of immune-suppressing mucosal DCs are CD103$^+$ DCs, which can induce antigen-specific FoxP3$^+$ Tregs[69,70]. Collectively, these studies show that there are several subtypes of APCs in the liver, spleen, and oral and mucosal lymphoid tissues which are specialized to induce tolerance and are therefore attractive for targeting nanoparticles. We will highlight several strategies by which this targeting can be achieved.

Passive Targeting

2.1 Physicochemical properties

2.1.1 Charge

One of the great advantages of nanoparticles is that their physicochemical properties can be optimized to the application. Depending on their physicochemical properties, nanoparticles can elicit different immune responses. In the case of pro-inflammatory responses, this is generally achieved by mimicking pathogens[41]. However, for tolerance induction, an attractive strategy would be to mimic apoptotic cells. Such apoptotic-like particles would efficiently be taken up directly or via the protein corona and processed through the tolerance-promoting effector mechanisms in the spleen and liver. Nanoparticle charge is one of the easiest and most effective ways to achieve this. When cells undergo apoptosis, they express the negatively charged phospholipid phosphatidylserine (PS) on their surface[71], which is recognized by receptors on efferocytes, such as stabilin-2[72], TIM-4[73], and CD300f[74,75], as shown in *Figure 1*. The role of PS in apoptosis is extensively reviewed by Birge et al. and several types of nanoparticles containing PS have taken advantage of this pathway to induce tolerance in autoimmune models[76,77]. Unfortunately, empty PS liposomes have been shown to induce non-specific immune tolerance which may hamper clinical application[35,78]. Anionic phosphatidylglycerol (PG)-containing liposomes encapsulating an atherosclerosis-specific peptide significantly reduced disease progression in an atherosclerotic mouse model. However, the same liposomes without antigen

did not have any effect on the disease, meaning that this effect was antigen-specific. The PG-liposomes were more effective at inducing antigen-specific Treg responses than PS-liposomes, even though both had similar surface charges[79]. This was hypothesized to be due to the formation of a protein corona, specifically C1q binding to the PG-liposomes, which has been shown to have a tolerogenic effect intricately linked to the clearance of apoptotic cells by binding to scavenger receptors such as class F scavenger receptor (SR-F1)[41,75,80,81]. In subsequent studies, it was observed that the liposomes are selectively taken up by APCs in the liver and spleen (unpublished data). In another study, anionic poly(ethylene-co-maleic acid)-poly(lactic-co-glycolic acid) (PEMA-PLGA) nanoparticles encapsulating EAE peptides upregulated PD-L1 expression on liver CD103$^+$ DCs and Kupffer cells. This in turn led to antigen-specific lymphocyte unresponsiveness, as measured by reduced proliferation of lymphocytes and reduced production of IL-17, GM-CSF, and IFNγ. The nanoparticles could both prevent and treat EAE in mice. This effect was even observed after splenectomy, underlining the importance of the liver in this model[82]. Interestingly, protection against EAE was induced after i.v. injection, and to a lesser extent i.p. injection of nanoparticles, but not after oral or s.c. administration. This was hypothesized to be because these nanoparticles need to travel to the liver and spleen to exert their effects[83].

Splenic macrophages can also be targeted by negatively charged particles[47]. PLGA particles coupled to encephalitogenic peptides were localized to the splenic marginal zone after i.v. injection and prevented and treated EAE in mice[84]. I.v. injection of polystyrene beads coupled to zinc transporter 8- and islet-specific glucose-6-phosphatase catalytic subunit-related protein-derived peptides could induce antigen-specific tolerance in HLA-A2.1 transgenic mice (HHD) and prevent the development of diabetes in humanized NOD.β2m null HHD mice. The nanoparticles could also suppress antigen-specific CD8$^+$ T cell responses in PBMCs from individuals with type 1 diabetes. Specifically, Siglec-1$^+$ macrophages and marginal zone DCs produced CCL22 upon nanoparticle uptake, which mediated FoxP3$^+$ Treg and CD103$^+$ DC chemotaxis via CCR4[85].

LSECs can also be targeted by negatively charged particles since stabilin receptors on LSECs preferentially bind negatively charged particles[86,87]. For instance, Carambia et al. designed anionic iron oxide nanoparticles with a poly(maleic anhydride-alt-1-octadecene)-coat. The particles were rapidly cleared from the plasma after i.v. injection and accumulated together with their antigen load in LSECs which led to an increase in FoxP3$^+$ Tregs. The nanoparticles induced long-lasting protection to EAE and could even cure EAE in mice. The authors point out that in-depth analysis of the phenotype of nanoparticle-induced Tregs was lacking, as it was not possible to separate nanoparticle-induced Tregs from endogenously induced Tregs[88]. Similarly, Saito et al. showed that PEMA-coated polylactide (PLA) nanoparticles accumulated in the liver and spleen after i.v. injection and were mainly associated with Kupffer cells and LSECs in the liver, which showed a reduction in CD86 expression. *Ex vivo* restimulation of splenocytes with the relevant antigen ($PLP_{139-151}$) resulted in reduced production of IFNγ, IL-17, GM-CSF, TNFα, IL-2 and increased IL-10 in mice immunized with PLP nanoparticles as compared to mice injected with OVA nanoparticles. The PLP nanoparticles prevented EAE in mice[89].

The nanoparticle charge also influences targeting in mucosal tissues. Frome et al. developed anionic and cationic rod-shaped nanoparticles of 80 nm x 320 nm in size, functionalized to ovalbumin. After delivery into the lungs of mice by orotracheal instillation, anionic nanoparticles were immunologically inert, while the cationic particles were pro-inflammatory, as demonstrated by higher gene expression of CCL2, IL-10, IL-2, IL-6, CXCL10, IFNγ and IL-12β in homogenized lung cells. This was hypothesized to be because the anionic nanoparticles were taken up by alveolar macrophages, which maintain tissue homeostasis, while the cationic nanoparticles were taken up by lung DCs[90].

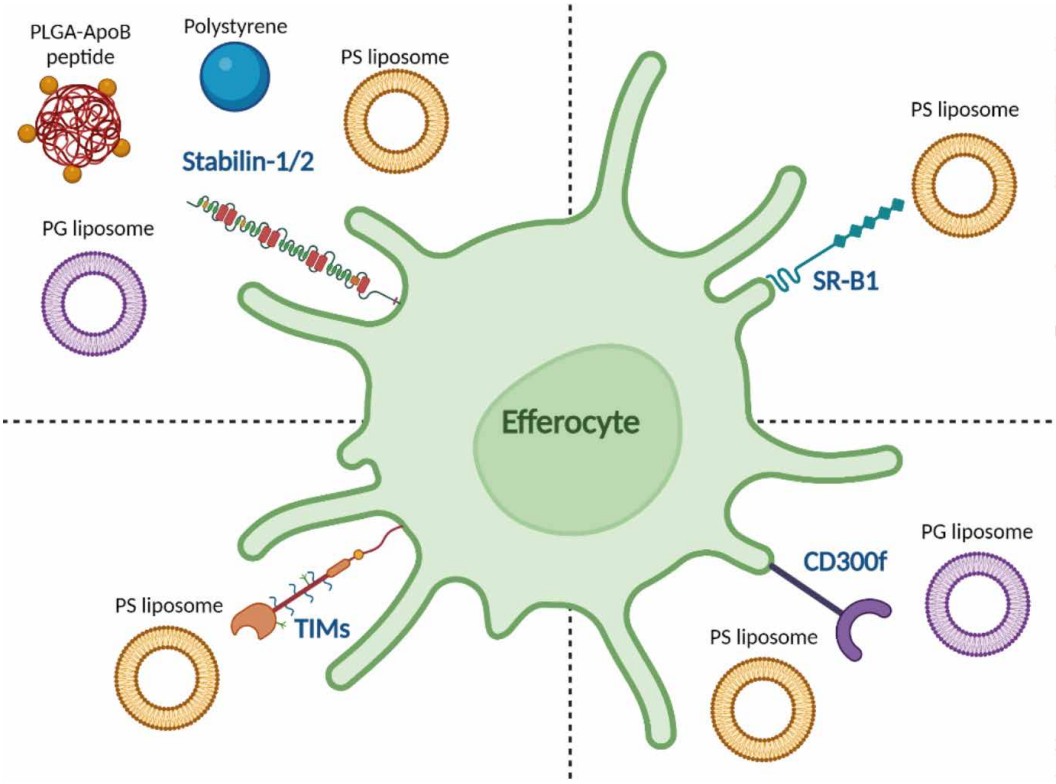


Figure 1. Reported binding of tolerance-inducing nanoparticles to efferocytic receptors on APCs.
The stabilin-1/2 receptor was reported to bind to anionic polystyrene beads, apolipoprotein B (ApoB)-peptide-functionalized poly(lactic-co-glycolic acid) (PLGA) nanoparticles, and anionic phosphatidylserine (PS) and phosphatidylglycerol (PG) liposomes. The T cell/transmembrane, immunoglobulin, and mucin (TIM) receptor family recognize PS liposomes as does scavenger receptor class B type 1 (SR-B1). CD300f recognizes both PS and PG liposomes.

2.1.2 Other physicochemical properties

Apart from particle charge, the size of the nanoparticles can also determine their biodistribution and uptake by immune cells, as reviewed elsewhere[91]. Specifically, concerning targeting tolerance-inducing cell subsets, it was shown that nanoparticles between 79 and 199 nm in size efficiently delivered siRNA to LSECs after i.v. injection, while 420 nm particles did not[92]. Particle size is also important for mucosal tolerance induction; i.v. injection of 15 µm, but not 400 nm-sized PLGA nanoparticles led to the retention of these particles in the lungs. While both particles encapsulating PLP were able to prevent EAE development in a mouse model compared to untreated control, the larger particles were more effective[93]. In addition, intratracheal administration of glycine-coated polystyrene nanoparticles of 50 nm in size, but not 500 nm were taken up by $CD103^+$ DCs in the lungs[26,94]. Finally, 300 nm PLGA nanoparticles encapsulating type II collagen were found in the Peyer's patches of mice after oral administration. The nanoparticles were able to significantly reduce arthritis scores in a collagen-induced arthritis mouse model. This coincided with reduced circulating anti-type II collagen IgG antibodies, reduced proliferation of draining lymph node lymphocytes after type II collagen restimulation, increased gene expression of TGF-β in Peyer's patches, and decreased gene expression of TNFα in draining lymph nodes in mice immunized with antigen-loaded nanoparticles compared to placebo[55].

The rigidity of nanoparticles is gaining increasing attention as another important physicochemical parameter for tolerance induction[95]. For instance, the FcγRIIB$^+$ DCs involved in mucosal tolerance[65,66] are sensitive to particle rigidity[96]. Particle rigidity also influences whether circulating particles can reach sterically obscured cells[97] such as LSECs.

Interestingly, polyethylene glycol (PEG)ylation of PLGA nanoparticles can have profound effects on tolerance induction. Comparing PLGA-PEG to PLGA nanoparticles with identical size and charge, Li et al. show that PEGylation induced lower complement activation, neutrophil recruitment, and co-stimulatory molecule expression on DCs around the injection site after s.c. injection[98].

A unique challenge for the induction of mucosal tolerance is that many antigens are not mucoadhesive. Mucoadhesiveness of antigens is highly dependent on several factors such as hydrophilicity, molecular weight, charge, and chemical structure[99]. Nanoparticles designed to be mucoadhesive can overcome this problem, and some of the properties that affect the mucoadhesion of formulations are charge, spreadability/rigidity, and ability to bind to the mucus substrate[100]. For example, sublingual administration of ovalbumin adsorbed on mucoadhesive polymerized maltodextrin nanoparticles showed therapeutic tolerance in an ovalbumin-induced allergic mouse model, which was not observed with free ovalbumin. Unfortunately, cellular mechanisms were not reported in this study[101]. Pulmonary administration of an EAE antigen with hyaluronic acid (a mucoadhesive[102]) abrogated EAE in mice[103], and peanut-induced anaphylaxis was only inhibited in mice receiving intranasal administration of a nanoemulsion with peanut extract, but not free extract[104]. Finally, while only antigen-loaded PLGA nanoparticles increased FoxP3 gene expression in cervical lymph nodes and suppressed delayed-type hypersensitivity response in mice, intranasal administration of both antigen-loaded PLGA and PLGA-TMC (N-trimethyl chitosan, a mucoadhesive) nanoparticles suppressed proteoglycan-induced arthritis in an antigen-specific manner. The authors hypothesize that the discrepancy between the models could be explained by the chronic nature of the arthritis model compared to the delayed-type hypersensitivity model[105]. Collectively, these studies show that the physicochemical properties of nanoparticles can be tuned to induce tolerance by targeting specific organs and/or cell subsets.

Active Targeting

Aside from targeting cell subsets via the physicochemical properties of nanoparticles, particles can be designed to actively target cells via the use of targeting moieties[106]. The use of targeting antibodies for tolerance induction was extensively reviewed by Castenmiller et-al.[28]. Functionalization of PLGA nanoparticles with an ApoB peptide (a ligand for the stabilin-1 and stabilin-2 receptors expressed on LSECs) led to higher uptake by LSECs *in vivo* as compared to bare PLGA nanoparticles or mannan (another ligand for LSECs) nanoparticles. I.v. injection of the ApoB nanoparticles induced high TGF-β production by LSECs which coincided with an increase in FoxP3$^+$ Tregs in the lungs. The nanoparticles could prevent and treat allergic symptoms in a pulmonary allergen sensitization model in mice[107]. Another cell type, CD8$^+$ CD205$^+$ DCs, can induce FoxP3$^+$ Tregs from FoxP3$^-$ precursors in mice in the presence of a low dose of antigen[108]. There is evidence that is important in the recognition of apoptotic cells[109]. Targeting antigens to CD205$^+$ cells in the spleen using anti-antibodies has shown to be effective at deletion of autoreactive CD8$^+$ T cells[110], and at inducing tolerance in several autoimmune disease models[111-112]. Targeting Siglec-1 and other C-type lectin receptors can be achieved by glycosylated molecules[113], as reviewed elsewhere[114]. Regarding mucosal tolerance, reports of specific targeting of antigens to CD103$^+$ DCs using antibodies are divided amongst both pro-and anti-inflammatory responses, and co-administration of a pro-inflammatory adjuvant abrogates the tolerogenic effects of CD103$^+$ DCs[115]. Targeting FcγRIIB with specific antibodies has been used to induce tolerance[116,117], but

so far these targeting antibodies have not been combined with nanoparticles, although this approach would be promising. Using targeting moieties is an attractive strategy to target APC subsets, but care must be taken to avoid the production of anti-drug antibodies against the targeting ligand[118].

Immunomodulators

It may not always be necessary to target antigens to a specific APC subset. After systemic injection, APCs rapidly take up nanoparticles. If a nanoparticle encapsulates an antigen together with an immunomodulator, the immunomodulator can direct the APC towards a tolerogenic phenotype. Several studies demonstrate that co-encapsulation of an antigen with an immunomodulator (e.g., rapamycin, calcitriol, aryl hydrocarbon receptor ligands, or NF-κB inhibitors) can ameliorate autoimmunity, whilst free antigen does not[119-129]. For example, s.c. injections of nanoparticles encapsulating MOG35-55 and dexamethasone significantly treated EAE in mice as compared to empty nanoparticles, dexamethasone nanoparticles, MOG_{35-55} nanoparticles, or free dexamethasone and MOG_{35-55}. After *ex vivo* restimulation of splenocytes with MOG_{35-55} only spleens of mice that received the nanoparticles encapsulating MOG_{35-55} and dexamethasone had reduced IL-17 and GM-CSF production[130]. In another study s.c. injection of retinoic acid/TGF-β/insulin peptide-encapsulating PLG A microparticles led to uptake by $CD11c^+$ splenic DCs, and a significant increase in $B220^+CD19^+CD1d^+CD5^+$ Bregs, but not $CD4^+CD25^+FoxP3^+$ Tregs in the mesenteric lymph nodes, as compared to control mice. These effects were acute (3 days after particle administration), and it is unknown how longlasting they are. The particles could prevent diabetes in NOD mice, while the administration of free retinoic acid or TGF-β could not[131]. There is also evidence that co-encapsulation is not always necessary. Lewis et al. prepared a mixture of distinct PLGA nano- and microparticles, namely vitamin D3 (1000 nm, phagocytosable), denatured human recombinant insulin (1000 nm), TGF-β1 (30 µm, non-phagocytosable), and GM-CSF (30 µm). When NOD mice were injected s.c. with the mix of particles, the phagocytosable particles were found in the paracortex of the draining lymph nodes and associated mainly with DCs. The particle-positive DCs expressed high PD-L1 and BTLA. In diabetic mice, the particle mixture enhanced $FoxP3^+$ Tregs in the spleen and pancreatic lymph nodes and increased PD-1 on $CD4^+$ and $CD8^+$ T cells. Treatment with all particles administered s.c. significantly prevented and treated diabetes in NOD mice[132]. All these studies demonstrate that the immunomodulator enables efficient tolerance induction even without active targeting specific organs or cellular subsets. This is underlined by the fact that these studies reported nanoparticle uptake by multiple subsets of DCs and macrophages in the spleen, lymph nodes, and liver, which gained a tolerogenic phenotype and were able to induce antigen-specific T cell tolerance.

While the current studies are very encouraging it is unclear whether immunomodulators that leak from formulations pose a safety concern by inducing non-specific immune suppression. Luo et al. proposed a nanoparticulate approach that could circumvent this problem. They prepared PLGA-PEG nanoparticles encapsulating the diabetes-specific peptide 2.5mi, together with a CRISPR-Cas9 plasmid and guide RNAs for CD80, CD86, and CD40. Upon i.v. administration, $MHC\text{-}II^+CD11c^+$ DCs in the lymph nodes, spleen, and blood took up these nanoparticles, and presented the peptide in the absence of costimulatory molecules, leading to an antigen-specific $FoxP3^+$ Treg response. Only the complete formulation significantly reduced diabetes incidence in NOD/Ltj mice[133]. In a similar but simpler approach, Krienke et al. made liposomes carrying mRNA coding for disease-relevant antigens that were specifically modified to suppress immune activation. The liposomes were taken up by different splenic $CD11c^+$ DCs after i.v. injection. The DCs presented the antigen to T cells in the absence of costimulatory molecules which resulted in antigen-specific $FoxP3^+$ Treg expansion and could prevent and treat EAE in mice. Specifically, MOG_{35-55} specific splenic $CD4^+$ T cells from mice treated with the liposomes had high

expression of inhibitory markers CD5, ICOS, LAG-3, PD-1, CTLA-4, TIGIT, and TIM-3[134]. These studies show that, if the nanoparticle is tolerance-inducing, either by the inclusion of immunomodulators or other methods, it can even skew pro-inflammatory APC subsets towards tolerance.

Translation to human

While there are many promising pre-clinical studies with nanoparticles, translation of animal models to human patients is a difficult challenge. It is not fully understood why the translation often fails but can be due to multiple factors. For instance, the protein corona that forms around a nanoparticle after administration can differ between species, which may affect the stability, toxicity, and biodistribution of the nanoparticles[135,136]. Furthermore, there may be differences in phagocytosis of nanoparticles among species; comparing dogs, humans, and several strains of rats and mice, it was found that opsonization of dextran-coated iron oxide nanoparticles occurred mainly via the alternative complement pathway in humans, while in the other species this was dependent on Ca^{2+}-sensitive pathways[137]. Another study tested a wide range of lipid nanoparticles containing mRNA in mice with humanized livers, primatized livers, or "murinized" livers. It was found that mRNA delivery was more efficient to human hepatocytes and primate hepatocytes compared to murine hepatocytes and that there was a discrepancy between the most efficient lipid nanoparticles for delivery to murine vs. human hepatocytes, leading to false positives or negatives.

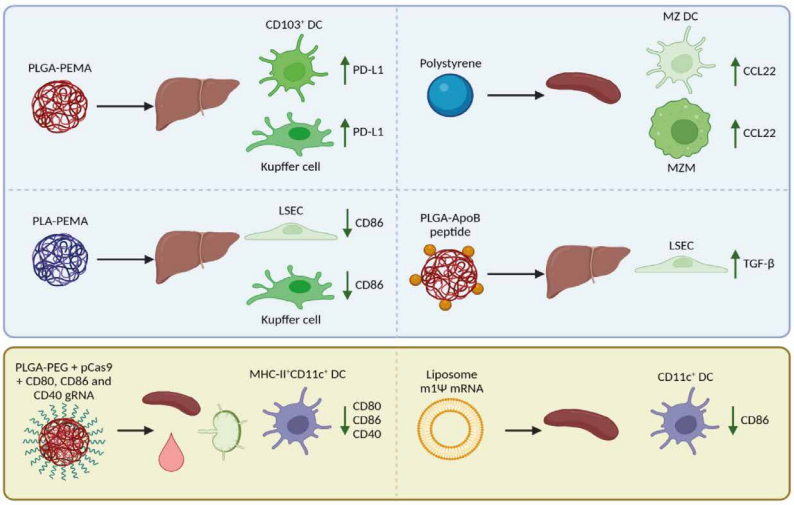


Figure 2. Reported biodistribution, cellular uptake, and FoxP3⁺ regulatory T cell (Treg)-inducing mechanisms in mice after i.v. injection of nanoparticles encapsulating an antigen (peptide/mRNA). The blue box (top) summarizes nanoparticles that target natural tolerogenic antigen-presenting cells in the liver and spleen including dendritic cells (DCs), macrophages, and liver sinusoidal endothelial cells (LSECs) to enhance tolerance. After uptake of nanoparticles, these cells upregulate inhibitory receptors, downregulate costimulatory molecules, produce chemokines that attract Tregs, or cytokines that induce Tregs. The yellow box (bottom) summarizes nanoparticles that are taken up by a wide range of APCs including DCs in the spleen, lymph nodes, and blood. The nanoparticles are designed to inhibit co-stimulation in target cells so that the antigen can be presented in a tolerogenic manner and induce Tregs. PLGA = poly(lactic-co-glycolic acid), PLA = polylactic acid, PEMA = poly(ethylene-co-maleic acid), ApoB = apolipoprotein B, PEG = polyethylene glycol, MZ = marginal zone, m1Ψ mRNA = 1 methylpseudouridine-modified messenger RNA, PD-L1 = programmed death-ligand 1, CCL22 = C-C motif chemokine 22, TGF-β = Transforming growth factor β.

Transcriptomic analysis revealed that in human hepatocytes clathrin-mediated endocytosis was increased while caveolin-mediated endocytosis was decreased after lipid nanoparticle administration,

while in mice this was reversed[138]. Finally, there are many different models for autoimmunity which have varying degrees of similarity to human patients, which can make the translation to humans difficult[6,139]. For translation from pre-clinical to clinical trials, humanized mice may be useful for studies with nanoparticles, and while some of the studies highlighted in this review use humanized mice[44,85], this is not standard practice.

To further complicate translation, immune cell number, phenotype, and function can differ between healthy individuals and patients. For example, the DCs in the pancreatic lymph nodes of type I diabetes patients may have reduced tolerogenic function than those of healthy controls, and there were even differences in lymph node cell composition when correcting for sex and age[140]. T cells, B cells, DCs, and other lymph node cells are also shown to be different in rheumatoid arthritis patients compared to healthy individuals[141-143]. Furthermore, efferocytosis can be defective in patients with autoimmune diseases[145,146] or obese individuals[146], so for these patients nanoparticles that aim to exploit efferocytosis may not be as effective. The tolerance-inducing strategy could be tailored towards each patient individually if the immune cell function of the patient was matched to the pathway by which the nanoparticles exert their immunomodulatory effects. Encouragingly, one study directly compared nanoparticles with a targeted approach (targeting to LSECs) to a general immune-suppressing approach (rapamycin nanoparticles) in a murine airway inflammation model, and found that both approaches similarly reduced antigen-specific allergic responses[147].

Despite all of these challenges, there have been several successful phase 1 and phase 2 clinical trials using antigen-carrying nanoparticles. Lutterotti et al. coupled myelin-derived peptides to apoptotic PBMCs derived from MS patients. In this phase 1 trial, the cells were well-tolerated, and at a dose >1 x 10^9 cells there was a decrease in T cell proliferation in response to restimulation with the peptides[148]. Another phase 1 clinical trial used CD206-targeting mannosylated liposomes carrying myelin-derived peptides for the treatment of MS. The liposomes were well-tolerated, and patients showed decreased serum concentrations of IL-7, IL-2, CCL2, and CCL4, while TNFα increased[149,150]. Finally, PLGA nanoparticles encapsulating gluten protein were tested in phase 1 and phase 2a trials in patients with celiac disease. The nanoparticles were well-tolerated and significantly reduced antigen-specific IFNγ production. After oral gluten challenge, the patients that received the nanoparticles showed reduced percentages of circulating $CD4^+CD38^+\alpha4\beta7^+$, $CD8^+CD38^+\alpha E\beta7^+$, and $\gamma\delta^+CD38^+\alpha E\beta7^+$ T cells compared to patients that received placebo[151]. These promising results show that there are good prospects for antigen-carrying nanoparticles to treat autoimmunity in human patients.

Table 1. Overview of antigen-carrying nanoparticles that resulted in immune suppression in autoimmune disease models

Composition	Type	Physico-chemical properties	Antigen	Immuno-modulator	Targe-ting mo-lecule	Route	Disease model	Ref
Soybean oil, cetylpyridinium chloride	Nanoemulsion	350-400 nm	Peanut extract	-	-	i.n.	C3H/HeJ mice sensitized to peanut	104
PLGA	Polymeric	320 nm, -48.2 mV	Hsp70-mB29a	-	-	i.n.	BALB/c mice with proteoglycan-induced arthritis	105
PLGA-N-trimethyl chitosan	Polymeric	448 nm, 24.5 mV	Hsp70-mB29a	-	-	i.n.	BALB/c mice with proteoglycan-induced arthritis	105
DOPS[a], DLPC[b] and cholesterol	Liposome	628 to 712 nm, -44.9 to -46.6 mV	$Insulin_{90–110}$ (A chain) and $Insulin_{25–54}$ (B chain	-	-	i.p.	Diabetes-prone NOD mic	77
DOTMA[c] and DOPE[d]	Liposome	300 nm, -30 mV	MOG_{35-55} -encoding mRNA	-	-	i.v.	C57BL/6 mice with MOG_{35-55}–induced EAE	134
DSPC[e], DSPG[f] and cholesterol	Liposome	168.9 nm, -55.9 mV	$ApoB100_{3500-3514}$	-	-	i.p.	LDLr-/- mice with western-type diet-induced atherosclerosis	79
PLA-PEMA	Polymeric	443.2 nm, -40.2 mV	$PLP_{139-151}$	-	-	i.v.	SJL/J mice with $LP_{139-151}$-induced EAE	89
PLGA	Polymeric	397.5 to 605 nm, -38 to -42.8 mV	$PLP_{139-151}$	-	-	i.v.	SJL/J mice with $LP_{139-151}$-induced EAE	27, 84
PLGA-PEMA	Polymeric	377.9 to 695.6 nm, -46.9 to -72.7 mV	Several peptides/proteins	-	-	i.v.	Diabetes-prone NOD mice, C57BL/6 mice with gliadin-induced celiac disease, SJL/J mice with $LP_{139-151}$-induced EAE	2,83, 153, 154
Polystyrene	Polymeric	500 nm, anionic	A mixture of HLAA*02:01-restricted epitopes	-	-	i.v.	Humanized diabetes-prone NOD.β2m-deficient HHD mice	85
PLGA	Polymeric	299.7 nm, anionic	Type II collagen	-	-	oral	DBA/1 mice with collagen-induced arthritis	55
LysoPS[g] and DMPC[h]	Liposome	169.7 nm, -14.96 mV	FVIII protein	-	-	s.c.	Hemophilia A mice	155
PLGA-PEG	Polymeric	286 nm, -23.2 mV	MOG_{35-55}	-	-	s.c.	C57BL/6 mice with MOG_{35-55}–induced EAE	98
Maltodextrin	Polysaccharidic	60 nm, cationic	Ovalbumin	-	-	sublingual	Ovalbumin-sensitized Balb/c mice	101
PLGA	Polymeric	270 nm,-8.63 to -4.56 mV	$OVA_{323-339}$	-	ApoB peptide	i.v.	C57BL/6 mice with OVA-induced allergy	107
Hyaluronic acid	Polysaccharidic	3 to 10 nm, anionic	$PLP_{139-151}$	-	LABL peptide	pulmonary	SJL/J mice with $PLP_{139-151}$-induced EAE	103
Superparamagnetic iron oxide core with poly(maleic anhydrie-alt-1-octadecene) coat	Metal	10 nm, -61.6 mV	MBP or MOG peptide	-	poly(-maleic anhydrie-alt-1-tadecene)	i.v.	B10.PL and tg4 mice with MBP peptide-induced EAE and C57BL/6 mice with MOG-induced EAE	88

Composition	Type	Physico-chemical properties	Antigen	Immuno-modulator	Targe-ting mo-lecule	Route	Disease model	Ref
L-α-egg phosp-hatidylcholine and L-α-egg phosphatidyl-glycerol	Liposome	105 nm, -55 mV	Several pep-tides	Vitamin D3	-	s.c. and i.v.	BALB/c mice with proteoglycan-induced arthritis or HLA-DR15–transgenic, MHC class II$^{-/-}$Fcgr2b$^{-/-}$ mice with $\alpha3_{135–145}$ -induced autoimmune Goodpasture's vasculitis, Diabetes-prone NOD mice	125, 126
Acetalated dextran	Polysaccha-ridic	111 to 127 nm	MOG_{35-55}	Dexame-thasone	-	s.c.	C57Bl/6 mice with MOG_{35-55}-induced EAE	130
PEG-Gold	Metal	60 nm	MOG_{35-55}	ITE	-	i.p. and i.v.	C57BL/6 mice with MOG_{35-55}-induced EAE, diabetes-prone NOD mice	123, 124
Egg phosphati-dylcholine	Liposome	Not reported	mBSA	NF-KB inhibitor	-	s.c.	C57BL/6 mice with mB-SA-induced arthritis	122
PLGA-PEG and N,N-bis(2-hy-droxyethy-l)-N-met-hyl-N-(2-chole-steryoxycarbo-nyl-aminoethyl) ammonium bromide	Lipid-assisted polymeric	138 nm, 23 mV	2.5mi	pCas9 and gRNAs targeting CD80, CD86, and CD40	-	i.v.	Diabetes-prone NOD mice	123
Calcium phosphate and dioleoylphosp-hatydic acid nanoparticles coated with DO-PE-PEG, DOPC[i], and cholesterol	Lipid-coated calcium phosphate	180 nm, -6 mV	Citrullinated peptides deri-ved from type II collagen, fibrinogen, vimentin, and fibronectin	Rapamycin	-	i.v.	Wistar rats with colla-gen-induced arthritis	129
PLGA and PLA-PEG	Polymeric	Not reported	Several pepti-des/proteins	Rapamycin	-	i.v.	SJL/J mice with $PLP_{139-151}$-induced EAE, BALB/c mice with OVA-in-duced allergy, and FVIII$^{-/-}$ hemophilic mice	119-121
Surface nic-kel-formulated PLGA	Polymeric	1088.6 nm	Insulin $_{B9-23}$	Retinoic acid, TGF-β	-	s.c.	Diabetes-prone NOD mice	131
PLGA	Polymeric	30 μm and 1 μm	Denatured insulin	Vitamin D3, TGF-β1, GM-CSF	-	s.c.	Diabetes-prone NOD mice	132

[a] 1,2-dioleoyl-sn-glycero-3-phospho-l-serine

[b] 1,2-didodecanoyl-sn-glycero-3-phosphocholine

[c] 1,2-di-O-octadecenyl-3-trimethylammonium propane

[d] 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine

[e] 1,2-distearoyl-sn-glycero-3-phosphocholine

[f] 1,2-distearoyl-sn-glycero-3-phosphoglycerol

[g] Lyso-phosphatidylserine

[h] Dimyristoyl phosphatidylcholine

[i] 1,2-dioleoyl-sn-glycero-3-phosphocholin

Discussion

Current treatments of autoimmune diseases are focused on the management of symptoms using non-antigen-specific anti-inflammatory drugs. These drugs require life-long adherence and can be expensive, especially in the case of the biologicals, while potentially causing severe side effects[152]. So far there is no cure for such diseases. Therefore, there is an urgent need to develop antigen-specific treatments for patients, which can induce long-lasting immune tolerance without causing general immune suppression. In this review, we have highlighted different strategies in which nanoparticles can be used to induce antigen-specific tolerance to treat autoimmune diseases and summarized these in *Table 1*. The most commonly used nanoparticles for passive targeting of antigens to APCs are polymeric nanoparticles and liposomes. The most well-studied biodegradable polymeric particles are (PEGylated) PLGA nanoparticles. Several promising studies also use PEMA coating to enhance the efferocytic effects. In studies that prevent and/or treat autoimmunity, the polymeric nanoparticles are anionic and generally around 300 nm in size. For liposomal nanoparticles that carry antigens, again anionic charge, provided by e.g. PG or PS is necessary for tolerance induction. The liposomes further generally contain the zwitterionic helper lipid PC and may contain cholesterol. They can range in size from around 150 nm up to 700 nm. On the other hand, mRNA requires cationic lipids for complexation, so for mRNA delivery, cationic lipids such as DOTMA and DOTAP are commonly used.

Once a targeting molecule is included in the formulation, the physicochemical properties of the nanoparticles may be less important. Similarly, for nanoparticles that co-encapsulate antigens and immunomodulators, there is a wider range of materials and physicochemical properties that can be used, as targeting specific APC subsets is not the goal. Here, the main focus is on the stability of the particle and the successful encapsulation and retention of the cargo inside the particle.

There have been reports of tolerance induction using antigen-free nanoparticles[35,78,94], which can be unfavorable if it leads to general immune suppression. Furthermore, while the incorporation of immunomodulators into nanoparticles is effective, care should be taken that the immunomodulator does not leak from the nanoparticle before reaching the site of action. Therefore, nanoparticle immunotherapy should always be tested for non-antigen-specific effects, and in the case of co-encapsulation of an immunomodulator *in vivo*, stability of the immunomodulator should be evaluated. In the case of using targeting ligands on the surface of nanoparticles, there is a risk for the production of anti-drug antibodies against the targeting ligand, which can abrogate the targeting effects of thenanoparticle, or in severe cases even lead to an unwanted immune response[118]. In this case, the prevention of the production of such antibodies e.g. by optimal dosing schemes needs to be taken into account[156].

Where reported in *in vivo* studies, we have outlined the biodistribution of nanoparticles, uptake by APC subsets, and immunological mechanisms for tolerance induction (summarized in *Figure 2*). However, not all studies have examined these aspects, and there is still much to be gained from research that describes well-characterized nanoparticles and their effects on the uptake by APC subsets and subsequent induced immune responses.

Author Contributions

Conceptualization	Naomi Benne, **Daniëlle ter Braake**, Arie Jan Stoppelenburg, Femke Broere
Funding Acquisition	Femke Broere
Literature Review	Naomi Benne, **Daniëlle ter Braake**, Arie Jan Stoppelenburg
Supervision	Femke Broere
Visualization	Naomi Benne
Writing – Original Draft Preparation	Naomi Benne, **Daniëlle ter Braake**, Arie Jan Stoppelenburg,
Writing – Review & Editing	Naomi Benne, **Daniëlle ter Braake**, Arie Jan Stoppelenburg, Femke Broere

Contribution **Author**

References

1. Chaplin, D. D. Overview of the immune response. J Allergy Clin Immunol 125, S3-23 (2010). https://doi.org:10.1016/j.jaci.2009.12.980.
2. Pozsgay, J., Szekanecz, Z. & Sarmay, G. Antigen-specific immunotherapies in rheumatic diseases. Nature reviews. Rheumatology 13, 525-537 (2017). https://doi.org:10.1038/nrrheum.2017.107.
3. Avdeeva, A., Rubtsov, Y., Dyikanov, D., Popkova, T. & Nasonov, E. Regulatory T cells in patients with early untreated rheumatoid arthritis: Phenotypic changes in the course of methotrexate treatment. *Biochimie* 174, 9-17 (2020). https://doi.org:10.1016/j.biochi.2020.03.014.
4. Jordan, M. S. et al. Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat Immunol* 2, 301-306 (2001). https://doi.org:10.1038/86302.
5. Schmitt, E. G. & Williams, C. B. Generation and function of induced regulatory T cells. *Front Immunol* 4, 152 (2013). https://doi.org:10.3389/fimmu.2013.00152.
6. Hahn, S. A., Bellinghausen, I., Trinschek, B. & Becker, C. Translating Treg Therapy in Humanized Mice. *Front Immunol* 6, 623 (2015). https://doi.org:10.3389/fimmu.2015.00623.
7. Ohkura, N. & Sakaguchi, S. Transcriptional and epigenetic basis of Treg cell development and function: its genetic anomalies or variations in autoimmune diseases. *Cell Res* 30, 465-474 (2020). https://doi.org:10.1038/s41422-020-0324-7.
8. Shevyrev, D. & Tereshchenko, V. Treg Heterogeneity, Function, and Homeostasis. *Front Immunol* 10, 3100 (2019). https://doi.org:10.3389/fimmu.2019.03100.
9. Gagliani, N. et al. Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells. *Nat Med* 19, 739-746 (2013). https://doi.org:10.1038/nm.3179.
10. Carrier, Y., Yuan, J., Kuchroo, V. K. & Weiner, H. L. Th3 cells in peripheral tolerance. I. Induction of FoxP3-positive regulatory T cells by Th3 cells derived from TGF-beta T cell-transgenic mice. J *Immunol* 178, 179-185 (2007). https://doi.org:10.4049/jimmunol.178.1.179.
11. Ran, Z., Yue-Bei, L., Qiu-Ming, Z. & Huan, Y. Regulatory B Cells and Its Role in Central Nervous System Inflammatory Demyelinating Diseases. *Front Immunol* 11, 1884 (2020). https://doi.org:10.3389/fimmu.2020.01884.
12. Catalan, D. et al. Immunosuppressive Mechanisms of Regulatory B Cells. *Front Immunol* 12, 611795 (2021). https://doi.org:10.3389/fimmu.2021.611795.
13. McInnes, I. B. & Schett, G. The pathogenesis of rheumatoid arthritis. *N Engl J Med* 365, 2205-2219 (2011). https://doi.org:10.1056/NEJMra1004965.
14. Schinnerling, K., Aguillon, J. C., Catalan, D. & Soto, L. The role of interleukin-6 signalling and its therapeutic blockage in skewing the T cell balance in rheumatoid arthritis. *Clin Exp Immunol* 189, 12-20 (2017). https://doi.org:10.1111/cei.12966.
15. Kerschbaumer, A. et al. Efficacy of pharmacological treatment in rheumatoid arthritis: a systematic literature research informing the 2019 update of the EULAR recommendations for management of rheumatoid arthritis. *Ann Rheum Dis* 79, 744-759 (2020). https://doi.org:10.1136/annrheumdis-2019-216656.
16. Wang, W., Zhou, H. & Liu, L. Side effects of methotrexate therapy for rheumatoid arthritis: A systematic review. *Eur J Med Chem* 158, 502-516 (2018). https://doi.org:10.1016/j.ejmech.2018.09.027.
17. Fang, G., Zhang, Q., Pang, Y., Thu, H. E. & Hussain, Z. Nanomedicines for improved targetability to inflamed synovium for treatment of rheumatoid arthritis: Multi-functionalization as an emerging strategy to optimize therapeutic efficacy. *J Control Release* 303, 181-208 (2019). https://doi.org:10.1016/j.jconrel.2019.04.027.

18. Keijzer, C., van der Zee, R., van Eden, W. & Broere, F. Treg inducing adjuvants for therapeutic vaccination against chronic inflammatory diseases. *Front Immunol* 4, 245 (2013). https://doi.org:10.3389/fimmu.2013.00245.
19. Griffin, J. D., Song, J. Y., Sestak, J. O., DeKosky, B. J. & Berkland, C. J. Linking autoantigen properties to mechanisms of immunity. *Adv Drug Deliv Rev* 165-166, 105-116 (2020). https://doi.org:10.1016/j.addr.2020.04.005.
20. Umeshappa, C. S. et al. Suppression of a broad spectrum of liver autoimmune pathologies by single peptide-MHC-based nanomedicines. *Nat Commun* 10, 2150 (2019). https://doi.org:10.1038/s41467-019-09893-5.
21. Serra, P. & Santamaria, P. Peptide-MHC-Based Nanomedicines for the Treatment of Autoimmunity: Engineering, Mechanisms, and Diseases. *Front Immunol* 11, 621774 (2020). https://doi.org:10.3389/fimmu.2020.621774.
22. Smith, D. M., Simon, J. K. & Baker, J. R., Jr. Applications of nanotechnology for immunology. *Nat Rev Immunol* 13, 592-605 (2013). https://doi.org:10.1038/nri3488.
23. Tibbitt, M. W., Dahlman, J. E. & Langer, R. Emerging Frontiers in Drug Delivery. *J Am Chem Soc* 138, 704-717 (2016). https://doi.org:10.1021/jacs.5b09974.
24. Benne, N., van Duijn, J., Kuiper, J., Jiskoot, W. & Slutter, B. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. *J Control Release* 234, 124-134 (2016). https://doi.org:10.1016/j.jconrel.2016.05.033.
25. Manolova, V. et al. Nanoparticles target distinct dendritic cell populations according to their size. *Eur J Immunol* 38, 1404-1413 (2008). https://doi.org:10.1002/eji.200737984.
26. Hardy, C. L. et al. Differential uptake of nanoparticles and microparticles by pulmonary APC subsets induces discrete immunological imprints. *J Immunol* 191, 5278-5290 (2013). https://doi.org:10.4049/jimmunol.1203131.
27. Pearson, R. M. et al. Controlled Delivery of Single or Multiple Antigens in Tolerogenic Nanoparticles Using Peptide-Polymer Bioconjugates. *Mol Ther* 25, 1655-1664 (2017). https://doi.org:10.1016/j.ymthe.2017.04.015.
28. Castenmiller, C., Keumatio-Doungtsop, B. C., van Ree, R., de Jong, E. C. & van Kooyk, Y. Tolerogenic Immunotherapy: Targeting DC Surface Receptors to Induce Antigen-Specific Tolerance. *Front Immunol* 12, 643240 (2021). https://doi.org:10.3389/fimmu.2021.643240.
29. Rao, J. P. & Geckeler, K. E. Polymer nanoparticles: Preparation techniques and size-control para meters. *Prog Polym Sci* 36, 887-913 (2011). https://doi.org:10.1016/j.progpolymsci.2011.01.001
30. Kelly, K. L., Coronado, E., Zhao, L. L. & Schatz, G. C. The Optical Properties of Metal Nanoparticles: The Influence of Size, Shape, and Dielectric Environment. *The Journal of Physical Chemistry B* 107, 668-677 (2003). https://doi.org:10.1021/jp026731y.
31. Pattni, B. S., Chupin, V. V. & Torchilin, V. P. New Developments in Liposomal Drug Delivery. *Chem Rev* 115, 10938-10966 (2015). https://doi.org:10.1021/acs.chemrev.5b00046.
32. Hawkins, M. J., Soon-Shiong, P. & Desai, N. Protein nanoparticles as drug carriers in clinical medicine. *Adv Drug Deliv Rev* 60, 876-885 (2008). https://doi.org:10.1016/j.addr.2007.08.044.
33. Song, W., Musetti, S. N. & Huang, L. Nanomaterials for cancer immunotherapy. *Biomaterials* 148, 16-30 (2017). https://doi.org:10.1016/j.biomaterials.2017.09.017.
34. Tiegs, G. & Lohse, A. W. Immune tolerance: what is unique about the liver. *J Autoimmun* 34, 1-6 (2010). https://doi.org:10.1016/j.jaut.2009.08.008.
35. Hosseini, H. et al. Phosphatidylserine liposomes mimic apoptotic cells to attenuate atherosclerosis by expanding polyreactive IgM producing B1a lymphocytes. *Cardiovasc Res* 106, 443-452 (2015). https://doi.org:10.1093/cvr/cvv037.

36. Abdolmaleki, F. et al. The Role of Efferocytosis in Autoimmune Diseases. *Front Immunol* 9, 1645 (2018). https://doi.org:10.3389/fimmu.2018.01645.
37. Doran, A. C., Yurdagul, A., Jr. & Tabas, I. Efferocytosis in health and disease. *Nat Rev Immunol* 20, 254-267 (2020). https://doi.org:10.1038/s41577-019-0240-6.
38. Parcina, M., Schiller, M., Gierschke, A., Heeg, K. & Bekeredjian-Ding, I. PDC expressing CD36, CD61 and IL-10 may contribute to propagation of immune tolerance. *Autoimmunity* 42, 353-355 (2009). https://doi.org:10.1080/08916930902831969.
39. Pujol-Autonell, I. et al. Efferocytosis promotes suppressive effects on dendritic cells through prostaglandin E2 production in the context of autoimmunity. *PLoS One* 8, e63296 (2013). https://doi.org:10.1371/journal.pone.0063296.
40. Ip, W. K. & Lau, Y. L. Distinct maturation of, but not migration between, human monocyte-derived dendritic cells upon ingestion of apoptotic cells of early or late phases. *J Immunol* 173, 189-196 (2004). https://doi.org:10.4049/jimmunol.173.1.189.
41. Ramirez-Ortiz, Z. G. et al. The scavenger receptor SCARF1 mediates the clearance of apoptotic cells and prevents autoimmunity. *Nat Immunol* 14, 917-926 (2013). https://doi.org:10.1038/ni.2670.
42. Waterborg, C. E. J. et al. Protective Role of the MER Tyrosine Kinase via Efferocytosis in Rheumatoid Arthritis Models. *Front Immunol* 9, 742 (2018). https://doi.org:10.3389/fimmu.2018.00742.
43. Watkins, E. A. et al. Persistent antigen exposure via the eryptotic pathway drives terminal T cell dysfunction. *Sci Immunol* 6, eabe1801 (2021). https://doi.org:10.1126/sciimmunol.abe1801.
44. Pilon, C. et al. Human Apoptotic Cells, Generated by Extracorporeal Photopheresis, Modulate Allogeneic Immune Response. *Front Immunol* 10, 2908 (2019). https://doi.org:10.3389/fimmu.2019.02908.
45. Miyake, Y. et al. Critical role of macrophages in the marginal zone in the suppression of immune responses to apoptotic cell-associated antigens. *J Clin Invest* 117, 2268-2278 (2007). https://doi.org:10.1172/JCI31990.
46. Bronte, V. & Pittet, M. J. The spleen in local and systemic regulation of immunity. *Immunity* 39, 806-818 (2013). https://doi.org:10.1016/j.immuni.2013.10.010.
47. McGaha, T. L., Chen, Y., Ravishankar, B., van Rooijen, N. & Karlsson, M. C. Marginal zone macrophages suppress innate and adaptive immunity to apoptotic cells in the spleen. *Blood* 117, 5403-5412 (2011). https://doi.org:10.1182/blood-2010-11-320028.
48. Ravishankar, B. et al. Marginal zone CD169+ macrophages coordinate apoptotic cell-driven cellular recruitment and tolerance. *Proc Natl Acad Sci U S A* 111, 4215-4220 (2014). https://doi.org:10.1073/pnas.1320924111.
49. van Dinther, D. et al. Functional CD169 on Macrophages Mediates Interaction with Dendritic Cells for CD8(+) T Cell Cross-Priming. *Cell Rep* 22, 1484-1495 (2018). https://doi.org:10.1016/j.celrep.2018.01.021.
50. Qiu, C. H. et al. Novel subset of CD8{alpha}+ dendritic cells localized in the marginal zone is responsible for tolerance to cell-associated antigens. *J Immunol* 182, 4127-4136 (2009). https://doi.org:10.4049/jimmunol.0803364.
51. Xia, S. et al. Hepatic microenvironment programs hematopoietic progenitor differentiation into regulatory dendritic cells, maintaining liver tolerance. *Blood* 112, 3175-3185 (2008). https://doi.org:10.1182/blood-2008-05-159921.
52. Bamboat, Z. M. et al. Human liver dendritic cells promote T cell hyporesponsiveness. *J Immunol* 182, 1901-1911 (2009). https://doi.org:10.4049/jimmunol.0803404.

53. Xu, X. et al. Liver sinusoidal endothelial cells induce tolerance of autoreactive CD4+ recent thymic emigrants. *Sci Rep* 6, 19861 (2016). https://doi.org:10.1038/srep19861.
54. You, Q., Cheng, L., Kedl, R. M. & Ju, C. Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. *Hepatology* 48, 978-990 (2008). https://doi.org:10.1002/hep.22395.
55. Kim, W. U. et al. Suppression of collagen-induced arthritis by single administration of poly (lactic-co-glycolic acid) nanoparticles entrapping type II collagen: a novel treatment strategy for induction of oral tolerance. *Arthritis Rheum* 46, 1109-1120 (2002). https://doi.org:10.1002/art.10198.
56. Trentham, D. E. et al. Effects of oral administration of type II collagen on rheumatoid arthritis. *Science* 261, 1727-1730 (1993). https://doi.org:10.1126/science.8378772.
57. Buettner, M., Bornemann, M. & Bode, U. Skin tolerance is supported by the spleen. *Scand J Immunol* 77, 238-245 (2013). https://doi.org:10.1111/sji.12034.
58. Hammerschmidt, S. I. et al. Stromal mesenteric lymph node cells are essential for the generation of gut-homing T cells in vivo. *J Exp Med* 205, 2483-2490 (2008). https://doi.org:10.1084/jem.20080039.
59. Wolvers, D. A. W. et al. Intranasally induced immunological tolerance is determined by characteristics of the draining lymph nodes: Studies with OVA and human cartilage gp-39. *Journal of Immunology* 162, 1994-1998 (1999).
60. Min, S. Y. et al. Antigen-induced, tolerogenic CD11c+,CD11b+ dendritic cells are abundant in Peyer's patches during the induction of oral tolerance to type II collagen and suppress experimental collagen-induced arthritis. *Arthritis Rheum* 54, 887-898 (2006). https://doi.org:10.1002/art.21647.
61. Suzuki, T., Chow, C. W. & Downey, G. P. Role of innate immune cells and their products in lung immunopathology. *The international journal of biochemistry & cell biology* 40, 1348-1361 (2008). https://doi.org:10.1016/j.biocel.2008.01.003.
62. Takaki, H., Ichimiya, S., Matsumoto, M. & Seya, T. Mucosal Immune Response in Nasal-Associated Lymphoid Tissue upon Intranasal Administration by Adjuvants. *Journal of innate immunity* 10, 515-521 (2018). https://doi.org:10.1159/000489405.
63. Wawrzyniak, M., O'Mahony, L. & Akdis, M. Role of Regulatory Cells in Oral Tolerance. Allergy *Asthma Immunol Res* 9, 107-115 (2017). https://doi.org:10.4168/aair.2017.9.2.107.
64. Fujihashi, K. et al. Peyer's patches are required for oral tolerance to proteins. *Proceedings of the National Academy of Sciences of the United States of America* 98, 3310-3315 (2001). https://doi.org:10.1073/pnas.061412598.
65. Samsom, J. N. et al. Fc gamma RIIB regulates nasal and oral tolerance: a role for dendritic cells. *J Immunol* 174, 5279-5287 (2005). https://doi.org:10.4049/jimmunol.174.9.5279.
66. van Montfoort, N. et al. Fcgamma receptor IIb strongly regulates Fcgamma receptor-facilitated T cell activation by dendritic cells. *J Immunol* 189, 92-101 (2012). https://doi.org:10.4049/jimmunol.1103703.
67. Guilliams, M., Bruhns, P., Saeys, Y., Hammad, H. & Lambrecht, B. N. The function of Fcgamma receptors in dendritic cells and macrophages. *Nat Rev Immunol* 14, 94-108 (2014). https://doi.org:10.1038/nri3582.
68. McGaha, T. L., Karlsson, M. C. & Ravetch, J. V. FcgammaRIIB deficiency leads to autoimmunity and a defective response to apoptosis in Mrl-MpJ mice. *J Immunol* 180, 5670-5679 (2008). https://doi.org:10.4049/jimmunol.180.8.5670.
69. Khare, A. et al. Cutting edge: inhaled antigen upregulates retinaldehyde dehydrogenase in lung CD103$^+$ but not plasmacytoid dendritic cells to induce FoxP3 de novo in CD4$^+$ T cells and promo

te airway tolerance. *J Immunol* 191, 25-29 (2013). https://doi.org:10.4049/jimmunol.1300193.
70. Coombes, J. L. et al. A functionally specialized population of mucosal CD103+ DCs induces FoxP3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med* 204, 1757-1764 (2007). https://doi.org:10.1084/jem.20070590.
71. Huynh, M.-L. N., Fadok, V. A. & Henson, P. M. Phosphatidylserine-dependent ingestion of apoptotic cells promotes TGF-β1 secretion and the resolution of inflammation. *Journal of Clinical Investigation* 109, 41-50 (2002). https://doi.org:10.1172/jci200211638.
72. Park, S. Y. et al. Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor. *Cell Death Differ* 15, 192-201 (2008). https://doi.org:10.1038/sj.cdd.4402242
73. Miyanishi, M. et al. Identification of Tim4 as a phosphatidylserine receptor. *Nature* 450, 435-439 (2007). https://doi.org:10.1038/nature06307.
74. Choi, S. C. et al. Cutting edge: mouse CD300f (CMRF-35-like molecule-1) recognizes outer membrane-exposed phosphatidylserine and can promote phagocytosis. *J Immunol* 187, 3483-3487 (2011). https://doi.org:10.4049/jimmunol.1101549.
75. Nagata, S., Hanayama, R. & Kawane, K. Autoimmunity and the clearance of dead cells. *Cell* 140, 619-630 (2010). https://doi.org:10.1016/j.cell.2010.02.014.
76. Rodriguez-Fernandez, S. et al. Phosphatidylserine-Liposomes Promote Tolerogenic Features on Dendritic Cells in Human Type 1 Diabetes by Apoptotic Mimicry. *Front Immunol* 9, 253 (2018). https://doi.org:10.3389/fimmu.2018.00253.
77. Villalba, A. et al. Preclinical evaluation of antigen-specific nanotherapy based on phosphatidylserine-liposomes for type 1 diabetes. *Artif Cells Nanomed Biotechnol* 48, 77-83 (2020). https://doi.org:10.1080/21691401.2019.1699812.
78. Ramos, G. C. et al. Apoptotic mimicry: phosphatidylserine liposomes reduce inflammation through activation of peroxisome proliferator-activated receptors (PPARs) in vivo. *Br J Pharmacol* 151, 844-850 (2007). https://doi.org:10.1038/sj.bjp.0707302.
79. Benne, N. et al. Anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) liposomes induce antigen-specific regulatory T cells and prevent atherosclerosis in mice. *J Control Release* 291, 135-146 (2018). https://doi.org:10.1016/j.jconrel.2018.10.028.
80. Clarke, E. V., Weist, B. M., Walsh, C. M. & Tenner, A. J. Complement protein C1q bound to apoptotic cells suppresses human macrophage and dendritic cell-mediated Th17 and Th1 T cell subset proliferation. *J Leukoc Biol* 97, 147-160 (2015). https://doi.org:10.1189/jlb.3A0614-278R
81. Wicker-Planquart, C. et al. Molecular and Cellular Interactions of Scavenger Receptor SR-F1 With Complement C1q Provide Insights Into Its Role in the Clearance of Apoptotic Cells. *Front Immunol* 11, 544 (2020). https://doi.org:10.3389/fimmu.2020.00544.
82. McCarthy, D. P. et al. An antigen-encapsulating nanoparticle platform for TH1/17 immune tolerance therapy. *Nanomedicine* 13, 191-200 (2017). https://doi.org:10.1016/j.nano.2016.09.007.
83. Hunter, Z. et al. A biodegradable nanoparticle platform for the induction of antigen-specific immune tolerance for treatment of autoimmune disease. *ACS Nano* 8, 2148-2160 (2014). https://doi.org:10.1021/nn405033r.
84. Getts, D. R. et al. Microparticles bearing encephalitogenic peptides induce T-cell tolerance and ameliorate experimental autoimmune encephalomyelitis. *Nat Biotechnol* 30, 1217-1224 (2012). https://doi.org:10.1038/nbt.2434.
85. Xu, X. et al. Multipeptide-coupled nanoparticles induce tolerance in 'humanised' HLA-transgenic mice and inhibit diabetogenic CD8(+) T cell responses in type 1 diabetes. *Diabetologia* 60, 2418-2431 (2017). https://doi.org:10.1007/s00125-017-4419-8.

86. Campbell, F. et al. Directing Nanoparticle Biodistribution through Evasion and Exploitation of Stab2-Dependent Nanoparticle Uptake. *ACS Nano* 12, 2138-2150 (2018). https://doi.org:10.1021/acsnano.7b06995.
87. Arias-Alpizar, G. et al. Stabilin-1 is required for the endothelial clearance of small anionic nanoparticles. *Nanomedicine* 34, 102395 (2021). https://doi.org:10.1016/j.nano.2021.102395.
88. Carambia, A. et al. Nanoparticle-based autoantigen delivery to Treg-inducing liver sinusoidal endothelial cells enables control of autoimmunity in mice. *J Hepatol* 62, 1349-1356 (2015). https://doi.org:10.1016/j.jhep.2015.01.006.
89. Saito, E. et al. Design of biodegradable nanoparticles to modulate phenotypes of antigen-presenting cells for antigen-specific treatment of autoimmune disease. *Biomaterials* 222, 119432 (2019). https://doi.org:10.1016/j.biomaterials.2019.119432.
90. Fromen, C. A. et al. Nanoparticle surface charge impacts distribution, uptake and lymph node trafficking by pulmonary antigen-presenting cells. *Nanomedicine* 12, 677-687 (2016). https://doi.org:10.1016/j.nano.2015.11.002.
91. Hoshyar, N., Gray, S., Han, H. & Bao, G. The effect of nanoparticle size on in vivo pharmacokinetics and cellular interaction. *Nanomedicine (Lond)* 11, 673-692 (2016). https://doi.org:10.2217/nnm.16.5.
92. Sato, Y., Hatakeyama, H., Hyodo, M. & Harashima, H. Relationship Between the Physicochemical Properties of Lipid Nanoparticles and the Quality of siRNA Delivery to Liver Cells. *Mol Ther* 24, 788-795 (2016). https://doi.org:10.1038/mt.2015.222.
93. Saito, E. et al. Modulating lung immune cells by pulmonary delivery of antigen-specific nanoparticles to treat autoimmune disease. *Sci Adv* 6 (2020). https://doi.org:10.1126/sciadv.abc9317.
94. Mohamud, R. et al. Synthetic Nanoparticles That Promote Tumor Necrosis Factor Receptor 2 Expressing Regulatory T Cells in the Lung and Resistance to Allergic Airways Inflammation. *Front Immunol* 8, 1812 (2017). https://doi.org:10.3389/fimmu.2017.01812.
95. Benne, N. et al. Atomic force microscopy measurements of anionic liposomes reveal the effect of liposomal rigidity on antigen-specific regulatory T cell responses. *J Control Release* 318, 246-255 (2020). https://doi.org:10.1016/j.jconrel.2019.12.003.
96. Beningo, K. A. & Wang, Y. L. Fc-receptor-mediated phagocytosis is regulated by mechanical properties of the target. *J Cell Sci* 115, 849-856 (2002).
97. Myerson, J. W. et al. Flexible Nanoparticles Reach Sterically Obscured Endothelial Targets Inaccessible to Rigid Nanoparticles. *Adv Mater* 30, e1802373 (2018). https://doi.org:10.1002/adma.201802373.
98. Li, P. Y. et al. PEGylation enables subcutaneously administered nanoparticles to induce antigen-specific immune tolerance. *J Control Release* 331, 164-175 (2021). https://doi.org:10.1016/j.jconrel.2021.01.013.
99. Shaikh, R., Raj Singh, T. R., Garland, M. J., Woolfson, A. D. & Donnelly, R. F. Mucoadhesive drug delivery systems. *J Pharm Bioallied Sci* 3, 89-100 (2011). https://doi.org:10.4103/0975-7406.76478.
100. Boddupalli, B. M., Mohammed, Z. N., Nath, R. A. & Banji, D. Mucoadhesive drug delivery system: An overview. *J Adv Pharm Technol Res* 1, 381-387 (2010). https://doi.org:10.4103/0110-5558.76436.
101. Razafindratsita, A. et al. Improvement of sublingual immunotherapy efficacy with a mucoadhesive allergen formulation. *J Allergy Clin Immunol* 120, 278-285 (2007). https://doi.org:10.1016/j.jaci.2007.04.009.

102. Sandri, G. et al. Mucoadhesive and penetration enhancement properties of three grades of hyaluronic acid using porcine buccal and vaginal tissue, Caco-2 cell lines, and rat jejunum. *The Journal of pharmacy and pharmacology* 56, 1083-1090 (2004). https://doi.org:10.1211/0022357044085.
103. Thati, S. et al. Routes of administration and dose optimization of soluble antigen arrays in mice with experimental autoimmune encephalomyelitis. *J Pharm Sci* 104, 714-721 (2015). https://doi.org:10.1002/jps.24272.
104. O'Konek, J. J. et al. Nanoemulsion adjuvant-driven redirection of TH2 immunity inhibits allergic reactions in murine models of peanut allergy. *J Allergy Clin Immunol* 141, 2121-2131 (2018). https://doi.org:10.1016/j.jaci.2018.01.042.
105. Keijzer, C. et al. PLGA, PLGA-TMC and TMC-TPP nanoparticles differentially modulate the outcome of nasal vaccination by inducing tolerance or enhancing humoral immunity. *PLoS One* 6, e26684 (2011). https://doi.org:10.1371/journal.pone.0026684.
106. Bottger, R. et al. Lipid-based nanoparticle technologies for liver targeting. *Adv Drug Deliv Rev* 154-155, 79-101 (2020). https://doi.org:10.1016/j.addr.2020.06.017.
107. Liu, Q. et al. Use of Polymeric Nanoparticle Platform Targeting the Liver To Induce Treg-Mediated Antigen-Specific Immune Tolerance in a Pulmonary Allergen Sensitization Model. *ACS Nano* 13, 4778-4794 (2019). https://doi.org:10.1021/acsnano.9b01444.
108. Yamazaki, S. et al. CD8+ CD205+ splenic dendritic cells are specialized to induce FoxP3+ regulatory T cells. *J Immunol* 181, 6923-6933 (2008). https://doi.org:10.4049/jimmunol.181.10.6923.
109. Shrimpton, R. E. et al. CD205 (DEC-205): a recognition receptor for apoptotic and necrotic self. *Mol Immunol* 46, 1229-1239 (2009). https://doi.org:10.1016/j.molimm.2008.11.016.
110. Mukhopadhaya, A. et al. Selective delivery of beta cell antigen to dendritic cells in vivo leads to deletion and tolerance of autoreactive CD8+ T cells in NOD mice. *Proc Natl Acad Sci U S A* 105, 6374-6379 (2008). https://doi.org:10.1073/pnas.0802644105.
111. Spiering, R. et al. DEC205+ Dendritic Cell-Targeted Tolerogenic Vaccination Promotes Immune Tolerance in Experimental Autoimmune Arthritis. *J Immunol* 194, 4804-4813 (2015). https://doi.org:10.4049/jimmunol.1400986.
112. Wadwa, M., Klopfleisch, R., Buer, J. & Westendorf, A. M. Targeting Antigens to Dec-205 on Dendritic Cells Induces Immune Protection in Experimental Colitis in Mice. *European journal of microbiology & immunology* 6, 1-8 (2016). https://doi.org:10.1556/1886.2015.00048.
113. Nijen Twilhaar, M. K. et al. Optimization of Liposomes for Antigen Targeting to Splenic CD169(+) *Macrophages. Pharmaceutics* 12, 1138 (2020). https://doi.org:10.3390/pharmaceutics12121138.
114. Busold, S. et al. Various Tastes of Sugar: The Potential of Glycosylation in Targeting and Modulating Human Immunity via C-Type Lectin Receptors. *Front Immunol* 11, 134 (2020). https://doi.org:10.3389/fimmu.2020.00134.
115. Semmrich, M. et al. Directed antigen targeting in vivo identifies a role for CD103+ dendritic cells in both tolerogenic and immunogenic T-cell responses. *Mucosal Immunol* 5, 150-160 (2012). https://doi.org:10.1038/mi.2011.61.
116. Veri, M. C. et al. Monoclonal antibodies capable of discriminating the human inhibitory Fcgamma-receptor IIB (CD32B) from the activating Fcgamma-receptor IIA (CD32A): biochemical, biological and functional characterization. *Immunology* 121, 392-404 (2007). https://doi.org:10.1111/j.1365-2567.2007.02588.x.
117. Horton, H. M. et al. Antibody-mediated coengagement of FcgammaRIIb and B cell receptor complex suppresses humoral immunity in systemic lupus erythematosus. *J Immunol* 186, 4223-4233 (2011). https://doi.org:10.4049/jimmunol.1003412.

118. Baker, M. P., Reynolds, H. M., Lumicisi, B. & Bryson, C. J. Immunogenicity of protein therapeutics: The key causes, consequences and challenges. *Self Nonself* 1, 314-322 (2010). https://doi.org:10.4161/self.1.4.13904.
119. Maldonado, R. A. et al. Polymeric synthetic nanoparticles for the induction of antigen-specific immunological tolerance. *Proc Natl Acad Sci U S A* 112, E156-165 (2015). https://doi.org:10.1073/pnas.1408686111.
120. LaMothe, R. A. et al. Tolerogenic Nanoparticles Induce Antigen-Specific Regulatory T Cells and Provide Therapeutic Efficacy and Transferrable Tolerance against Experimental Autoimmune Encephalomyelitis. *Front Immunol* 9, 281 (2018). https://doi.org:10.3389/fimmu.2018.00281.
121. Zhang, A. H., Rossi, R. J., Yoon, J., Wang, H. & Scott, D. W. Tolerogenic nanoparticles to induce immunologic tolerance: Prevention and reversal of FVIII inhibitor formation. *Cell Immunol* 301, 74-81 (2016). https://doi.org:10.1016/j.cellimm.2015.11.004.
122. Capini, C. et al. Antigen-specific suppression of inflammatory arthritis using liposomes. *J Immunol* 182, 3556-3565 (2009). https://doi.org:10.4049/jimmunol.0802972.
123. Yeste, A. et al. Tolerogenic nanoparticles inhibit T cell-mediated autoimmunity through SOCS2. *Sci Signal* 9, ra61 (2016). https://doi.org:10.1126/scisignal.aad0612.
124. Yeste, A., Nadeau, M., Burns, E. J., Weiner, H. L. & Quintana, F. J. Nanoparticle-mediated codelivery of myelin antigen and a tolerogenic small molecule suppresses experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 109, 11270-11275 (2012). https://doi.org:10.1073/pnas.1120611109.
125. Galea, R. et al. PD-L1- and calcitriol-dependent liposomal antigen-specific regulation of systemic inflammatory autoimmune disease. *JCI Insight* 4 (2019). https://doi.org:10.1172/jci.insight.126025.
126. Bergot, A. S. et al. Regulatory T Cells Induced by Single-Peptide Liposome Immunotherapy Suppress Islet-Specific T Cell Responses to Multiple Antigens and Protect from Autoimmune Diabetes. *J Immunol* 204, 1787-1797 (2020). https://doi.org:10.4049/jimmunol.1901128.
127. Sands, R. W. et al. Steroid-Peptide Immunoconjugates for Attenuating T Cell Responses in an Experimental Autoimmune Encephalomyelitis Murine Model of Multiple Sclerosis. *Bioconjug Chem* 31, 2779-2788 (2020). https://doi.org:10.1021/acs.bioconjchem.0c00582.
128. Kenison, J. E. et al. Tolerogenic nanoparticles suppress central nervous system inflammation. Proc *Natl Acad Sci U S A* 117, 32017-32028 (2020). https://doi.org:10.1073/pnas.2016451117.
129. Chen, X. Y. et al. Restoring immunological tolerance in established experimental arthritis by combinatorial citrullinated peptides and immunomodulatory signals. *Nano Today* 41, 101307 (2021). https://doi.org:ARTN 10130710.1016/j.nantod.2021.101307.
130. Peine, K. J. et al. Treatment of experimental autoimmune encephalomyelitis by codelivery of disease associated Peptide and dexamethasone in acetalated dextran microparticles. *Mol Pharm* 11, 828-835 (2014). https://doi.org:10.1021/mp4005172.
131. Phillips, B. E. et al. Arrest in the Progression of Type 1 Diabetes at the Mid-Stage of Insulitic Autoimmunity Using an Autoantigen-Decorated All-trans Retinoic Acid and Transforming Growth Factor Beta-1 Single Microparticle Formulation. *Front Immunol* 12, 586220 (2021). https://doi.org:10.3389/fimmu.2021.586220.
132. Lewis, J. S. et al. Dual-Sized Microparticle System for Generating Suppressive Dendritic Cells Prevents and Reverses Type 1 Diabetes in the Nonobese Diabetic Mouse Model. *ACS Biomater Sci Eng* 5, 2631-2646 (2019). https://doi.org:10.1021/acsbiomaterials.9b00332.
133. Luo, Y. L. et al. An All-in-One Nanomedicine Consisting of CRISPR-Cas9 and an Autoantigen Peptide for Restoring Specific Immune Tolerance. *ACS Appl Mater Interfaces* 12, 48259-48271

(2020). https://doi.org:10.1021/acsami.0c10885.
134. Krienke, C. et al. A noninflammatory mRNA vaccine for treatment of experimental autoimmune encephalomyelitis. *Science* 371, 145-153 (2021). https://doi.org:10.1126/science.aay3638
135. Solorio-Rodríguez, A. et al. A comparison of the human and mouse protein corona profiles of functionalized SiO2 nanocarriers. *Nanoscale* 9, 13651-13660 (2017). https://doi.org:10.1039/C7NR04685E.
136. Müller, L. K. et al. The Transferability from Animal Models to Humans: Challenges Regarding Aggregation and Protein Corona Formation of Nanoparticles. *Biomacromolecules* 19, 374-385 (2018). https://doi.org:10.1021/acs.biomac.7b01472.
137. Li, Y. et al. Complement opsonization of nanoparticles: Differences between humans and preclinical species. *Journal of Controlled Release* 338, 548-556 (2021). https://doi.org/10.1016/j.jconrel.2021.08.048.
138. Hatit, M. Z. C. et al. Species-dependent in vivo mRNA delivery and cellular responses to nanoparticles. *Nature Nanotechnology* (2022). https://doi.org:10.1038/s41565-021-01030-y.
139. Alves da Costa, T., Lang, J., Torres, R. M. & Pelanda, R. The development of human immune system mice and their use to study tolerance and autoimmunity. *J Transl Autoimmun* 2, 100021 (2019). https://doi.org:10.1016/j.jtauto.2019.100021.
140. Postigo-Fernandez, J., Farber, D. L. & Creusot, R. J. Phenotypic alterations in pancreatic lymph node stromal cells from human donors with type 1 diabetes and NOD mice. *Diabetologia* 62, 2040-2051 (2019). https://doi.org:10.1007/s00125-019-04984-w.
141. van Baarsen, L. G. et al. The cellular composition of lymph nodes in the earliest phase of inflammatory arthritis. *Ann Rheum Dis* 72, 1420-1424 (2013). https://doi.org:10.1136/annrheumdis-2012-202990.
142. Hahnlein, J. S. et al. Impaired lymph node stromal cell function during the earliest phases of rheumatoid arthritis. *Arthritis Res Ther* 20, 35 (2018). https://doi.org:10.1186/s13075-018-1529-8.
143. Ramwadhdoebe, T. H. et al. Myeloid Dendritic Cells Are Enriched in Lymph Node Tissue of Early Rheumatoid Arthritis Patients but not in At Risk Individuals. *Cells* 8, 756 (2019). https://doi.org:10.3390/cells8070756.
144. Suresh Babu, S. et al. MicroRNA-126 overexpression rescues diabetes-induced impairment in efferocytosis of apoptotic cardiomyocytes. *Sci Rep* 6, 36207 (2016). https://doi.org:10.1038/srep36207.
145. Gaipl, U. S. et al. Clearance of apoptotic cells in human SLE. *Current directions in autoimmunity* 9, 173-187 (2006). https://doi.org:10.1159/000090781.
146. Fernandez-Boyanapalli, R. et al. Obesity impairs apoptotic cell clearance in asthma. *J Allergy Clin Immunol* 131, 1041-1047, 1047 e1041-1043 (2013). https://doi.org:10.1016/j.jaci.2012.09.028.
147. Liu, Q. et al. Antigen- and Epitope-Delivering Nanoparticles Targeting Liver Induce Comparable Immunotolerance in Allergic Airway Disease and Anaphylaxis as Nanoparticle-Delivering Pharmaceuticals. *ACS Nano* 15, 1608-1626 (2021). https://doi.org:10.1021/acsnano.0c09206.
148. Lutterotti, A. et al. Antigen-specific tolerance by autologous myelin peptide-coupled cells: a phase 1 trial in multiple sclerosis. *Sci Transl Med* **5**, 188ra175 (2013). https://doi.org:10.1126/scitranslmed.3006168.
149. Lomakin, Y. et al. Administration of Myelin Basic Protein Peptides Encapsulated in Mannosylated Liposomes Normalizes Level of Serum TNF-α and IL-2 and Chemoattractants CCL2 and CCL4 in Multiple Sclerosis Patients. *Mediators Inflamm* 2016, 2847232-2847232 (2016). https://doi.org:10.1155/2016/2847232.

150. Belogurov, A., Jr. et al. CD206-Targeted Liposomal Myelin Basic Protein Peptides in Patients with Multiple Sclerosis Resistant to First-Line Disease-Modifying Therapies: A First-in-Human, Proof-of-Concept Dose-Escalation Study. *Neurotherapeutics* 13, 895-904 (2016). https://doi.org:10.1007/s13311-016-0448-0.
151. Kelly, C. P. et al. TAK-101 Nanoparticles Induce Gluten-Specific Tolerance in Celiac Disease: A Randomized, Double-Blind, Placebo-Controlled Study. *Gastroenterology* 161, 66-80 e68 (2021). https://doi.org:10.1053/j.gastro.2021.03.014.
152. Prasad, S. et al. Tolerogenic Ag-PLG nanoparticles induce tregs to suppress activated diabetogenic CD4 and CD8 T cells. *J Autoimmun* 89, 112-124 (2018). https://doi.org:10.1016/j.jaut.2017.12.010.
153. Freitag, T. L. et al. Gliadin Nanoparticles Induce Immune Tolerance to Gliadin in Mouse Models of Celiac Disease. *Gastroenterology* 158, 1667-1681 e1612 (2020). https://doi.org:10.1053/j.gastro.2020.01.045.
154. Glassman, F. Y. & Balu-Iyer, S. V. Subcutaneous administration of Lyso-phosphatidylserine nanoparticles induces immunological tolerance towards Factor VIII in a Hemophilia A mouse model. *Int J Pharm* 548, 642-648 (2018). https://doi.org:10.1016/j.ijpharm.2018.07.018.
155. Domogalla, M. P., Rostan, P. V., Raker, V. K. & Steinbrink, K. Tolerance through Education: How Tolerogenic Dendritic Cells Shape Immunity. *Front Immunol* 8, 1764 (2017). https://doi.org:10.3389/fimmu.2017.01764.
156. Jiskoot, W., Rispens, T. & Kijanka, G. in *Pharmaceutical Biotechnology: Fundamentals and Applications* (eds Daan J. A. Crommelin, Robert D. Sindelar, & Bernd Meibohm) 139-150 (Springer International Publishing, 2019).

Retinoic Acid-Containing Liposomes for the Induction of Antigen-Specific Regulatory T Cells as a Treatment for Autoimmune Diseases

Retinoic Acid-Containing Liposomes for the Induction of Antigen-Specific Regulatory T Cells as a Treatment for Autoimmune Diseases

Daniëlle ter Braake [1,†], Naomi Benne [1,†], Chun Yin Jerry Lau [2], Enrico Mastrobattista [2], Femke Broere [1]

[1] Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, 3584 CL Utrecht, The Netherlands
[2] Utrecht Institute for Pharmaceutical Sciences, Department of Pharmaceutics, Faculty of Science, Utrecht University, 3584 CG Utrecht, The Netherlands
[†] Authors contributed equally

Pharmaceutics (2021), Volume 13
DOI: 10.3390/pharmaceutics13111949

Abstract

Current treatment of autoimmune and chronic inflammatory diseases entails systemic immune suppression, which is associated with increased susceptibility to infections. To restore immune tolerance and reduce systemic side-effects, a targeted approach using tolerogenic dendritic cells (tolDCs) is being explored. TolDCs are characterized by the expression of CD11c, major histo-compatibility complex (MHC)II and low levels of co-stimulatory molecules CD40 and CD86. In this study tolDCs are generated using a human-proteoglycan-derived peptide (hPG) and all-trans retinoic acid (RA). RA-tolDCs not only display a tolerogenic phenotype but also can induce an antigen-specific regulatory T cell (Treg) response *in vitro*. However, further analysis showed that RA-tolDCs make up a heterogeneous population of DCs, with only a small proportion being antigen-associated tolDCs. To increase the homogeneity of this population, 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG)-containing liposomes were used to encapsulate the relevant antigen together with RA. These liposomes greatly enhanced the proportion of antigen-associated tolDCs in culture. In addition, in mice we show that liposomal co-delivery of antigen and RA can be a more targeted approach to induce antigen-specific tolerance compared to injection of RA-tolDCs, and that these liposomes can stimulate the generation of antigen-specific Tregs. This work highlights the importance of co-delivery of an antigen and immunomodulator to minimize off-target effects and systemic side-effects and provides new insights in the use of RA for antigen-specific immunotherapy for autoimmune and chronic inflammatory diseases.

Introduction

Autoimmune diseases and chronic inflammatory diseases are major public health concerns in Europe[1]. As there is no cure for these diseases, patients often require life-long treatment with immune-suppressing medication, which may be accompanied by severe side-effects. In addition, the use of immunosuppressive drugs can increase the risk of infection[2]. Therefore, there is a great need to develop more effective treatments for autoimmune and chronic inflammatory diseases. In several autoimmune disorders, a disbalance in immune homeostasis is observed. This disbalance can be attributed to a loss of function or reduced presence of antigen-specific suppressive immune cells, resulting in a breach of immune tolerance[3]. Immune tolerance is generally maintained by a variety of immune cells, including subsets of dendritic cells (DCs), T, and B cells[4–6]. In autoimmune disorders, these cells recognize autoantigens as non-self and elicit a pro-inflammatory immune response. To date, several autoimmune disorders have been linked to specific autoantigens[7]. Antigen recognition is mediated by antigen-presenting cells (APCs), such as DCs. These cells are continuously sensing their environment through the pattern recognition receptors on their cell surface[8]. Under inflammatory conditions, detection of an antigen by these receptors causes DCs to become activated and migrate to draining lymph nodes. This dendritic cell maturation results in an upregulation of the antigen-presenting MHC molecules, chemokine receptors and an increase in pro-inflammatory cytokine secretion[9]. Naive T cells reside in the draining lymph nodes, where DCs can initiate effector T cell responses through antigen presentation, co-stimulation and cytokine secretion[10]. However, some specialized sub-types of immune cells, such as tolerogenic dendritic cells (tolDCs) can help maintain immune tolerance. TolDCs are derived from immature dendritic cells, upon encountering a tolerogenic stimulus and an activation stimulus[11,12]. These tolDCs can induce T cell anergy, inhibit proliferation of effector T cells (such as the pro-inflammatory $CD4^+$ T helper subsets Th1, Th2 and Th17) and can promote regulatory T cell (Treg) differentiation[13]. TolDCs are characterized by a semi-mature phenotype, in which they show a reduced expression of co-stimulatory molecules (CD40, CD86) as compared to mature DCs (mDCs) on their cell surface and can secrete anti-inflammatory molecules, such as IL-10, to mediate immune suppression. Therefore, these cells are of great interest when developing treatments for autoimmune and chronic inflammatory diseases, in which immune tolerance needs to be restored. Previously our lab and others have cultured tolDCs using a variety of immunomodulators, including dexamethasone and vitamin D3[11]. In this study, we use all-trans retinoic acid (RA) for the culture of tolDCs, as has been described before[14-16].

RA is an active metabolite of vitamin A (all-trans retinol) that has been shown to play a significant role in the induction and maintenance of gut immune tolerance. The gut is host to a subpopulation of specialized DCs, which are able to metabolize food-derived vitamin A to RA[17]. RA can prime other gut-associated DCs to become RA-producing $CD103^+$ DCs[18]. These DCs subsequently can convert naive T cells into Tregs[19-21]. This tolerance-inducing ability makes tolDCs interesting targets for the development of antigen-specific immunotherapy for autoimmune and chronic inflammatory diseases. *Ex vivo* culturing of patient DCs, converting them to tolDCs that present a disease-specific antigen, and reinjecting them into the patient has already shown to be promising in clinical trials for several auto-immune diseases[22]. However, the process of isolating DC precursors from patients, stimulating the cells *ex vivo* and injecting them back into patients will remain not only labor intensive, but is also restricted to highly specialized cell culture facilities, thereby limiting the number of patients to be treated. Therefore, we propose that a delivery system such as nanoparticles can be a suitable alternative to tolDC culture. The use of nanoparticles, such as liposomes, shows great promise for *in vivo* immunomodulation, and liposomes have been widely used as a delivery vehicle for antigens and adjuvants[23,24].

In this study, we selected anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG)-containing liposomes of around 200 nm in size as a delivery system, since these liposomes were shown to be inherently tolerogenic[25], and RA is lipophilic so would be readily encapsulated into the lipid bilayer. To assess the antigen-specificity of these nanocarriers, a mouse model in which the T cells of the animal only express T cell receptors specific for human proteoglycan (hPG) was used. The hPG antigen has been widely used for the induction of proteoglycan-induced arthritis (PGIA) in mice[12], which is a model for autoimmunity. This study aimed to see whether liposomes can be a suitable carrier for the hPG antigen and RA and if these liposomes are as effective *in vivo* as using antigen-specific RA tolDCs.

Materials and Methods

Synthesis of peptides and conjugates

Dimethylformamide, N, N'-diisopropyl carbodiimide, piperidine, and acetonitrile were purchased from Biosolve BV, Netherlands. 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids, Fmoc-Lys(Boc)-Wang resin, and trifluoroacetic acid were purchased from Novabiochem GmbH, Germany. The peptide epitope sequences were synthesized by microwave-assisted solid-phase peptide synthesis using an H12 liberty blue peptide synthesizer (CEM Corporation, USA). Dimethylformamide was used as the coupling and washing solvent for the whole synthesis process. For each coupling step, Fmoc-protected amino acids were activated by five eq of Oxyma pure (Manchester Organics, UK) and N, N'-diisopropyl carbodiimide to react with the free N-terminal amino acids in preloaded resin for 1 minute at 90˚C. After each coupling step, the Fmoc group was removed by treatment with 20% piperidine for 1 minute at 90˚C. Fluorescein (FAM, ThermoFisher, Netherlands) was coupled to the N-terminal of the peptide as with other Fmoc-protected amino acids. Trifluoroacetic acid/water/triisopropylsilane (Sigma-Aldrich, Netherlands) (95/2.5/2.5) was used to simultaneously cleave the peptide off the resin and remove the side chain protecting groups. Peptides were purified by Prep-HPLC using a Reprosil-Pur C18 column (10 µm, 250 × 22 mm) eluted with water-acetonitrile gradient 5% to 80% acetonitrile (0.1% formic acid, Sigma-Aldrich, Netherlands) in 30 minutes at a flowrate of 15.0 mL/min with UV detection at 220 nm. Mass spectrometry analysis was performed using a Bruker micrOTOF-Q instrument in positive mode to confirm the identity of the synthetic products (*Figure S1*). The epitope was derived from the hPG antigen, with sequence ATEGRVRVNSAYQDK. For coupling to FAM for flow cytometry and microscopy experiments, a lysine tetramer linker was added to the N-terminal of the sequence to compensate for the reduced solubility caused by dye conjugation, (i.e., hPG-FAM: FAM-KKKKATEGRVRVNSAYQDK).

Liposome preparation

The phospholipids 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG), were purchased from Avanti Polar Lipids, USA). Cholesterol (CHOL) and RA were purchased from Sigma-Aldrich, Netherlands. Liposomes were prepared using the thin film dehydration-rehydration method, as described previously[25]. Briefly, phospholipids and CHOL (40 µmol, 4 mL) were dissolved in chloroform and methanol 1:1 and mixed in a 100 mL round-bottom flask at a molar ratio of 4:1:2 DSPC:DSPG:CHOL. To prepare RA encapsulating liposomes, 60 nmol of RA was added in this step. To prepare fluorescently labeled liposomes, 0.02 mol% of total lipid of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine, 4-chlorobenzenesulfonate salt (DiD, ThermoFisher, Netherlands) was added in this step. The solvents were evaporated under vacuum in a rotary evaporator for 1 h at 40 °C. The resulting lipid film was rehydrated with 1000 µg hPG, hPG-K4 or hPG-K4-FAM dissolved in 4 mL 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.2) buffer and homogenized by rotation in a water bath at 40 °C for 1 hour. The multilamellar vesicle suspension was sized by high-pressure extrusion (LIPEX Extruder, Northern Lipids Inc., Canada) by passing the dispersion four times through stacked 400-nm and 200-nm pore size membranes (Whatman® NucleoporeTM, GE Healthcare, UK). To separate non-encapsulated cargo from the liposomes, liposomes were pelleted by ultracentrifugation (Type 70.1 Ti rotor) for 50 minutes at 55,000 rpm at 4°C. This was repeated three times. Liposomes were stored at 4°C and their stability was measured periodically. Liposomes were determined to be unchanged for up to at least 1 year. Liposomes were used within 2 months for *in vitro* experiments, and within 2 weeks for *in vivo* experiments.

Liposome characterization

The Z-average diameter and polydispersity index (PDI) of the liposomes were measured by dynamic light scattering (DLS) using a NanoZS Zetasizer (Malvern Ltd., UK). ζ-potential was measured by laser Doppler electrophoresis (Malvern Ltd., UK). For this, the liposomes were diluted 100-fold in HEPES buffer pH 7.2 to a total volume of 1 mL. To determine the concentration of loaded hPG and RA, the content of the liposomes was measured using RP-UPLC. For this, 10 µL of liposome suspension was dissolved in 190 µL of methanol, and the sample was vortexed. Sample injections were 5 µL and the column used was a 1.7 µm BEH C18 column (2.1 × 50 mm, Waters ACQUITY UPLC, Waters, MA, USA). Column and sample temperatures were 40°C and 20°C, respectively. The mobile phases were Milli-Q water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). For separation, the mobile phases were applied in a linear gradient from 5% to 95% solvent B over 6.5 min at a flow rate of 0.25 mL/min. hPG was detected by absorbance at 280 nm using an ACQUITY UPLC TUV detector (Waters ACQUITY UPLC, Waters, MA, USA). RA was detected at 351 nm.

Mice

Female Balb/cAnNCrl mice were purchased from Charles River Laboratories (France). Female mice were used as they are more susceptible to develop arthritis in the proteoglycan induced arthritis model[26]. hPG T cell receptor (TCR) transgenic Thy1.1$^+$ mice[27] were bred in-house at Utrecht University under standard laboratory conditions. Mice were provided with food and water ad libitum. All animal work was performed in compliance with the Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. Experiments were approved by the Ethics Committee for Animal Experiments of Utrecht University.

Bone marrow-derived DC (BMDC) culture

Penicillin, streptomycin, and β-mercaptoethanol were purchased from Gibco (Thermo Fisher Scientific, United States). Bone marrow was obtained from the femurs and tibias of Balb/cAnNCrl mice. Cells were cultured at 37°C, 5% CO2 in a 6-well plate (Corning, United States), at a density of 900,000 cells/mL, in IMDM (Gibco, Thermo Fisher Scientific, United States), supplemented with 10% FCS (Bodinco, The Netherlands), 100 units/mL penicillin, 100 µg/mL streptomycin and 0.5 µM β-mercaptoethanol in the presence of 20ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF, in house produced). Fresh medium and GM-CSF was added on day 2 and extra GM-CSF was supplemented to the culture on day 5. On day 7 cells were matured in the presence of 10 ng/mL lipopolysaccharide (LPS, O111:B4; Sigma-Aldrich, Netherlands) and treated with free or encapsulated RA and hPG antigen, or controls. After 16 hours DCs were harvested for phenotypic characterization by flow cytometry or microscopy, for co-culture with T cells, or for *in vivo* transfer in mice.

T cell isolation and co-culture with BMDCs

Single-cell suspensions of splenocytes were obtained by mashing spleens of hPG TCR transgenic mice through a 70 µM filter and erythrocytes were lysed with Ammonium-Chloride-Potassium (ACK) lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na2EDTA; pH 7.3). Subsequently, CD4$^+$ T cells were negatively selected using Dynabeads™ (sheep anti-rat IgG, Invitrogen, United States) and anti-CD8 (YTS169), anti-CD11b (M1/70), anti-MHCII (M5/114) and anti-B220 (RA3-6B2) as described previously[12]. Enriched CD4$^+$ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE, 0.5 nM) according to manufacturer's protocol (Invitrogen, United States). Selected T cells were co-cultured in a 2:1 ratio with DCs for 3 days at 37°C and 5% CO2 and subsequently harvested for phenotypic characterization.

Adoptive transfer of hPG TCR-specific T cells

$CD4^+CD25^-$ T cells were magnetically isolated from spleens of hPG TCR transgenic $Thy1.1^+$ Balb/cAnNCrl mice as described above, with the addition of anti-CD25 (PC61, in-house produced) to the antibody mix to deplete activated T cells[28]. Enriched $CD4^+CD25^-$ T cells were labelled with 0.5nM CFSE (Invitrogen), resuspended in 200 µL phosphate-buffered saline (PBS), and 300,000 cells were injected intravenously via the tail vein into wildtype Balb/cAnNCrl acceptor mice within 1 hour. After 24 hours, 1×10^6 DCs pulsed with RA and hPG (tolDCs), free RA and hPG or liposomes encapsulating RA and hPG were injected into the acceptor mice. The concentration of hPG and RA were 1 nmol and 0.2 nmol, respectively. After 72 hours, acceptor mice were sacrificed, and spleens were harvested.

Flow cytometry

For all flow cytometry experiments, the cell suspension was first blocked with Fc Block (2.4G2, in house produced). Extracellular staining was performed with a cocktail of antibodies in FACS Buffer (1X PBS supplemented with 2% FCS). For intracellular staining, the FoxP3 transcription factor staining set was used (eBioscience, United States). For all flow cytometric analyses, appropriate single stain and fluorescence minus one controls were used. Flow cytometry was performed using the Beckman Coulter Cytoflex LX at the Flow Cytometry and Cell Sorting Facility at the Faculty of Veterinary Medicine at Utrecht University.

On day 8 BMDCs were stained with monoclonal antibodies CD11c-APC (N418, eBioscience, Thermo Fisher Scientific, United States), MHCII-eFluor450 (M5/114.15.2, eBioscience, Thermo Fisher Scientific, United States), CD40-PE (3/23, BD Biosciences, United States), CD86-FITC (GL-1, BD Biosciences, United States) and ViaKrome808 (Beckman Coulter, United States).

For the co-cultures, $CD4^+$ T cells were harvested and transferred to a 96-well V-bottom plate for staining. T cells were stained with monoclonal antibodies CD4-BV785 (RM4-5, BioLegend, United States), CD25-PerCPCy5.5 (PC61.5, eBioscience, Thermo Fisher Scientific, United States), CD49b-APC (DX5, BioLegend, United States), Lag-3-PE (C9B7W, eBioscience, Thermo Fisher Scientific, United States), FoxP3-eFluor450 (FJK-16s, eBioscience, Thermo Fisher Scientific, United States) and ViaKrome808 (Beckman Coulter, United States).

For adoptive T cell transfer experiments, spleens of acceptor mice were harvested 72 hours post-treatment, as described above. Spleens were mashed through a 70 µM filter and erythrocytes were lysed with ACK lysis buffer. Acquired splenocytes were stained with monoclonal antibodies CD4-BV785 (RM4-5, BioLegend, United States), Thy1.1 (CD90.1)-PerCP-Cy5.5 (HIS51, eBioscience, Thermo Fisher Scientific, United States), CD44-APC (IM7, eBioscience, Thermo Fisher Scientific, United States), CD62L-PE (MEL-14, BD Biosciences, United States), CTLA-4 (CD152)-BV605 (UC10-4B9, eBioscience, Thermo Fisher Scientific, United States), FoxP3-eFluor450 (FJK-16s, eBioscience, Thermo Fisher Scientific, United States), RORγT-PE (AFKJS-9, eBioscience, Thermo Fisher Scientific, United States), GATA-3-PE-Cy7 (TWAJ, eBioscience, Thermo Fisher Scientific, United States), T-Bet-APC (4B10, eBioscience, Thermo Fisher Scientific, United States), CD11c-APC (N418, eBiosciences, Thermo Fisher Scientific, United States), CD11b-PerCP-Cy5.5 (M1/70, eBioscience, Thermo Fisher Scientific, United States), MHCII(I-A/I-E)-PE-Cy5 (M5/114.15.2, eBioscience, Thermo Fisher Scientific, United States), CD40-PE (3/23, BD Biosciences, United States), CD8α-V500 (53-6.7, BD Biosciences, United States) and ViaKrome808 (Beckman Coulter, United States), in different flow cytometry panels to avoid spectral overlap.

Live cell imaging

On day 7, BMDCs were harvested and 75,000 cells per well were added to 35 mm glass-bottom cell culture dishes (CELLview™, Greiner Bio-One, Austria). 1 µg/mL hPG-FAM, free or in liposomes, or controls were added to the cells, together with 10 ng/mL LPS. Cells were cultured overnight at 37°C and 5% CO2. Before imaging, cells were carefully washed to remove unbound liposomes and/or hPG-FAM. 5 µg/mL membrane permeable DNA stain Hoechst 33342 (Thermo Fisher, The Netherlands) was added to each well shortly before imaging. Microscopy and analysis were performed at the Center for Cell Imaging at the Faculty of Veterinary Medicine at Utrecht University. Images were acquired using a NIKON A1R confocal microscope with a 40x Plan Apo objective (NA 1.3). Standard lasers and filter settings were used to detect Hoechst, FAM and DiD. Representative images were processed in NIS elements 5.02 (Nikon Microsystems, Europe).

Results

RA induces a tolDC phenotype in BMDCs in vitro

TolDCs are generally described as having an immature phenotype and express the pan-DC marker CD11c, the antigen-presenting molecule MHCII, and have low expression of the co-stimulatory molecules CD40 and CD86. To assess whether the vitamin A-derived RA could induce this tolDC phenotype *in vitro*, we tested different concentrations of RA on BMDCs. Cells were simultaneously incubated with LPS. The expression of each marker is presented as the geometric mean fluorescence intensity (MFI) and was determined by flow cytometry analysis. The addition of RA results in a significant decrease in the expression of MHC II (*Figure 1A*) and co-stimulatory molecules CD40 (*Figure 1B*) and CD86 (*Figure 1C*) on the cell surface of BMDCs. Increasing the dose of RA that was administered to the cells 10-fold did not result in significant reductions in the expression of the measured surface proteins compared to the lower concentrations of RA (*Figure 1*). Addition of RA to a BMDC culture therefore seems to be a promising way of inducing tolDCs.

Table 1. properties of liposomes, means ± SD.

Formulation	Z-average diameter (nm)	ζ-potential (mV)	PDI	Encapsulation hPG (%)	Encapsulation RA (%)
hPG	186.8 ± 11.2	-47.7 ± 2.1	0.10 ± 0.05	57.3 ± 3.3	-
hPG/RA	183.7 ± 4.9	-45.9 ± 0.9	0.07 ± 0.01	43.9 ± 4.5	79.5 ± 29.0

RA can be efficiently encapsulated into liposomes and retains tolDC inducing effects

To assess whether RA remains able to induce tolDCs when encapsulated in liposomes, DSPC:DSPG:CHOL liposomes were loaded with hPG with or without RA. The liposomes were characterized using DLS and laser Doppler electrophoresis (*table 1*). To assess liposome uptake by DCs, *in vitro* liposomes were fluorescently labeled using DiD and added to BMDCs. After 24 hours of incubation, around 30% of all BMDCs were able to take up hPG-loaded liposomes or hPG/RA-loaded liposomes (*Figure 2A*). To address whether the liposomes were able to induce phenotypic tolDCs, BMDCs were stimulated in the presence of LPS with free hPG, free hPG and RA, hPG-loaded liposomes, and hPG/RA-loaded liposomes. DCs treated with RA free or in liposomes show a reduced expression of CD86 (*Figure 2B*) and CD40 (*Figure 2C*) on their cell surface compared to the control hPG, indicating inhibition of maturation. The expression of MHCII (*Figure 2D*) and PD-L1, which is involved in T cell suppression[29] (*Figure 2E*) seemed to remain similar for all DCs regardless of stimulation. The expression of chemokine receptor CCR7, essential for homing to secondary lymph nodes[30], was upregulated in tolDCs generated with free RA or RA liposomes (*Figure 2F*).

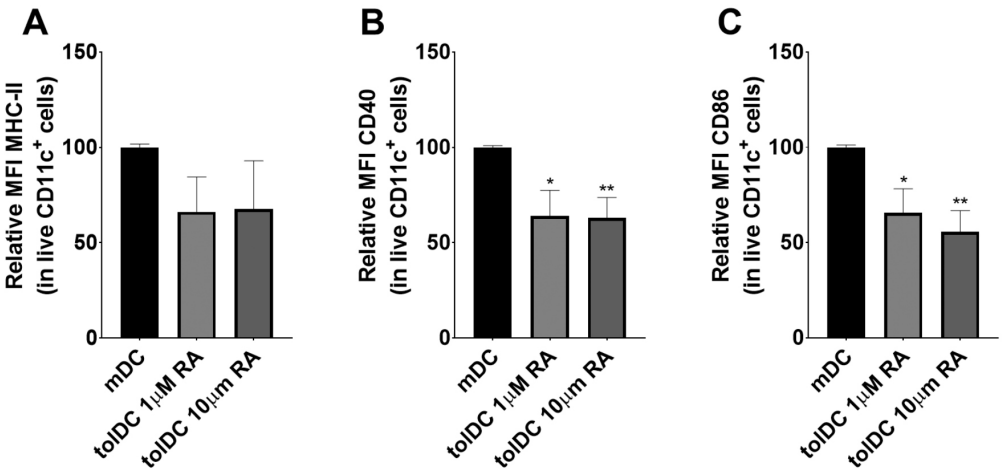


Figure 1. Addition of RA results in a tolDC phenotype *in vitro*. BMDCs were cultured from the bone marrow of Balb/c mice. BMDCs were stimulated with LPS (mDC) or LPS and different concentrations of RA (tolDC; 1 or 10uM RA). The concentration of LPS was constant for all groups. After 24 hours of incubation, cells were washed thoroughly to remove stimuli and analyzed via flow cytometry. Relative MFI normalized to mDC of (A) MHCII expression (B) CD40 expression, and (C) CD86 expression on live $CD11c^+$ BMDCs. Combined data of three independent experiments. n = 3. Means + SD, *$p < 0.05$, **$p < 0.01$ compared to mDC, as determined by one-way ANOVA and Tukey's multiple comparisons test.

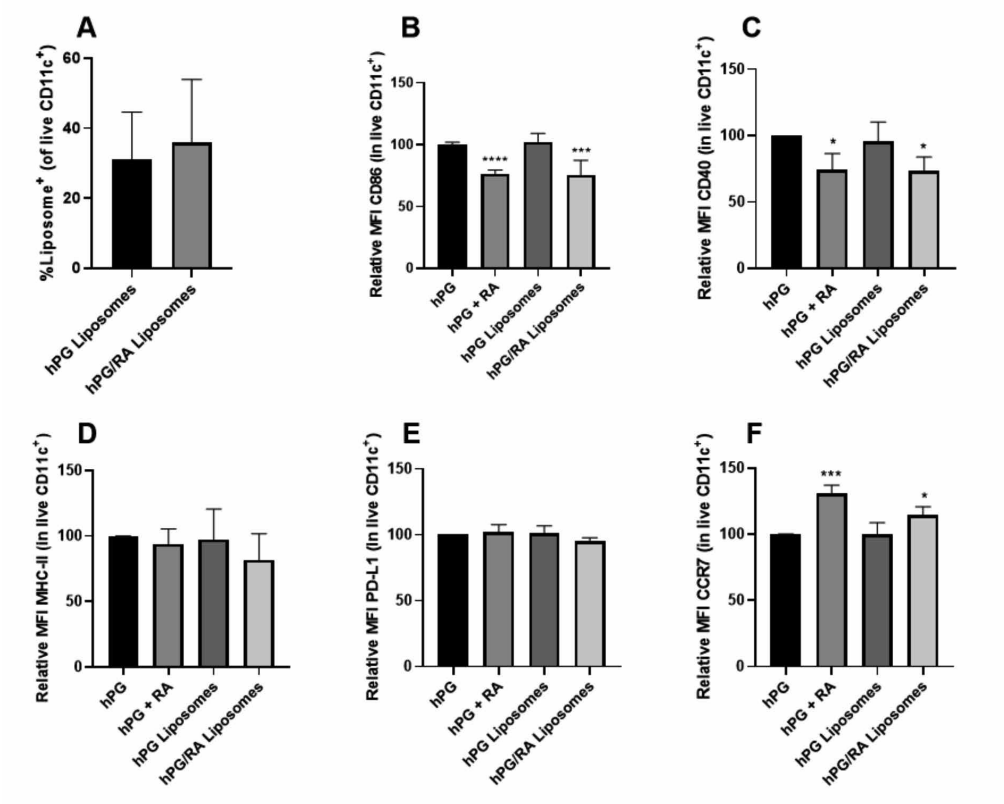


Figure 2. RA, free or encapsulated in liposomes, induces a tolDC phenotype in BMDCs. BMDCs were stimulated with LPS and cultured in the presence of hPG, hPG + RA, hPG liposomes, or hPG/RA liposomes. The hPG and RA concentrations were constant in all groups. After 24 hours of incubation, cells were washed thoroughly to remove unbound liposomes and analyzed via flow cytometry. (A) Percentage of live BMDCs positive for the fluorescent label in the liposomes. (B-F) Relative MFIs (compared to hPG control) of several DC markers. Combined data of four independent experiments. Means + SD, ****$p < 0.0001$, ***$p < 0.001$, *$p < 0.05$ compared to free hPG determined by mixed-effects analysis and Dunnett's multiple comparisons test.

Liposomal co-delivery of hPG and RA leads to antigen-associated tolDC induction in vitro

To assess whether the liposomes affected antigen delivery, the hPG antigen was modified to include the fluorescent label FAM. Liposomes were prepared using hPG-FAM, and this modification did not affect liposomal properties (*table S1*). BMDCs were incubated with LPS in the presence of hPG-FAM, hPG-FAM and RA, hPG-FAM liposomes, or hPG-FAM/RA liposomes for 24 hours. The free hPG-FAM readily associated with cells (*Figure 3C*), and microscopy showed that most of the antigen was located at the surface of the BMDCs (*Figure 4*). In contrast, while flow cytometry showed that fewer cells were positive for the FAM label when the antigen was encapsulated in liposomes (*Figure 3C*), microscopy showed that the FAM label was present mostly inside the cells (*Figure 4*). Interestingly, the presence of RA reduces the uptake of the antigen (*Figure 3C*). Next, we aimed to determine whether antigen-associated cells were also phenotypically tolDCs. For this, the expression of CD86 and CD40 were measured in the hPG-FAM$^+$ DCs. hPG-FAM liposomes and hPG-FAM/RA Liposomes induced more tolDCs (as defined by CD40$^-$ or CD86$^-$CD11c$^+$ DCs) than free hPG-FAM or free hPG-FAM and RA, within antigen-associated cells (*Figure 3A* and *3B*). Interestingly, only the free hPG-FAM and RA increased non-antigen-loaded tolDCs (*Figure3D* and *3E*).

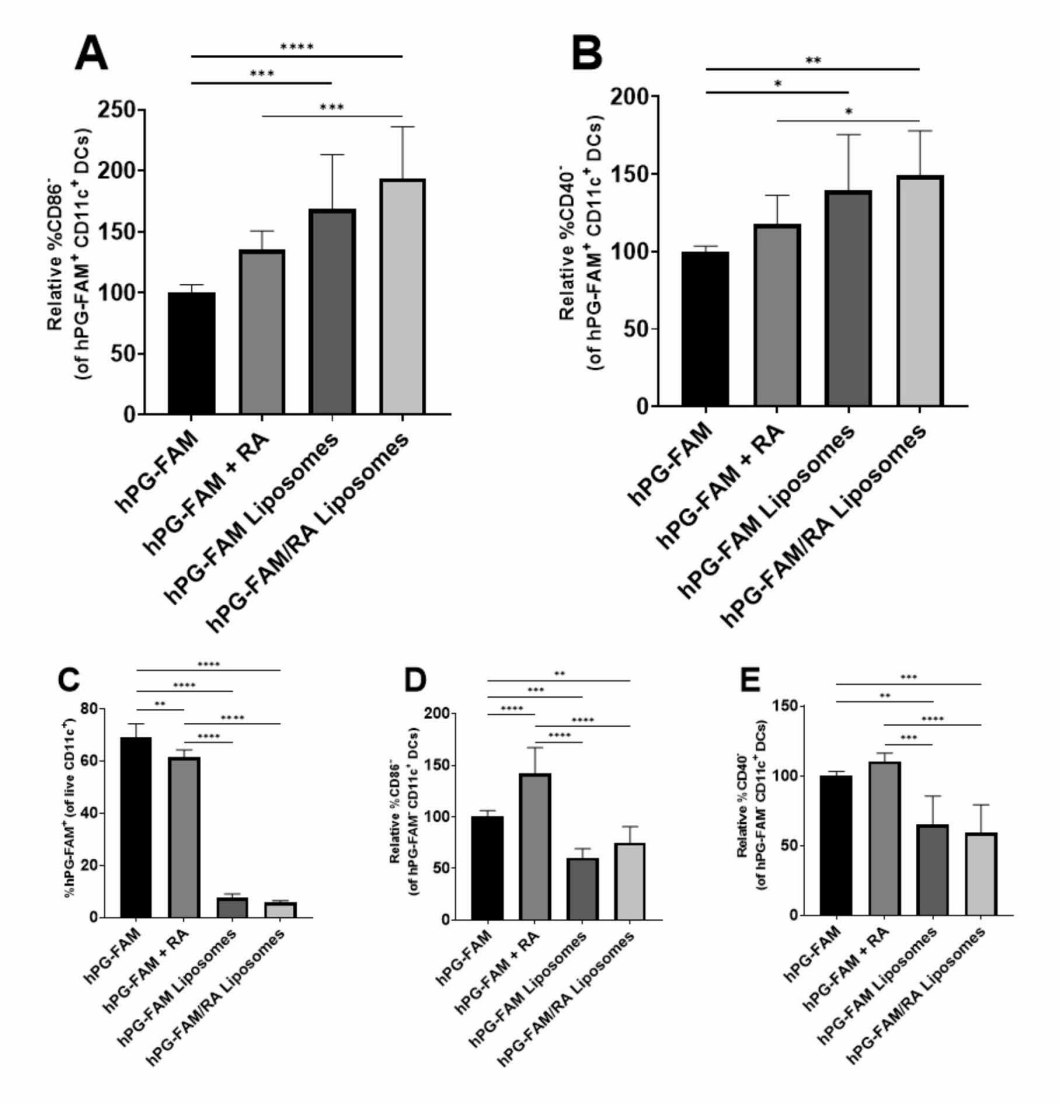


Figure 3. Encapsulation of hPG in liposomes skews towards tolDCs within antigen-associated BMDCs. BMDCs were stimulated with LPS and cultured in the presence of hPG-FAM, hPG-FAM + RA, hPG-FAM liposomes, or hPG-FAM/RA liposomes. After 24 hours of incubation, cells were washed thoroughly and analyzed by flow cytometry. (A) Relative %CD86$^-$ (normalized to hPG-FAM control) and (B) CD40$^-$ tolDCs within hPG-FAM$^+$ cells. (C) Percentage of live BMDCs positive for FAM. (D) Relative %CD86$^-$ (normalized to hPG-FAM control) and (E) CD40$^-$ tolDCs within hPG$^-$FAM- cells. Means + SD, ****p < 0.0001, ***p < 0.001, ** p < 0.01 compared to hPG-FAM determined by one-way ANOVA and Tukey's multiple comparisons test. The data shown are combined normalized data from three independent experiments.

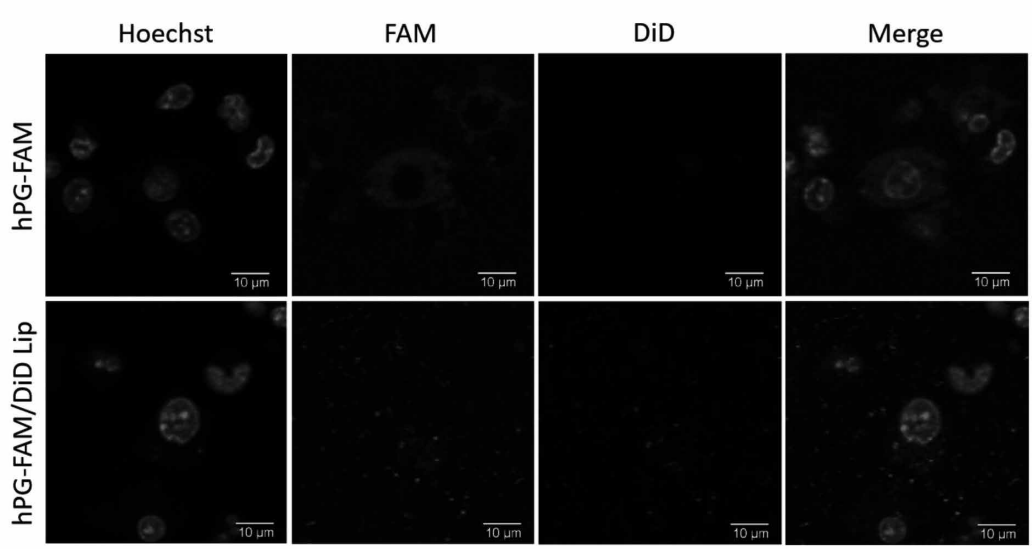


Figure 4. Fluorescence microscopy. BMDCs were stimulated with LPS and cultured in the presence of hPG-FAM added freely or encapsulated in DiD-labelled liposomes. After 24 hours of incubation, cells were washed to remove unbound antigen and liposomes. The blue signal shows the Hoechst staining, the green signal indicates the presence of hPG-FAM, and in red is the liposomal dye. The scale bar shows 10 µm. N = 1.

TolDCs generated with hPG/RA liposomes skew T cells towards a regulatory phenotype in vitro

hPG/RA liposomes can inhibit CD40 and CD86 expression in DCs to the same extent as free hPG and RA, but the question about the functionality of these DCs remained. To assess the ability of these liposome-induced tolDCs to reduce effector T cells (Teff) and stimulate regulatory T cell (Treg) proliferation, purified hPG-TCR transgenic $CD4^+$ T cells were co-cultured with DCs pulsed with different conditions. TolDCs generated using free hPG and RA, and DCs that were induced through hPG/RA liposome stimulation induced significantly less $CD4^+$ T cell proliferation compared to the pro-inflammatory mDCs control (*Figure 5A*). All groups induced Tregs (*Figure 5B*), as shown by the increase in expression of $CD25^+$ $FoxP3^+$ T cells. Additionally, reduced populations of $Tbet^+$ Teffs (*Figure 5C*) were observed in all treatments compared to the control group, suggesting a decrease in the inflammatory Th1 cell
population.

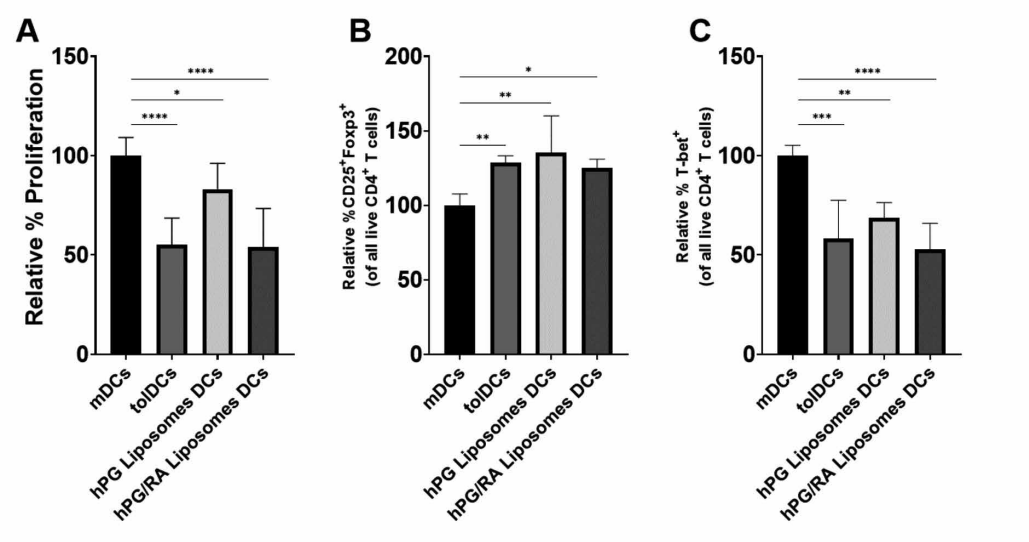


Figure 5. RA, free or encapsulated in liposomes, skew T cells towards a regulatory phenotype. BMDCs were stimulated with LPS and cultured in the presence of hPG (mDCs), hPG + RA (tolDCs), or hPG-containing liposomes with or without RA (hPG Liposomes DCs and hPG/RA Liposomes DCs, respectively). The hPG and RA concentrations were constant in all groups. After 24 hours of incubation, the BMDCs were washed and hPG-TCR specific $CD4^+$ T cells were added. T cell activation was analyzed by flow cytometry after 72 hours of co-culture. (A) Relative % proliferated cells compared to mDC control. (B) Relative $\%CD25^+Foxp3^+$ and (C) $\%T\text{-}bet^+$ in all $CD4^+$ T cells, normalized to mDCs. Means + SD, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ compared to mDC determined by one-way ANOVA and Tukey's multiple comparisons test. The data shown are combined normalized data from three independent experiments.

hPG and RA delivered by tolDCs, liposomes or free affect splenic $CD11c^+$ cell populations in vivo

To observe whether hPG and RA-induced tolDCs, hPG/RA liposomes, or free hPG/RA had effects on splenic DC population phenotype, mice were injected intravenously with the different formulations. Three days after injection, we characterized $CD11c^+$ cells in the spleen using flow cytometry. Within splenic $CD11c^+$ cells, we found no differences in the % of $CD11b^{hi}$ and MHC-II^{lo} cells (*Figure 6B and 6C*). However, $\%CD8\alpha^+CD11c^+$ cells were significantly increased in mice receiving tolDCs compared to mice receiving hPG/RA liposomes (*Figure 6D*). Furthermore, mice receiving the liposomes had significantly fewer MHC-$II^{hi}CD40^{hi}$ activated cells compared to mice that received free hPG/RA (*Figure 6E*).

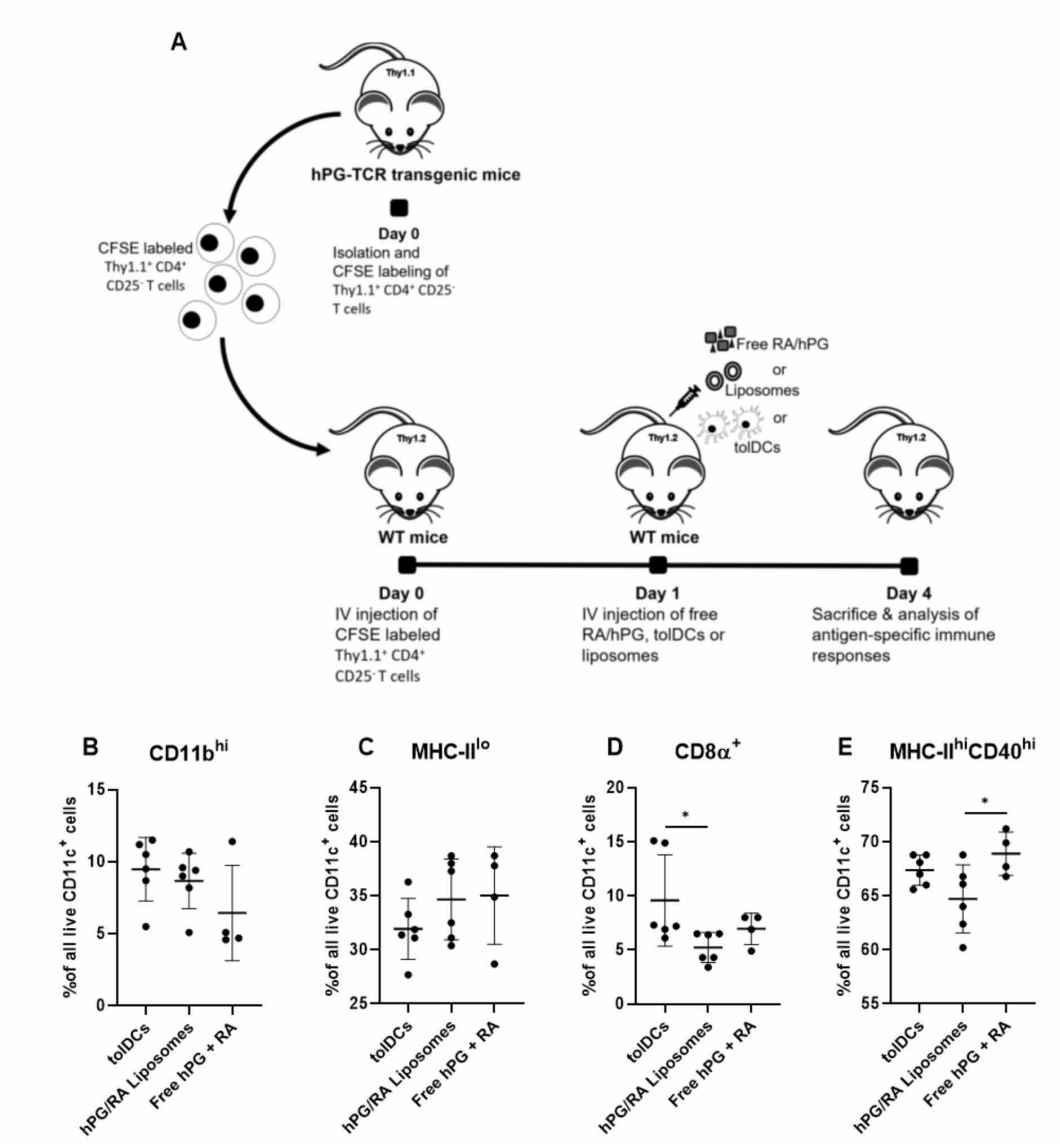


Figure 6. Effect of RA and hPG delivery on splenic DCs *in vivo*. (A) After adoptive transfer of Thy1.1$^+$CD4$^+$CD25$^-$ T cells, mice received intravenous injections of either tolDCs pulsed with hPG + RA (tolDCs), liposomes encapsulating hPG + RA (hPG/RA Liposomes), or free hPG + RA (Free hPG + RA). Three days after injection, splenic DC populations (in live CD11c$^+$ cells) were assessed by flow cytometry. The % of (B) CD11b^{hi} DCs, (C) MHC-II^{lo} DCs, (D) CD8α$^+$ DCs, and (E) MHC-II^{hi}CD40^{hi} DCs were determined. n = 6 for tolDCs and hPG/RA Liposomes, n = 4 for free hPG + RA control. Means ± SD, *$p < 0.05$ determined by one-way ANOVA and Tukey's multiple comparisons test.

hPG and RA delivered by tolDCs, liposomes or free affect splenic CD4$^+$ T cell populations in vivo

To assess the effect on antigen specific T cell responses by hPG and RA administration, we performed an *in vivo* adoptive transfer study. Mice received CFSE-labelled Thy1.1$^+$CD4$^+$CD25$^-$ T cells isolated from hPG-TCR transgenic mice, followed by an intravenous injection of tolDCs, hPG/RA liposomes or free hPG/RA. Both the Thy1.1$^+$ hPG-specific and the bystander Thy1.1$^-$ CD4$^+$ T cell populations were evaluated by flow cytometry. Strikingly, tolDCs showed the highest activation of antigen-specific

CD4$^+$ T cells, as measured by % Thy1.1$^+$ cells, % CFSE$^-$ cells and % CD25$^+$ cells (*Figure 7A*, *B* and *C*). This proliferation was due to the presence of the antigen, since in mice that received only PBS show no or hardly any CFSE$^-$ Thy1.1$^+$ CD4$^+$ T cells (data not shown). Within memory T cell subsets, there were no differences between the groups within the antigen-specific T cell populations (*Figure 7D*, *E*, and *F*). However, in the Thy1.1$^-$ population, tolDCs reduced the % of naïve CD4$^+$ T cells compared to the other groups (*Figure 7D*). Within the antigen-specific immune cell subsets, there were no differences in the induction of CD25$^+$FOXP3$^+$ (Treg), T-bet$^+$ (Th1), GATA-3$^+$ (Th2), and RORγT$^+$ (Th17) CD4$^+$ T cells (*Figure 8A*, *B*, *C*, and *D*). However, the %CTLA-4$^+$ cells were significantly lower in the tolDC group compared to the other groups (*Figure 8E*). CTLA-4 is a negative regulator of T cell activation[31]. Comparing the Thy1.1$^+$ vs Thy1.1$^-$ effects within each group, we observed increased CD25$^+$FOXP3$^+$ and CTLA-4$^+$ cells regardless of delivery method (*Figure 8A* and *E*), and tolDCs reduced T-bet+ cells (*Figure 8B*). Interestingly, bystander RORγT cells were significantly higher in mice receiving tolDCs compared to mice receiving liposomes (*Figure 8D*).

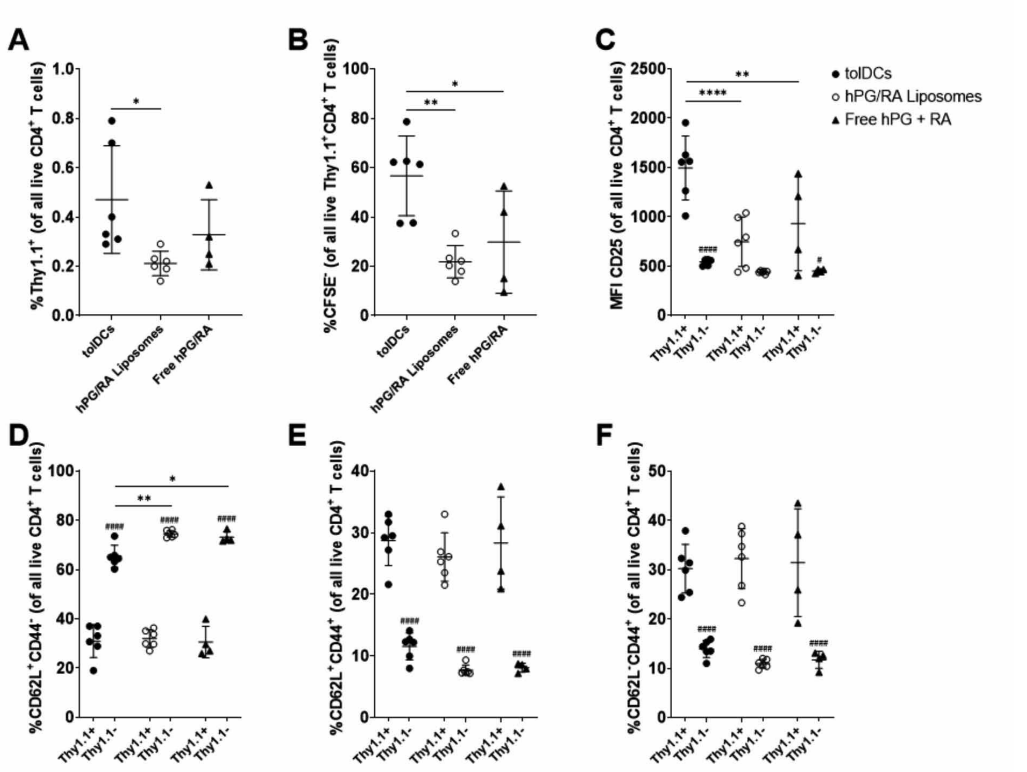


Figure 7. Effect of RA and hPG delivery on the activation of splenic CD4$^+$ T cells *in vivo*. WT Balb/c mice received CFSE-labelled Thy1.1$^+$CD4$^+$CD25$^-$ T cells isolated from the spleens of hPG-TCR transgenic mice via intravenous injection. 24 hours after injection, mice received intravenous injections of either tolDCs pulsed with hPG and RA (tolDCs), liposomes encapsulating hPG and RA (hPG/RA Liposomes), or free hPG + RA. Three days after injection, splenic CD4$^+$ T cells were assessed by flow cytometry. (A) % of antigen-specific Thy1.1$^+$ CD4$^+$ T cells, and (B) % of proliferated CFSE$^-$ cells within this population. (C) CD25$^+$, (D) naïve CD62L$^+$CD44$^-$, (E) central memory CD62L$^+$CD44$^+$, (F) and effector CD62L$^-$CD44$^+$ cells within the Thy1.1$^+$ and Thy1.1$^-$ CD4$^+$ T cell populations. n = 6 for tolDCs and hPG/RA Liposomes, n = 4 for free hPG + RA control. Means ± SD, **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$. #### $p < 0.0001$, # $p < 0.05$ comparing Thy1.1$^+$ to Thy1.1$^-$. Statistics were performed by one-way or two-way ANOVA and Bonferroni's multiple comparisons test.

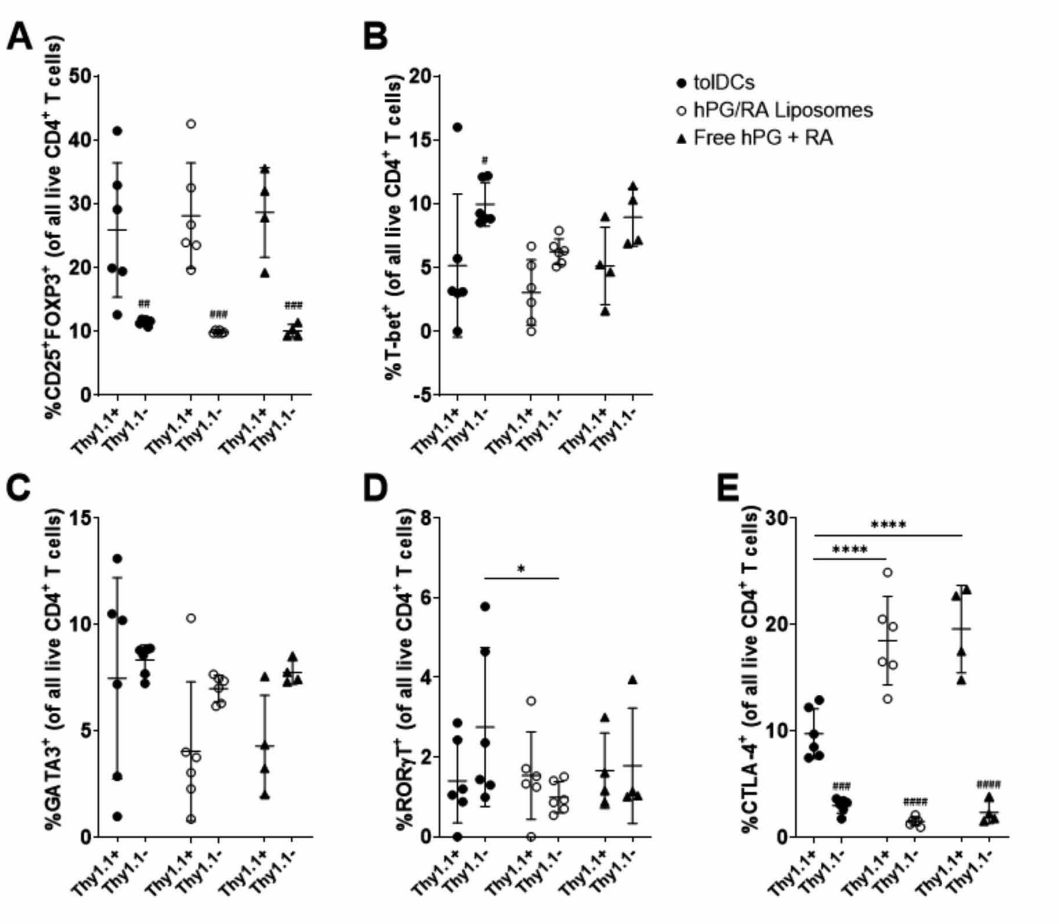


Figure 8. Effect of RA and hPG delivery on the splenic CD4⁺ T cell subsets *in vivo*. WT Balb/c mice received CFSE-labelled Thy1.1⁺CD4⁺CD25⁻ T cells isolated from the spleens of hPG-TCR transgenic mice via intravenous injection. 24 hours after injection, mice received intravenous injections of either tolDCs pulsed with hPG and RA (tolDCs), liposomes encapsulating hPG and RA (hPG/RA Liposomes), or free hPG + RA. Three days after injection, splenic CD4⁺ T cells were assessed by flow cytometry. (A) %CD25⁺FOXP3⁺ (B) %T-bet⁺ (C) %GATA3⁺, (D) %RORγT⁺ (E), and % CTLA-4⁺ cells within the Thy1.1⁺ and Thy1.1⁻ CD4⁺ T cell populations. n = 6 for tolDCs and hPG/RA Liposomes, n = 4 for free hPG + RA control. Means ± SD, **** $p < 0.0001$, *$p < 0.05$. #### $p < 0.0001$, ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$ comparing Thy1.1⁺ to Thy1.1⁻. Statistics performed by two-way ANOVA and Bonferroni's multiple comparisons test.

Discussion

The restoration of antigen-specific tolerance is essential for the development of a curative therapy for autoimmune diseases. TolDCs, have shown promising results in the induction of antigen-specific tolerance in animal models and shown positive results in clinical trials[22]. Here, we focused on the naturally-occurring tolerance-inducing compound RA, and evaluated several methods for delivering RA to dendritic cells *in vitro* and *in vivo*. *In vitro*, we show that RA can induce a semi-mature phenotype that is characteristic of tolDCs (*Figures 1 and 2*), which is in line with other studies[14-16]. These tolDCs were functional and could inhibit antigen-specific T cell proliferation while increasing the relative population of Tregs and reducing the Th1 population (*Figure 5*). Uptake studies with fluorescently labelled hPG-FAM in BMDCs revealed that incubation of these cells with free hPG-FAM and RA leads to a heterogeneous population (*Figure 3*), suggesting that not all cells that take up antigen also become tolerogenic, and vice versa. When this heterogeneous population of cultured RA-tolDCs was injected intravenously in mice, we observed not only antigen-specific effects, but also found changes in non-antigen-specific T cell subsets (*Figure 7*). We also studied several subsets of splenic DCs that might give more insight into the general immune environment of the spleen after the different treatments. We found no differences in the immunosuppressive $CD11b^{+}CD11c^{+}$ population[32], however, injection of tolDCs increased the proportion of $CD8\alpha^{+}CD11c^{+}$ cells in the spleens of mice (*Figure 6D*). $CD8\alpha^{+}$ DCs have been described to take up apoptotic cells in lymphoid tissues and are highly efficient at cross-presentation in MHC-I[33], which is important for the induction of CD8 T cells. $CD8\alpha^{+}$ DCs are also considered to be vital for maintaining immune tolerance[34,35], however, there is a report of these cells accelerating the progression of collagen-induced arthritis in mice[36], which is a murine model for autoimmune disease. Further studies on the involvement of different DC subsets in the regulation of autoimmune diseases are needed to clarify the role of these cells in tolerance induction. To mitigate the observed effect on non-antigen-specific T cells, we theorized that co-delivery of the antigen and RA by a nanoparticle, such as a liposome, would be a better strategy for inducing antigen-specific tolerance and limiting off-target effects.

The liposomes we selected have been previously shown to induce Tregs *in vivo*[25,37], but their effect on DCs had not been studied. BMDCs incubated with LPS and hPG behaved similar to cells incubated with LPS and hPG-containing liposomes (*Figure 2*). These hPG-containing liposomes only had a small effect on $CD4^{+}$ T cell proliferation in a co-culture assay (*Figure 5A*). However, we did observe an *in vitro* effect of liposome-pulsed BMDCs on the induction of antigen-specific Tregs and Th1 cells (*Figure 5B and C*). Furthermore, after *in vitro* incubation with BMDCs, we saw striking differences between hPG-FAM that was given freely to these cells and hPG-FAM encapsulated in liposomes (*Figure 3 and 4*). Liposomal delivery of the antigen reduced the % of cells which had taken up antigen from 69 ± 5% to 8 ± 1% ($p < 0.0001$) , which was possibly due to the cationic charge of the antigen (isoelectric point 10.88) compared to the anionic charge of the liposomes[38]. Even without the addition of RA, the hPG-containing liposomes themselves can induce antigen-specific tolDCs and Tregs (*Figure 5*). This was also observed in previous studies whereby antigen-loaded DSPC:DSPG:CHOL liposomes induced antigen-specific Tregs and mitigated the progression of atherosclerosis in mice[25]. While this is promising, we hypothesized that the addition of RA in the hPG liposomes would enhance tolerance induction even further.

The co-encapsulation of RA and hPG in liposomes did not alter their physicochemical properties as compared to hPG alone (*Table 1*). This is likely because the hPG is localized in the aqueous core of the liposomes, while the hydrophobic RA is incorporated into the lipid bilayer of the liposomes.

RA induced tolDCs equally efficiently when given freely to BMDCs or when encapsulated in liposomes (*Figure 2*). This suggested that while liposomes do not affect the ability of RA to induce tolDCs, they had no advantage over free RA. This was also reflected in *in vitro* co-culture assays with antigen-specific $CD4^+$ T cells; free RA had the same effects as RA encapsulated in liposomes (*Figure 5*). Capurso et al. similarly observed an equivalent effect between free RA or RA encapsulated in poly(lactic-co-glycolic acid) nanoparticles[39]. These results are expected since the *in vitro* system cannot reproduce the complex parameters that direct the (co-)delivery of compounds to APCs *in vivo*, such as administration route, clearance rate, biodistribution, and stability. Within the antigen-associated subset of cells, liposomal delivery of RA led to a significantly higher proportion of tolDCs *in vitro* as compared to free RA (*Figure 3A and B*), while the opposite was observed in non-antigen-specific tolDCs (*Figure 3D and E*). Comparing hPG/RA liposomes with hPG liposomes, the hPG/RA liposomes caused a larger decline in proliferated $CD4^+$ T cells (*Figure 5*). In the *in vivo* experiment, the liposomes had the lowest %MHC-$II^{hi}CD40^{hi}$ cells in the $CD11c^+$ population in the spleen, suggesting that the liposomes are (indirectly) interacting with splenic $CD11c^+$ cells to inhibit their activation. In addition, injection of hPG/RA liposomes mitigated bystander effects in non-antigen-specific T cells (*Figure 7 and 8*) but had no effect compared to the other groups on antigen-specific T cells. Similarly, Phillips et al. found that, after subcutaneous injection of microparticles consisting of human insulin peptide $B_{9\text{-}23}$, RA and TGF-β, there was no change in Tregs in mice compared to controls. However, they did find a significant increase in regulatory B cells in the mesenteric lymph nodes 3 days after microparticle injection, and the mice in this group had a significant reduction in diabetes progression[40]. These data combined show that nanoparticle delivery of RA can be a more specific method to induce antigen-specific tolerance compared to tolDCs.

While there were some differences in T cell subsets between the groups *in vivo*, it should be stressed that the proportion of antigen-specific Tregs in all mice were significantly enhanced compared to the background and effector T cells (*Figure 8*). While Treg induction was the main goal of the current study, it would be interesting to further study in more detail the mechanisms whereby tolDCs and liposomes induce tolerance. While our hPG-FAM experiments do give some insights into this, the effect will likely be different in an *in vivo* system. This could be achieved by injecting fluorescently labelled (antigens in) tolDCs and liposomes and tracking their biodistribution over time. Tracking tolDCs or liposomes would also give more information about the *in vivo* phenotypical stability of the tolDCs, and the phenotype of antigen- or liposome-associated cells *in vivo*. Finally, other dosage schemes or administration routes could improve the effects of RA liposomes on Treg induction.

In conclusion, we show that RA is a potent immunomodulator for the induction of antigen-specific tolerance and that DSPC:DSPG:CHOL liposomes are a suitable carrier system for the co-delivery of an antigen with RA *in vitro*. Additionally, we show strong induction of antigen-specific Tregs, with no off-target effects when using these liposomes. Although the *in vitro* data seems very promising, generating the same effects *in vivo* remains challenging. In this work, we have looked at the heterogeneous populations of DCs that arise in a tolDC culture *in vitro*, and the bystander effect of immunosuppressive therapy *in vivo*. This stresses the importance of not only measuring antigen-specific effects, but also considering off-target effects. Optimization of *in vivo* administration and thorough examination of off-target effects of RA-tolDC or RA-liposome treatment could provide new insights in the use of RA for antigen-specific immunotherapy for autoimmune and chronic inflammatory diseases.

Acknowledgements

The authors would sincerely like to thank L. van Bloois for assistance with liposome preparation, Dr. I.S. Ludwig for assistance with animal studies, and E. van 't Veld and Dr. R. Wubbolts from the Center for Cell Imaging for assistance with microscopy.

Funding

This work was funded in part by the DC4Balance collaboration project which is co-funded by the PPP Allowance made available by Health~Holland, Top Sector Life Sciences & Health, to the Dutch Cooperation of Health Foundations (SGF) and the Dutch Arthritis Foundation.

Conflicts of Interests

The authors declare no conflict of interests.

Author Contributions

Contribution	Author
Conceptualization	**Daniëlle ter Braake**, Naomi Benne, Femke Broere
Formal Analysis	**Daniëlle ter Braake**, Naomi Benne, Chun Yin Jerry Lau
Funding Acquisition	Femke Broere, Enrico Mastrobattista
Investigation	**Daniëlle ter Braake**, Naomi Benne, Chun Yin Jerry Lau
Methodology	**Daniëlle ter Braake**, Naomi Benne, Chun Yin Jerry Lau
Project Administration	**Daniëlle ter Braake**, Naomi Benne
Resources	Femke Broere, Enrico Mastrobattista
Supervision	Naomi Benne, Femke Broere, Enrico Mastrobattista
Validation	**Daniëlle ter Braake**, Naomi Benne
Visualization	**Daniëlle ter Braake**, Naomi Benne
Writing – Original Draft Preparation	**Daniëlle ter Braake**, Naomi Benne
Writing – Review & Editing	**Daniëlle ter Braake**, Naomi Benne, Chun Yin Jerry Lau, Enrico Mastrobattista, Femke Broere

References

1. Willame, C., et al., *Incidence Rates of Autoimmune Diseases in European Healthcare Databases: A Contribution of the ADVANCE Project*. Drug Saf, 2021. 44(3): p. 383-395.
2. Wang, W., H. Zhou, and L. Liu, *Side effects of methotrexate therapy for rheumatoid arthritis*: A systematic review. Eur J Med Chem, 2018. 158: p. 502-516.
3. Chaplin, D.D., *Overview of the immune response*. J Allergy Clin Immunol, 2010. 125(2 Suppl 2): p. S3-23.
4. Audiger, C., et al., *The Importance of Dendritic Cells in Maintaining Immune Tolerance*. J Immunol, 2017. 198(6): p. 2223-2231.
5. Peng, B., Y. Ming, and C. Yang, R*egulatory B cells: the cutting edge of immune tolerance in kidney transplantation*. Cell Death Dis, 2018. 9(2): p. 109.
6. Sakaguchi, S., et al., Regulatory *T cells and immune tolerance*. Cell, 2008. 133(5): p. 775-87.
7. Riedhammer, C. and R. Weissert, Antigen Presentation, Autoantigens, and Immune Regulation in Multiple Sclerosis and Other Autoimmune Diseases. Front Immunol, 2015. 6(322): p. 322.
8. Padovan, E., R.M. Landmann, and G. De Libero, *How pattern recognition receptor triggering influences T cell responses: a new look into the system*. Trends Immunol, 2007. 28(7): p. 308-14.
9. Thery, C. and S. Amigorena, *The cell biology of antigen presentation in dendritic cells*. Curr Opin Immunol, 2001. 13(1): p. 45-51.
10. Worbs, T., S.I. Hammerschmidt, and R. Forster, *Dendritic cell migration in health and disease*. Nat Rev Immunol, 2017. 17(1): p. 30-48.
11. Anderson, A.E., et al., *LPS activation is required for migratory activity and antigen presentation by tolerogenic dendritic cells*. J Leukoc Biol, 2009. 85(2): p. 243-50.
12. Jansen, M.A.A., et al., *Matured Tolerogenic Dendritic Cells Effectively Inhibit Autoantigen Specific CD4(+) T Cells in a Murine Arthritis Model*. Front Immunol, 2019. 10: p. 2068.
13. van Eden, W., et al., *The Immunomodulatory Potential of tolDCs Loaded with Heat Shock Proteins*. Front Immunol, 2017. 8: p. 1690.
14. Garcia-Gonzalez, P., et al., *Tolerogenic dendritic cells for reprogramming of lymphocyte responses in autoimmune diseases*. Autoimmun Rev, 2016. 15(11): p. 1071-1080.
15. Bhatt, S., et al., *All-trans retinoic acid induces arginase-1 and inducible nitric oxide synthase-producing dendritic cells with T cell inhibitory function*. J Immunol, 2014. 192(11): p. 5098-108.
16. Koprivica, I., et al., *Orally delivered all-trans-retinoic acid- and transforming growth factor-beta-loaded microparticles ameliorate type 1 diabetes in mice*. Eur J Pharmacol, 2019. 864: p. 172721.
17. Iwata, M. and A. Yokota, *Retinoic acid production by intestinal dendritic cells*. Vitam Horm, 2011. 86: p. 127-52.
18. Theodosiou, M., V. Laudet, and M. Schubert, *From carrot to clinic: an overview of the retinoic acid signaling pathway*. Cell Mol Life Sci, 2010. 67(9): p. 1423-45.
19. Benson, M.J., et al., *All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation*. J Exp Med, 2007. 204(8): p. 1765-74.
20. Sun, C.M., et al., *Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid*. J Exp Med, 2007. 204(8): p. 1775-85.
21. Coombes, J.L., et al., *A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism*. J Exp Med, 2007. 204(8): p. 1757-64.
22. Suuring, M. and A. Moreau, *Regulatory Macrophages and Tolerogenic Dendritic Cells in Myeloid Regulatory Cell-Based Therapies*. Int J Mol Sci, 2021. 22(15).
23. Tandrup Schmidt, S., et al., *Liposome-Based Adjuvants for Subunit Vaccines: Formulation Strategies for Subunit Antigens and Immunostimulators*. Pharmaceutics, 2016. 8(1): p. 7.

24. Krienke, C., et al., *A noninflammatory mRNA vaccine for treatment of experimental autoimmune encephalomyelitis*. Science, 2021. 371(6525): p. 145-153.
25. Benne, N., et al., *Anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) liposomes induce antigen-specific regulatory T cells and prevent atherosclerosis in mice*. J Control Release, 2018. 291: p. 135-146.
26. Angum, F.; Khan, T.; Kaler, J.; Siddiqui, L.; Hussain, A. The Prevalence of Autoimmune Disorders in Women: A Narrative Review. Cureus 2020, 12, e8094-e8094.
27. Berlo, S.E., et al., *Naive transgenic T cells expressing cartilage proteoglycan-specific TCR induce arthritis upon in vivo activation*. J Autoimmun, 2005. 25(3): p. 172-80.
28. Reddy, M.; Eirikis, E.; Davis, C.; Davis, H.M.; Prabhakar, U. Comparative analysis of lymphocyte activation marker expression and cytokine secretion profile in stimulated human peripheral blood mononuclear cell cultures: an in vitro model to monitor cellular immune function. Journal of Im munological Methods 2004, 293, 127-142,
29. Peng, Q., et al., *PD-L1 on dendritic cells attenuates T cell activation and regulates response to immune checkpoint blockade*. Nat Commun, 2020. 11(1): p. 4835.
30. Braun, A., et al., *Afferent lymph-derived T cells and DCs use different chemokine receptor CCR7-dependent routes for entry into the lymph node and intranodal migration*. Nat Immunol, 2011. 12(9): p. 879-87.
31. Ueda, H., et al., *Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease*. Nature, 2003. 423(6939): p. 506-11.
32. Li, H., et al., *CD11c+CD11b+ dendritic cells play an important role in intravenous tolerance and the suppression of experimental autoimmune encephalomyelitis*. J Immunol, 2008. 181(4): p. 2483-93.
33. Dudziak, D., et al., *Differential antigen processing by dendritic cell subsets in vivo*. Science, 2007. 315(5808): p. 107-11.
34. Belz, G.T., et al., *The CD8alpha(+) dendritic cell is responsible for inducing peripheral self-tolerance to tissue-associated antigens*. J Exp Med, 2002. 196(8): p. 1099-104.
35. Bilsborough, J., et al., *Mucosal CD8alpha+ DC, with a plasmacytoid phenotype, induce differentiati on and support function of T cells with regulatory properties*. Immunology, 2003. 108(4): p. 481-92.
36. Jung, Y.O., et al., *CD8alpha+ dendritic cells enhance the antigen-specific CD4+ T-cell response and accelerate development of collagen-induced arthritis*. Immunol Lett, 2007. 111(2): p. 76-83.
37. Benne, N., et al., *Atomic force microscopy measurements of anionic liposomes reveal the effect of liposomal rigidity on antigen-specific regulatory T cell responses*. J Control Release, 2020. 318: p. 246-255.
38. Nagy, N.A., et al., *Therapeutic Liposomal Vaccines for Dendritic Cell Activation or Tolerance*. Front Immunol, 2021. 12: p. 674048.
39. Capurso, N.A., et al., *Development of a nanoparticulate formulation of retinoic acid that suppresses Th17 cells and upregulates regulatory T cells*. Self Nonself, 2010. 1(4): p. 335-340.
40. Phillips, B.E., et al., *Arrest in the Progression of Type 1 Diabetes at the Mid-Stage of Insulitic Autoimmunity Using an Autoantigen-Decorated All-trans Retinoic Acid and Transforming Growth Factor Beta-1 Single Microparticle Formulation*. Front Immunol, 2021. 12: p. 586220.

Autoantigen-Dexamethasone Conjugate-Loaded Liposomes Halt Arthritis Development in Mice

Autoantigen-Dexamethasone Conjugate-Loaded Liposomes Halt Arthritis Development in Mice

Naomi Benne, PhD[1*], Daniëlle ter Braake, MSc[1*], Deja Porenta, MSc[1,2], Chun Yin Jerry Lau, PhD[2,3], Enrico Mastrobattista, PhD[2], Femke Broere, PhD[1]

* Authors contributed equally

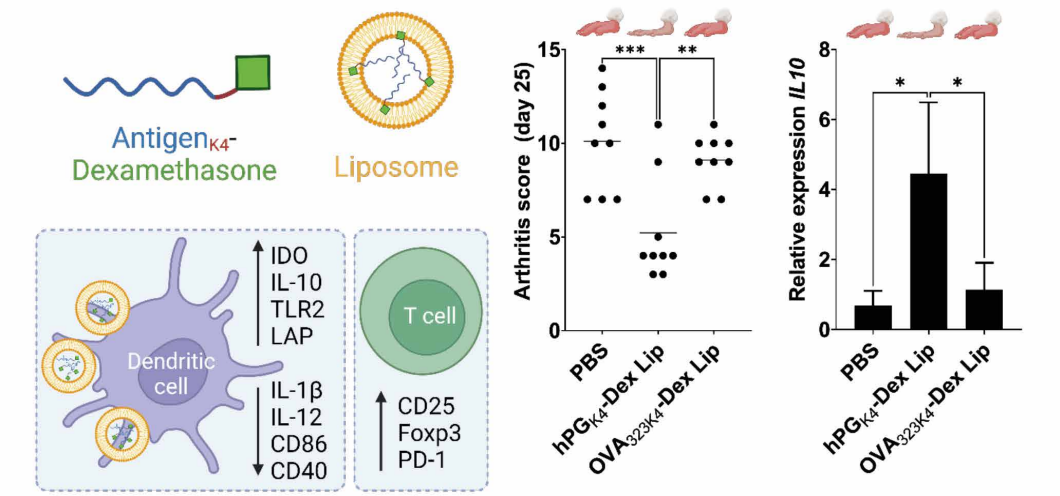
1 Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, 3584 CL Utrecht, The Netherlands
2 Utrecht Institute for Pharmaceutical Sciences, Department of Pharmaceutics, Faculty of Science, Utrecht University, 3584 CG Utrecht, The Netherlands
3 Department of Materials Engineering, Graduate School of Engineering, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656, Japan

Advanced Healthcare Materials (2024)
DOI: 10.1002/adhm.202304238

Abstract

There is no curative treatment for autoimmune or chronic inflammatory diseases such as rheumatoid arthritis, and current treatments can induce off-target side effects due to systemic immune suppression. We have previously shown that dexamethasone-pulsed tolerogenic dendritic cells loaded with the RA-specific antigen human proteoglycan can suppress arthritis development in a proteoglycan-induced arthritis mouse model. To circumvent *ex vivo* dendritic cell culture, and enhance antigen-specific effects, drug delivery vehicles, such as liposomes, provide an interesting approach. Here, we use anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol liposomes with enhanced loading of human proteoglycan-dexamethasone conjugates by cationic lysine tetramer addition. Antigen-pulsed tolerogenic dendritic cells induced by liposomal dexamethasone *in vitro* enhanced antigen-specific regulatory T cells to a similar extent as dexamethasone-induced tolerogenic dendritic cells. In an inflammatory adoptive transfer model, mice injected with antigen-dexamethasone liposomes had significantly higher antigen-specific type 1 regulatory T cells than mice injected with antigen only. The liposomes significantly inhibited the progression of arthritis compared to controls in preventative and therapeutic proteoglycan-induced arthritis mouse models. This coincided with systemic tolerance induction and an increase in IL10 expression in the paws of mice. In conclusion, a single administration of autoantigen and dexamethasone-loaded liposomes seems to be a promising antigen-specific treatment strategy for arthritis in mice.

Graphical Abstract



Keywords

Dexamethasone, Liposomes, Rheumatoid Arthritis, Autoimmunity, Immunotherapy

Abbreviations

Abbreviation	Meaning
RA	Rheumatoid Arthritis
DMARDS	Disease-modifying antirheumatic drugs
NSAIDS	Non-steroidal anti-inflammatory agents
TolDC	Tolerogenic dendritic cells
Tregs	Regulatory T cells
Tr1	Type 1 regulatory T cells
Th	T helper cells
Dex	Dexamethasone
DSPG	1,2-distearoyl-sn-glycero-3-phosphoglycerol
APCs	Antigen-presenting cells
GR	Glucocorticoid receptor
hPG	Human proteoglycan
TFA	Trifluoroacetic acid
DMF	Dimethylformamide
DCM	Dichloromethane
DIC	N, N'-diisopropyl carbodiimide
HPLC	High performance liquid chromatography
TIPS	Triisopropylsilane
DMAP	4-Dimethylaminopyridine
MS	Mass spectrometry
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
CHOL	Cholesterol
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PDI	Polydispersity index
DLS	Dynamic Light Scattering
PGIA	Proteoglycan-Induced Arthritis
FCS	Fetal Calf Serum
BMDC	Bone marrow-derived dendritic cells
GM-CSF	Granulocyte-macrophage colony-stimulating factor
LPS	Lipopolysaccharide
PBMCs	Peripheral blood mononuclear cells
ELISA	Enzyme Linked Immune Sorbent Assay
ACK	Ammonium–Chloride–Potassium
CFSE	Carboxyfluorescein Succinimidyl Ester
IDO	Indoleamine 2,3-dioxygenase 1
HPRT	Hypoxanthine-guanine phosphoribosyl transferase
DDA	Dimethyldiotadecylammonium bromide
FMO	Fluorescence minus one
TLR	Toll-like receptor
LAP	Latency-associated peptide
TGFβ	Transforming growth factor beta
DiD	1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine
MPO	Myeloperoxidase
EAE	Experimental autoimmune encephalomyelitis

Introduction

Rheumatoid arthritis (RA) is a common auto-immune disease with a worldwide prevalence of 0.46%[1]. RA is characterized by an influx of pro-inflammatory immune cells into the synovium, resulting in synovial lining hyperplasia and the destruction of bone and articular cartilage[2], leading to pain and disability. No curative treatments are currently available for RA, and available treatment modalities such as disease-modifying antirheumatic drugs (DMARDs), non-steroidal anti-inflammatory agents (NSAIDS), and corticosteroids[3,4] often bring about off-target side effects as they are not antigen-specific and generally immunosuppressive. This leaves patients more susceptible to developing other complications, such as viral infections[5]. Recently, there have been developments in the field of antigen-specific immunotherapy for autoimmune diseases, specifically focusing on tolerogenic dendritic cells (tolDCs)[6]. These tolDCs are a specialized subset of DCs that can induce $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) and $CD49^+LAG\text{-}3^+$ type 1 regulatory T cells (Tr1) and can inhibit the proliferation of effector $CD4^+$ T cells (Teff), such as $Tbet^+$ T helper 1 (Th1), or $ROR\gamma T^+$ Th17 cells[7]. It has been shown that this skewing towards Tregs/Tr1 and away from other Th subsets is beneficial for the treatment of autoimmunity, including RA[8]. TolDCs are generally characterized by a semi-mature $CD11c^+MHCII^+CD40^{lo}CD86^{lo}$ phenotype, limiting their co-stimulatory abilities. Simultaneously a shift in cytokine production from pro-inflammatory (e.g., IL-12, TNFα) to anti-inflammatory (e.g., IL-10, TGF-β) occurs in these cells. The lack of co-stimulation results in effector T cell anergy[9–11], whilst also promoting Treg differentiation[9,12–15]. TolDCs can be induced through the addition of immunomodulators, such as dexamethasone (Dex)[16], vitamin D3[17], or retinoic acid[18]. A great advantage of using tolDCs for immunotherapy is the ability to load them with disease-relevant antigens, allowing for antigen-specific tolerance induction. This results in a suppressive response specifically towards the disease-causing antigen while avoiding systemic immune suppression that would result from suppressive responses towards other antigens.

At present, differentially generated tolDCs are tested in clinical trials worldwide to assess their efficacy and safety (NCT02903537[19], NCT01352858[20], NCT05251870), and results so far are promising[21,22]. However, just like other cell-based therapies, tolDC therapy is restricted to specialized cell culture and medical facilities, which makes this therapy costly and not widely accessible to patients. Therefore, there is a clear need for a new treatment strategy that allows for easy, low-cost production whilst simultaneously facilitating the beneficial effects also observed in current cell-based treatment options. The use of nanoparticles, such as liposomes, which encapsulate both an immunomodulator and an antigen is a promising strategy to circumvent these limitations[23–26].

Liposomes are drug-delivery vehicles that consist of one or more lipid bilayers that form around an aqueous core[27,28], allowing for the encapsulation of cargo with different physiochemical properties[23]. Depending on the properties of these liposomes, they can modulate immune responses to induce the desired T cell responses. We have previously shown that anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG)-containing liposomes can induce strong antigen-specific Treg responses[29]. These liposomes facilitate the efficient uptake of antigens by antigen-presenting cells (APCs), such as DCs[18]. When loaded with an atherosclerosis-specific antigen, these liposomes have been shown to reduce disease progression in an atherosclerotic mouse model[29], highlighting their therapeutic potential. Nanoparticles encapsulating both immunomodulators and disease-relevant antigens are potent tolerance inducers[30,31]. Therefore, we aimed to enhance the tolerogenic effect of the DSPG-liposomes by including an immunomodulator, such as Dex. Dex is a potent inducer of tolerance in DCs. Upon Dex interaction with the glucocorticoid receptor (GR), gene transcription of IL-10 is increased[32,33], and transcription of pro-inflammatory genes, such as IL-12, is inhibited[34].

Dex has been described as an effective treatment option for a plethora of inflammatory diseases, including rheumatoid arthritis[11]. Dex is poorly soluble in water (logP 1.68), which does lead to beneficial transmembrane transport and *in vivo* bioavailability, but limits encapsulation in liposomes containing high Tm lipids and cholesterol[35,36]. Additionally, when Dex is administered systemically, it can result in severe side effects owing to non-specific immune suppression, especially after long-term use[37]. Therefore, to minimize the risk of off-target effects and increase encapsulation in liposomes, Dex was linked to the antigen epitope via a biodegradable spacer to form a single entity for liposomal encapsulation. Furthermore, a lysine tetramer (K4) was added adjacent to the Dex, to provide a cationic moiety for electrostatic complexation with anionic DSPG, thereby facilitating liposomal loading. This study aimed to induce antigen-specific immune tolerance to an RA-causing antigen, thereby mitigating the progression of RA in preventative and curative mouse models for RA. We hypothesized that DSPG-liposomes loaded with autoantigen-human proteoglycan (hPG)-Dex conjugate can improve the induction of antigen-specific immune suppression.

We show that the antigen-Dex conjugates can be efficiently encapsulated in liposomes. hPG_{K4}-Dex liposomes induce IDO, TLR2, and IL-10 in DCs, which in turn can increase levels of antigen-specific Tr1 cells *in vitro* and *in vivo*. Subsequently, these hPG_{K4}-Dex liposomes can protect against arthritis development in a murine model for PGIA and even halt the progression of established disease. At the end of the therapeutic arthritis study, we observed a reduction of hPG-specific IgG1 autoantibodies and splenic $CD86^+CD11c^+$ cells, and we noticed an increase in splenic $CD25^+Foxp3^+$ and $PD\text{-}1^+CD4^+$ T cells in hPG_{K4}-Dex liposome treated mice compared to controls. In the paws of these mice, we measured high IL-10 which indicates local immune protection. This shows that hPG_{K4}-Dex liposomes are a promising treatment strategy for RA in mice.

Methods

Synthesis and characterization of Dex-peptide conjugates

Preloaded Fmoc-Lys(Boc)-Wang resin, Fmoc-Arg(Pbf)-Wang resin, 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids, and trifluoroacetic acid (TFA) were purchased from Novabiochem GmbH (Hohenbrunn, Germany). Peptide grade dimethylformamide (DMF), dichloromethane (DCM), piperidine, N, N'-diisopropyl carbodiimide (DIC), and high-performance liquid chromatography (HPLC) grade acetonitrile were purchased from Biosolve BV (Valkenswaard, Netherlands). Ethyl cyano-hydroxyiminoacetate (Oxyma pure) was purchased from Manchester Organics Ltd (Cheshire, UK). Triisopropylsilane (TIPS), BioUltra grade ammonium bicarbonate, succinyl anhydride, 4-dimethylaminopyridine (DMAP), and pyridine were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, Netherlands). Dex was purchased from Acros Organics BV (Hague, Netherlands).

Dex-peptide conjugates were synthesized as described previously[38]. In brief, the peptide epitope sequences were synthesized by microwave-assisted Fmoc-based chemistry using an H12 liberty blue peptide synthesizer (CEM Corporation, US). Dex succinate was coupled to the N-terminal of the peptide as with other Fmoc-protected amino acids. TFA/water/TIPS (95/2.5/2.5) was used to cleave the peptide off the resin and remove the side chain protecting groups. Peptides were purified by Prep-HPLC using Reprosil-Pur C18 column (10 µm, 250 × 22 mm). Mass spectrometry (MS) analysis was performed using a Bruker microTOF-Q instrument in positive mode to confirm the identity of the synthetic products. The epitope was derived from the hPG and Ovalbumin (OVA) antigens with the sequence ATEGRVRVNSAYQDK and ISQAVHAAHAEINEAGR, respectively. A lysine tetramer was added to the N-terminal of the epitope sequences for the introduction of a cationic charge. A biodegradable succinyl spacer was added to Dex before conjugation to the N-terminus of the peptide on resin. The Dex-peptide conjugates were cleaved and purified as described above for the peptides.

Liposome preparation and characterization

Liposomes were prepared using the thin film hydration followed by extrusion method.
The phospholipids 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG), were purchased from Avanti Polar Lipids, Birmingham, AL, USA. Cholesterol (CHOL) was purchased from Sigma-Aldrich. Briefly, 180 mg total of dry powder DSPC: DSPG:CHOL in a 4:1:2 molar ratio was weighed and transferred to a dry 100 mL round-bottom flask. The lipids were dissolved in 8 mL chloroform and 8 mL methanol. The solvents were evaporated under a vacuum in a rotary evaporator for 1 h at 40 °C, followed by an N2 stream for 30 min at RT. The resulting lipid film was rehydrated with 2000 µg of hPG, hPG_{K4}, hPG_{K4}-Dex or OVA_{323K4}-Dex dissolved in 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.2) buffer to a total volume of 4 mL and homogenized by rotation in a water bath at 40°C for 1 h. For empty liposomes, liposomes were rehydrated with 4 mL of 10mM HEPES buffer. The resulting suspension was sized by high-pressure extrusion (LIPEX Extruder, Northern Lipids Inc., Burnaby, BC, Canada) on a heating plate set at 60°C by passing the dispersion four times through stacked 400 nm and 200 nm pore-size membranes (Whatman® NucleoporeTM, GE Healthcare, Amersham, UK). To separate non-encapsulated cargo from the liposomes, liposomes were ultracentrifuged (Type 70.1 Ti rotor) for 35 min at 55,000 rpm at 4°C. Liposomes were washed with 10 mL HEPES buffer and centrifugation was repeated three times. Liposomes were stored at 4°C and their stability was measured periodically. Liposomes were used within 2 months for *in vitro* experiments and within 2 weeks for *in vivo* experiments.

The Z-average diameter and polydispersity index (PDI) of the liposomes were measured by dynamic light scattering (DLS) using a NanoZS Zetasizer (Malvern Ltd., Malvern, UK). For this, 10 µL of liposomes were diluted in 990 µL HEPES buffer pH 7.2. The ζ-potential was measured by laser Doppler electrophoresis (Malvern Ltd.) using a universal dip cell. To determine the concentration of loaded hPG_{K4}, hPG_{K4}-Dex, OVA_{323K4}, or OVA_{323K4}-Dex RP-UPLC was used. For this, 20 µL of liposome suspension was dissolved in 180 µL of methanol, and the sample was vortexed. Sample injections were 7.5 µL in volume and the column used was a 1.7 µm BEH C18 column (2.1 × 50 mm, Waters ACQUITY UPLC, Waters, MA, USA). Column and sample temperatures were 40 °C and 20 °C, respectively. The mobile phases were Milli-Q water with 0.1% TFA (solvent A) and acetonitrile with 0.1% TFA (solvent B). For separation, the mobile phases were applied in a linear gradient from 5% to 95% solvent B over 10 min at a flow rate of 0.25 mL/min. Peptide content was detected by absorbance at 280 nm, and Dex was detected at 240 nm[39] using an ACQUITY UPLC TUV detector (Waters ACQUITY UPLC, Waters, MA, USA). Peptide concentrations were calculated based on the respective calibration curves of antigen-Dex complexes dissolved in Milli-Q water.

Mice

For bone marrow isolation, 8-week-old WT mice on Balb/cAnNCrl background (male and female) were purchased from Charles River laboratories . Tyh1.1$^+$ hPG-TCR transgenic[40] mice were bred in-house at the central animal laboratory of Utrecht University, the Netherlands. For proteoglycan-induced arthritis (PGIA) studies, 16-week-old female Balb/cAnNCrl mice were purchased from Charles River laboratories. Mice were randomized into experimental groups based on weight or arthritis score using RandoMice[41]. Humane end-points were adhered to, and the physical discomfort of arthritic animals was relieved by providing easy-to-reach water and food, and additional soft bedding materials. Animals were kept under standard conditions of the animal facility and all experiments were approved by the Animal Experiment Committee of Utrecht University (AVD108002016467 and AVD10800202115687).

Murine bone marrow-derived dendritic cell (BMDC) isolation, dendritic cell culture, and stimulation

Bone marrow isolated from femurs and tibias of Balb/cAnNCrl WT mice were homogenized and seeded in 6-well plates at a cell density of 450,000 cells/mL in 2 mL IMDM (Gibco, ThermoFisher Scientific) supplemented with 10% FCS (Fetal Calf Serum; Bodinco, Alkmaar, The Netherlands), 100 units/mL of penicillin (Gibco, ThermoFisher Scientific, Landsmeer, The Netherlands), 100 µg/mL of streptomycin (Gibco, ThermoFisher Scientific, Landsmeer, The Netherlands) and 0.5 µM β-mercaptoethanol (Gibco, ThermoFisher Scientific, Landsmeer, The Netherlands). Cells were cultured at 37°C and 5% CO2 in the presence of 20 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF, in-house produced) for 6 days. On day 2, IMDM and 20 ng/mL GM-CSF were added to the wells. Extra GM-CSF (20 ng/mL) was supplemented on day 5. On day 6, cells were harvested by scraping and counted. For flow cytometry of DCs, and further co-culture with T cells, cells were transferred to a 96-well F-bottom plate at 50,000 cells/well. The cells were left to adhere for 2 hours. Cells were matured in the presence of 10 ng/mL lipopolysaccharide (LPS, O111:B4; Sigma-Aldrich) and treated with free or encapsulated hPG_{K4}, or free or encapsulated hPG_{K4}-Dex (200 µL/well). In all cases, the concentration of the peptide was 1 µg/mL. For Dex-containing groups, the concentration was 0.18 µg/mL Dex. After 16 h, DCs were harvested for phenotypic characterization by flow cytometry. For qPCR and ELISA, cells were plated out at 600,000 cells/well in an F-bottom 48-well plate. The cells were left to adhere for 2 hours. Cells were stimulated with the same conditions and concentrations as for flow cytometry, in a total volume of 600 µL/well.

Human monocyte isolation, monocyte-derived dendritic cell culture, and stimulation

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy human donors at Sanquin Blood Bank (Amsterdam, Netherlands). Informed consent was given in accordance with the Declaration of Helsinki and Dutch National and Sanquin Internal Ethic Boards. PBMCs were isolated by a Ficoll gradient, and subsequently, monocytes were isolated using anti-CD14 microbeads (Miltenyi Biotech) according to the manufacturer's instructions. Monocytes were seeded in 6-well plates at 2,000,000 cells/mL in 2 mL RPMI (Gibco) supplemented with 5% FCS (Bodinco, Alkmaar, The Netherlands), 100 units/mL of penicillin (Gibco, ThermoFisher Scientific, Landsmeer, The Netherlands), and 100 µg/mL of streptomycin (Gibco, ThermoFisher Scientific, Landsmeer, The Netherlands). To differentiate monocytes towards DCs, 50 ng/mL hGM-CSF (Miltenyi Biotech) and 50 ng/mL hIL-4 (Miltenyi Biotech) were added. On day 3 of culture, fresh medium, and cytokines were added. On day 6, cells were harvested by scraping, counted, and transferred to a 96-well F-bottom plate at 50,000 cells/well. The cells were left to adhere for 2 hours. Cells were matured in the presence of 100 ng/mL lipopolysaccharide (LPS, O111:B4; Sigma-Aldrich) and treated with free or encapsulated hPG_{K4}, or free or encapsulated hPG_{K4}-Dex (200 µL/well). In all cases, the concentration of the peptide was 1 µg/mL. For Dex-containing groups, the concentration was 0.18 µg/mL Dex. After 16h, DCs were harvested for phenotypic characterization by flow cytometry.

Enrichment of $CD4^+$ T cells from murine spleens and co-culture with BMDCs

Spleens were isolated from $Tyh1.1^+$ hPG-TCR mice. A single-cell suspension of splenocytes was obtained by mashing spleens through a 70 µM filter (Falcon, Corning, New York, USA). Erythrocytes were lysed with Ammonium–Chloride–Potassium (ACK) lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na2EDTA; pH 7.3). $CD4^+$ T cells were negatively selected by magnetic separation using Dynabeads™ (sheep anti-rat IgG, ThermoFisher) and anti-CD8 (YTS169), anti-CD11b (M1/70), anti-MHCII (M5/114) and anti-B220 (RA3-6B2, all in-house produced). The enriched $CD4^+$ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE, 0.5 nM) according to the manufacturer's protocol (ThermoFisher). BMDCs were plated out into a 96-well F-bottom plate (50,000 cells/well) and stimulated as described above. After 16h stimulation, cells were washed 4 times with 200 µL PBS/well to remove any free stimuli. To this, 100,000 cells/well of CFSE-labelled $CD4^+$ T cells suspended in 200 µL RPMI (Gibco), supplemented with 5% FCS (Bodinco, Alkmaar, The Netherlands), 100 units/mL of penicillin (Gibco, ThermoFisher Scientific, Landsmeer, The Netherlands), and 100 µg/mL of streptomycin (Gibco, ThermoFisher Scientific, Landsmeer, The Netherlands) were added and incubated for 3 days. Subsequently, $CD4^+$ T cells were harvested for phenotypic characterization by flow cytometry.

Inflammatory adoptive transfer in vivo

$CD4^+$ T cells were purified from the spleens and lymph nodes of $Thy1.1^+$ hPG-TCR transgenic mice as described above. At t0, WT Balb/cAnNCrl mice received an intramuscular injection of 50 µL PBS containing 100µg hPG protein to induce a strong inflammatory response against hPG. After 2 hours mice received 500,000 $CD4^+$ T cells intravenous via the tail vein. After 16 hours, mice were immunized intravenously with 200 µL PBS, 1 nmol free hPG_{K4}, 1 nmol hPG_{K4} liposomes, or 1 nmol hPG_{K4}-Dex liposomes. 3 days after immunization, mice were sacrificed, and spleens were removed and processed as described above.

Preventative arthritis study in vivo

To induce arthritis in mice, female Balb/cAnNCrl mice were injected on days 0 and 21 intraperitoneally with a mixture of 2 mg dimethyldiotadecylammonium bromide (DDA) and 250 µg human proteoglycan.

For the treatment of mice with hPG_{K4}-Dex tolDCs, BMDCs were cultured in 6-well plates as described above. On day 6, 40 µg/mL hPG_{K4}-Dex and 10 ng/mL LPS were added to the cells. DCs were harvested after 16 hours. The viability, purity, and phenotype of the DCs were confirmed using flow cytometry before injection in mice. Mice were treated on day 17 via an intravenous injection in the tail vein with 200 µL PBS, 200 µL 1 x 106 hPG_{K4}-Dex tolDCs (equivalent to 20 nmol of hPG_{K4}-Dex) in PBS, or 200 µL hPG_{K4}-Dex liposomes (2 nmol hPG_{K4}-Dex) in PBS. Arthritis scores were determined 3 times per week starting from day 21 until day 55 in a blinded fashion by two researchers independently using a visual scoring system based on swelling and redness of paws[42]. At the end of the experiment, mice were sacrificed by cervical dislocation.

Curative arthritis study in vivo

To induce arthritis in mice, female Balb/cAnNCrl mice were injected twice intraperitoneally with a mixture of 2 mg DDA and 250 µg human proteoglycan as described above. Arthritis scores were determined 3 times per week as described above. Mice were enrolled in the experiment (day -1) when they had a score of >2 for 2 consecutive scoring moments. Mice were treated on days 0 and 7 via intravenous injection in the tail vein with 200 µL PBS, 200 µL hPG_{K4}-Dex liposomes (2 nmol hPG_{K4}-Dex) in PBS, or 200 µL OVA_{K4}-Dex liposomes (2 nmol OVA_{323K4}-Dex) in PBS. Mice were scored during a period of 25 days after enrollment. At the end of the experiment, mice were sacrificed by cervical dislocation. Spleens were collected for flow cytometry, paws were collected for qPCR, and blood was collected for ELISA in 0.8 mL z-serum separation tubes (Greiner Bio-One, Kremsmünster, Austria). Serum was separated from cells by centrifuging the blood samples at 10,000 x g for 5 minutes at 4 °C, collected into separate tubes, and stored at –20°C.

ELISA of stimulated BMDCs

BMDCs were stimulated as described above and the supernatant was harvested and either used directly for ELISA or stored at -80°C for future analysis. IL-10 (U-CyTech, Utrecht, the Netherlands) and IL-12p70 (9A5 and C17.8, BD Biosciences) was measured in the supernatants by ELISA according to the manufacturer's instructions. Briefly, F-bottom Costar assay 96-well plates (Corning, Kennebunk, ME, USA) were coated with capture antibody at 4°C overnight. Plates were washed thoroughly with 0.01% Tween-20 in PBS and blocked with 1% BSA in PBS for 30 min at RT. Subsequently, plates were washed, and (diluted) samples and standard curves were incubated for 2h at RT. Then, plates were washed and the biotinylated detection antibody and streptavidin-HRP (BD Biosciences) were incubated for 1 hour at RT. Finally, plates were washed, and the samples were reacted with TMB substrate solution (BioLegend). The reaction was stopped with 2N H_2SO_4 solution, and the plates were measured using an iMark™ Microplate Absorbance Reader (Bio-Rad). Cytokine concentrations were calculated based on the respective calibration curves prepared with purified cytokines.

qPCR of stimulated BMDCs

BMDCs were stimulated as described above, and 350 µL RLT buffer (Qiagen Benelux B.V., Venlo, the Netherlands) was added to the cells. The lysate was either used directly for mRNA extraction or stored at -80°C for future analysis. Total mRNA was extracted from stimulated BMDCs using the RNeasy kit (Qiagen) according to the manufacturer's instructions. DNase treatment was performed on-column (Qiagen). The yield of mRNA extraction was measured using a Nanodrop (ThermoFisher). Transcription into cDNA was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). PCR and Real-Time detection were performed using a Bio-Rad MyiQ iCycler (Bio-Rad). Amplification was performed using IQ™ SYBR Green® Supermix (Bio-Rad) with 0.25 µM final concentrations of primers specific for IL1B (5'-TCC ATC TTC TTC TTT GGG TAT TG-3' and 5'-TTC CCG TGG

ACC TTC CAG-3') and Indoleamine 2,3-dioxygenase 1 (IDO) (5'-GCA GAC TGT GTC CTG GCA AAC T-3' and 5'-AGA GAC GAG GAA GAA GCC CTT G-3'), and hypoxanthine-guanine phosphoribosyl transferase (HPRT) (5'-CTG GTG AAA AGG ACC TCT CG-3' and 5'-TGA AGT ACT CAT TAT AGT CAA GGG CA-3'). The following PCR program was used: pre-soaking at 95 °C for 3 min, [denaturation at 95 °C for 20 sec, annealing at 59°C for 30 sec] repeated 40 times. Melting curves and primer efficiencies were measured for each sample. For each sample mRNA expression was normalized to the detected Ct value of HPRT and expressed relative to the average of the DCs incubated with hPG_{K4} + LPS.

qPCR of paws

Paws were harvested and pooled per mouse in 6-well plates containing ice-cold sterile PBS. The skin was removed using scissors and tweezers, and the paws were agitated to release synovial fluid. The resulting suspension was passed through a 70 µM filter (Falcon, Corning, New York, USA) and cells were pelleted by centrifugation. After the removal of supernatant, cells were lysed using 350 µL RLT buffer (Qiagen Benelux B.V., Venlo, the Netherlands). Total mRNA was immediately extracted using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Transcription into cDNA was performed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). PCR and Real-Time detection were performed using a Bio-Rad MyiQ iCycler (Bio-Rad). Amplification was performed using IQ™ SYBR Green® Supermix (Bio-Rad) with 0.25 µM final concentrations of primers specific for MPO (5'-GCT ACC CGC TTC TCC TTC TT-3' and 5'-GGT TCT TGA TTC GAG GGT CA-3'), IL1B (5'- TCC ATC TTC TTC TTT GGG TAT TG-3' and 5'-TTC CCG TGG ACC TTC CAG-3'), IL10 (5'-GGT TGC CAA GCC TTA TCG GA-3' and 5'-ACC TGC TCC ACT GCC TTG CT-3'), and hypoxanthine-guanine phosphoribosyl transferase (HPRT) (5'-CTG GTG AAA AGG ACC TCT CG-3' and 5'-TGA AGT ACT CAT TAT AGT CAA GGG CA-3'). The following PCR program was used: pre-soaking at 95 °C for 3 min, [denaturation at 95 °C for 20 sec, annealing at 59°C for 30 sec] repeated 40 times. Melting curves and primer efficiencies were measured for each sample. The Pfaffl method was used to calculate the gene expression ratio of each gene of interest vs. HPRT, using the PBS group as control.

ELISA of serum anti-hPG IgG1 and IgG2a

ELISA 96-well plates (Corning) were coated overnight with hPG (5µg/mL per well) in 0.1M Carbonate buffer (pH = 9.5). Subsequently, wells were blocked with a blocking buffer consisting of 1.5% milk powder (Campina, Zaltbommel, The Netherlands) dissolved in 1X PBS for 2 hours at RT. Mouse serum was added to the wells at different dilutions (IgG1: 1:12500, 1:25000, 1:50000; IgG2a: 1:500, 1:2500, 1:12500). On each plate, a standard curve composed of serum of a mouse that reached the humane endpoint for arthritis development (PGIA induction, no treatment) at dilutions 0, 1:6250, 1:12500, 1:25000, 1:50000, 1:100000 for IgG1 and 0, 1:250, 1:500, 1:1000, 1:2000, 1:4000 for IgG2a was included. After two hours, IgG1-HRP (X56; BD Biosciences) and IgG2a-HRP(19-15; BD Biosciences) antibodies were added to the wells in the blocking buffer at a 1:1000 dilution. After 1 hour of incubation at RT, wells were washed, and TMB (Thermo Fisher Scientific) was added. The reaction was stopped using 2M H2SO4. ELISA data was read on the using an iMark™ Microplate Absorbance Reader (Bio-Rad) at 450 nm. The background signal (550 nm) was subtracted and serum levels of anti-hPG IgG1 and IgG2a were calculated using the standard curve.

Flow cytometry

BMDCs or moDCs were stimulated as described above and harvested, washed 3 times with 200 µL of 4 mM EDTA and once with 200 µL PBS to remove any free antigen or liposomes, and transferred to a V-bottom 96-well plate. Co-cultured CFSE-labelled $CD4^+$ T cells were harvested and transferred to a V-bottom 96-well plate. For splenocytes from *in vivo* experiments, 2 x 10^6 splenocytes were plated out in 96-well U-bottom plates.

Cell suspensions were blocked for 15 min with 10 µg/mL Fc Block (2.4G2, in-house produced). BMDCs were stained with a monoclonal antibody mix of CD11c-APC (N418, eBioscience, Thermo Fisher Scientific), TLR2-FITC (6C2, eBioscience, Thermo Fisher Scientific), CD86-FITC (GL1, BD Biosciences), CD40-PE (3/23, BD Biosciences), LAP-PE (TW7-16B4, eBioscience, Thermo Fisher Scientific), MerTK-APC (2B10C42, BioLegend), and ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA) in FACS Buffer (1X PBS supplemented with 2% FCS, 0.01% sodium azide, and 2 mM EDTA). moDCs were stained with a monoclonal antibody mix of CD11c-PE (MJ4-27G12, Miltenyi), CD40 PE-Cy7 (5C3, eBioscience), CD86-BB515 (FUN-1, BD Biosciences), and ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA) in FACS Buffer. $CD4^+$ T cells were stained with a monoclonal antibody mix of CD4-BV785 (RM4-5, BioLegend, USA), LAG-3-PE (eBioC9B7W, eBioscience, Thermo Fisher Scientific, USA), CD49b-APC-Cy7 (DX5, BioLegend, USA), and CD25-PerCP-Cy5.5 (PC61.5, eBioscience, Thermo Fisher Scientific, USA), and ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA) in FACS Buffer. After 30 min incubation at 4°C in the dark, cells were washed with PBS, and fixed and permeabilized using the FoxP3 transcription factor staining set (eBioscience, San Diego, CA, USA). Subsequently, cells were stained intracellularly according to the manufacturer's instructions with FoxP3-eFluor450 (FJK-16s, eBioscience, Thermo Fisher Scientific) and T-Bet-APC (4B10, eBioscience, Thermo Fisher Scientific).

Splenocytes from the adoptive transfer experiment were stained with CD4-BV510 (RM4-5, BioLegend, USA), Thy1.1-PerCP-Cy5.5 (HIS51, eBioscience), Thy1.1-FITC (HIS51, eBioscience), LAG-3-APC (C9B7W, eBioscience), CD49b-APC-Cy7 (DX5, Biolegend), PD-L1-BV650 (10F.9G2, Biolegend), CD11c-FITC (N418, eBioscience), CD86-PE-Cy5 (GL1, eBioscience), and ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA) in FACS Buffer. After 30 min incubation at 4°C in the dark, cells were washed with PBS, and fixed and permeabilized using the FoxP3 transcription factor staining set (eBioscience, San Diego, CA, USA). Subsequently, cells were stained intracellularly according to the manufacturer's instructions with FoxP3-eFluor450 (FJK-16s, eBioscience, Thermo Fisher Scientific) and T-bet-APC (4B10, eBioscience). Splenocytes from the curative arthritis study experiment were stained with a monoclonal antibody mix of CD4-BV785 (RM4-5, BioLegend, USA) and CD25-PerCPCy5.5 (PC61.5, eBioscience, Thermo Fisher Scientific, USA), and ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA) in FACS Buffer. After 30 min incubation at 4°C in the dark, cells were washed with PBS, and fixed and permeabilized using the FoxP3 transcription factor staining set (eBioscience, San Diego, CA, USA). Subsequently, cells were stained intracellularly according to the manufacturer's instructions with FoxP-eFluor450 (FJK-16s, eBioscience, Thermo Fisher Scientific), RORγT-PE (AFKJS-9, eBioscience, Thermo Fisher Scientific), GATA-3-PE-Cy7 (TWAJ, eBioscience, Thermo Fisher Scientific) and T-Bet-APC (4B10, eBioscience, Thermo Fisher Scientific). After 30 min incubation at 4°C in the dark, cells were washed and resuspended in 100 µL PBS for measurement. To ensure correct analysis, relevant single-stain, and fluorescence minus one (FMO) controls were used. Samples were measured on a Beckman Coulter Cytoflex LX at the Flow Cytometry and Cell Sorting Facility located at the Faculty of Veterinary Medicine at Utrecht University. The total measured volume was 85 µL per sample, at a measurement speed of 60 µL/min. Acquired data were analyzed using FlowJo Software v.10.7 (FlowJo LLC, Ashland, OR, USA).

Statistical analysis

Statistical analysis was performed in GraphPad Prism v.9.3.1. Details of the analyses are indicated in the figure legends.

Results

Table 1. Physicochemical characterization of Dex-loaded liposomes. The Z_{ave} size, polydispersity index (PDI), ζ-potential and % antigen encapsulation in the liposomes.

Cargo	Z_{ave} (nm)[a]	PDI	Z-potential (mV)	% LE[b]
Empty	191.2 ± 6.3	0.11 ± 0.03	-54.1 ± 3.6	-
hPG	187.6 ± 14.1	0.06 ± 0.03	-47.7 ± 2.1	10.4 ± 6.6
hPG_{K4}	199.4 ± 6.2	0.06 ± 0.02	-51.3 ± 3.8	45.9 ± 11.4
hPG_{K4}-Dex	195.7 ± 21.4	0.08 ± 0.01	-54.9 ± 5.4	46.5 ± 10.8
OVA_{323K4}-Dex	192.3 ± 17.9	0.07 ± 0.03	-52.0 ± 2.9	49.6 ± 11.8

[a] Z-average diameter (Zave), mean ± SD, n = 3.

[b] %LE (loading efficiency) was calculated as the total amount of peptide before extrusion/total amount of peptide after purification * 100%.

Dex, free or encapsulated in liposomes, induces a tolerogenic phenotype in DCs in vitro

The arthritis-relevant MHC-II autoantigen hPG and the ovalbumin-derived MHC-II-restricted $OVA_{323-339}$ antigen were extended with a lysine tetramer to couple to Dex (forming hPG_{K4}-Dex and OVA_{323K4}-Dex, respectively). The antigens were encapsulated into anionic DSPG liposomes. The liposomes, with or without antigen loading, were below 200 nm in size and had a negative surface charge. The LE of the antigen-Dex complexes was between 46.5 and 49.6% (see *Table 1*). The addition of the lysine tetramer enhanced the LE of the antigen from 10.4%, and of Dex from 0.02% (*Figure S1*).

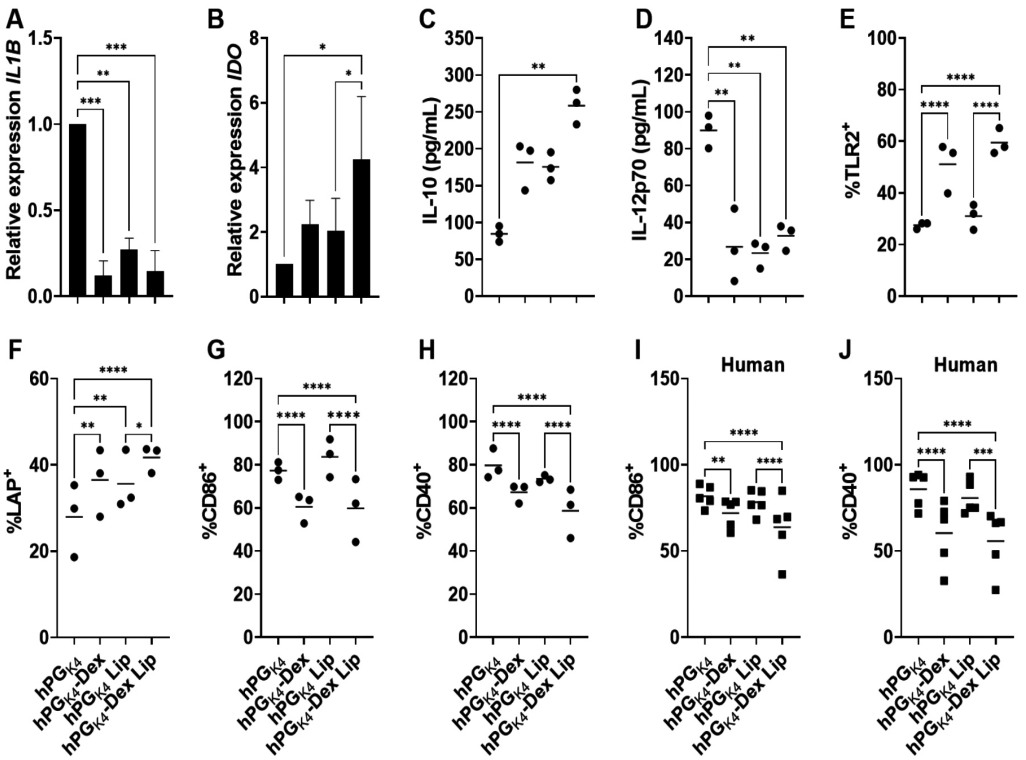


Figure 1. hPG$_{K4}$-Dex, free or encapsulated in liposomes, induces a tolerogenic phenotype in Balb/cAnNCrl BMDCs and human moDCs. Immature BMDCs cultured from the bone marrow of Balb/cAnNCrl mice or human moDCs cultured from CD14$^+$ cells isolated from buffy coats of healthy donors were stimulated overnight with hPG$_{K4}$ + LPS, hPG$_{K4}$-Dex + LPS, hPG$_{K4}$ Liposomes (Lip) + LPS, or hPG$_{K4}$-Dex Lip + LPS. Relative expression of (A) IL1B and (B) IDO, normalized based on HPRT expression and relative to the hPGK4 group, measured by qPCR. (C) IL-10 and (D) IL-12p70 concentration was measured in supernatants of BMDCs (pg/mL) using ELISA. (E) %TLR2$^+$, (F) %LAP$^+$, (G) %CD86$^+$, and (H) %CD40$^+$ in BMDCs, and (I) %CD86$^+$

<

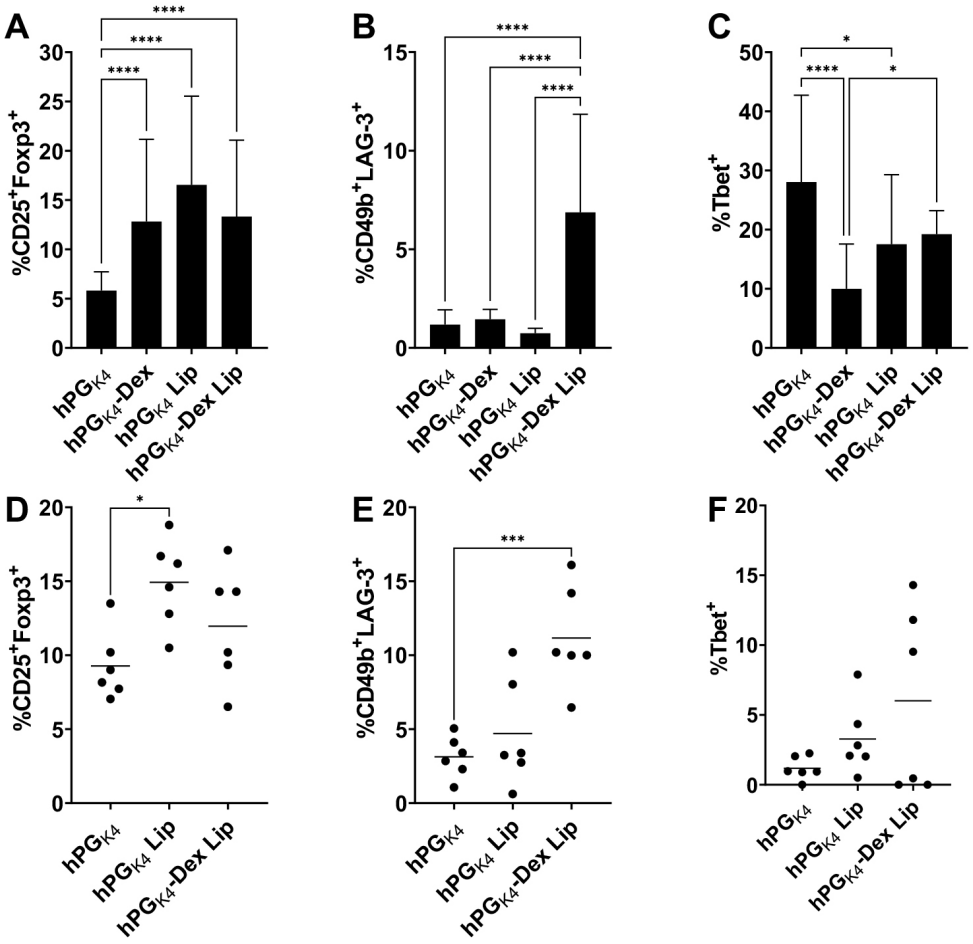


Figure 2. Antigen-specific T cell skewing by hPG$_{K4}$-Dex liposomes *in vitro and in vivo*. Immature BMDCs cultured from the bone marrow of Balb/cAnNCrl mice were stimulated overnight with hPG$_{K4}$ + LPS, hPG$_{K4}$-Dex + LPS, hPG$_{K4}$ Liposomes (Lip) + LPS, or hPG$_{K4}$-Dex Lip + LPS. Cells were washed and CFSE-labelled CD4$^+$ T cells from hPG-TCR mice were co-incubated for 3 days with the BMDCs. (A) %CD25$^+$Foxp3$^+$ (B) %CD49b$^+$LAG-3$^+$, and (C) %Tbet$^+$ cells of CFSE$^-$CD4$^+$ T cells, measured by flow cytometry, n = 3. Thy1.1$^-$ Balb/cAnNCrl mice were injected intramuscularly with hPG protein, followed intravenously with 500,000 Thy1.1$^+$ hPG-TCR CD4$^+$ T cells. This induces hPG-specific inflammatory responses and an expansion of the hPG-TCR CD4$^+$ T cells. 1 day later, mice were injected intravenously with 1 nmol hPG$_{K4}$ or 1 nmol hPG$_{K4}$-Dex encapsulated in liposomes. 3 days later, mice were sacrificed, and spleens were isolated for flow cytometry. (D) %CD25$^+$Foxp3$^+$ (E) %CD49b$^+$LAG-3$^+$, and (F) %Tbet$^+$ cells of Thy1.1$^+$CD4$^+$ T cells, n = 6. Means (+ SD), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, as determined by one-way ANOVA and Tukey's multiple comparisons test (*in vitro*) or Bonferroni's multiple comparisons test (*in vivo*).

To evaluate the tolerance induction by free or encapsulated Dex, immature BMDCs were stimulated overnight with LPS and either free or encapsulated hPG_{K4} and hPG_{K4}-Dex. Gene expression of IL1B was greatly reduced when BMDCs were incubated with hPG_{K4}-Dex, hPG_{K4} liposomes, or hPG_{K4}-Dex encapsulated in liposomes compared to the hPG_{K4} control (*Figure 1A*). Interestingly encapsulation in liposomes seems to enhance the tolerogenic capacity of hPG_{K4}-Dex, as evidenced by the increased gene expression of IDO (*Figure 1B*), and the release of IL-10 (*Figure 1C*). Secretion of IL-12p70 was also reduced in all groups compared to the positive control (*Figure 1D*). Dex, free or encapsulated, increased the expression of Toll-like receptor 2 (TLR2) (*Figure 1E, Figure S2A and S2B*), and latency-as-sociated protein (LAP, the membrane-bound form of TGF-β) (*Figure 1F and Figure S2C*). This coincided with a reduction in the expression of costimulatory molecules CD86 and CD40 in BMDCs and moDCs (*Figure 1G and Figure S2D, Figure 1H and Figure S2E, Figure 1I, and Figure 1J*). We also observed that Dex liposomes had more efficient uptake by BMDCs as compared to Dex-free liposomes, possibly due to an increase in the phagocytic receptor MerTK (*Figure S3*).

Dex-linked hPG induces antigen-specific Tregs in vitro and in vivo

As shown above, hPG_{K4}-Dex liposomes can induce phenotypical tolDCs with a cytokine secretion and gene expression profile that is hypothesized to promote tolerance in T cells. Next, the effect of these tolDCs on antigen-specific T cells was assessed *in vitro* and *in vivo*. Both free and encapsulated hPG_{K4}-Dex pulsed DCs increased antigen-specific $CD25^+Foxp3^+$ Tregs. hPG_{K4} liposomes without Dex also increase Tregs (*Figure 2A*). Only encapsulated Dex increased $CD49b^+LAG\text{-}3^+$ Tr1 cells (*Figure 2B*). Both free and encapsulated Dex decreased antigen-specific T-bet^+ Th1 cells, although free Dex was more potent than encapsulated Dex. Dex-free liposomes also reduced Th1 cells (*Figure 2C*). In a naïve adoptive transfer model, OVA_{323K4}-Dex liposomes were able to expand antigen-specific $CD25^+Foxp3^+$ Tregs (*Figure S4*). In an inflammatory *in vivo* adoptive transfer model, intravenous injection of hPG_{K4} liposomes induced antigen-specific $CD25^+Foxp3^+$ Tregs, while hPG_{K4}-Dex liposomes could not skew the $CD4^+$ T cell response towards $CD25^+Foxp3^+$ Tregs in the pro-inflammatory environment (*Figure 2D*). The hPG_{K4}-Dex liposomes did greatly enhance antigen-specific $CD49b^+LAG\text{-}3^+$ Tr1 cells (*Figure 2E*) compared to free antigen. $Tbet^+$ Th1 cell levels also seemed enhanced in the mice receiving hPG_{K4}-Dex liposomes, albeit non-specifically (*Figure 2F*). This shows that hPG_{K4}-Dex liposomes can induce strong Tr1 responses, even in an inflammatory environment, which is hypothesized to be necessary for suppressing the responses in an arthritis model.

Arthritis development in mice is inhibited by hPG_{K4}-Dex liposomes

Based on the results, we hypothesized that the hPG_{K4}-Dex liposomes would give the best protection in a model of arthritis. To assess the pre-clinical efficacy of the hPG_{K4}-Dex liposomes we employed the PGIA mouse model. First, we performed a preventative study, whereby mice were injected intravenously with PBS, 1×10^6 hPG_{K4}-Dex tolDCs (equivalent to 20 nmol of hPG_{K4}-Dex), or hPG_{K4}-Dex liposomes (2 nmol of hPG_{K4}-Dex) before they had developed arthritis (*Figure 3A*). Mice that were treated with hPG_{K4}-Dex liposomes developed significantly less arthritis compared to the PBS and even the hPG_{K4}-Dex-pulsed tolDC-treated mice, as shown by a lower arthritis score (*Figure 3A*).

Furthermore, while 100% of mice in the PBS group had developed arthritis (score of 2 or higher), 75% and 27% of mice in the hPG_{K4}-Dex-pulsed tolDC and hPG_{K4}-Dex liposomes groups, respectively, developed arthritis (*Table S1*). Next, we wanted to see whether the hPG_{K4}-Dex liposomes could halt the progression of arthritis in mice that had ongoing inflammation (*Figure 3B*). In this model, we observed that they could indeed stabilize arthritis in mice, compared to PBS and compared to OVA_{323K4}-Dex liposomes (*Figure 3B*). 100% of mice in the PBS and OVA_{323K4}-Dex liposomes groups had increased

arthritis scores compared to the day of the first injection, while 33% of mice treated with hPG_{K4}-Dex liposomes overall had lower amounts of anti-hPG IgG1 compared to the other groups, but not IgG2a (*Figure 3C and D*). compared to the start (*Table S1*). Anti-hPG IgG1 and IgG2a were measured in the serum of mice after sacrifice. Mice that received hPG_{K4}-Dex liposomes had a lower score at the end of the experiment.

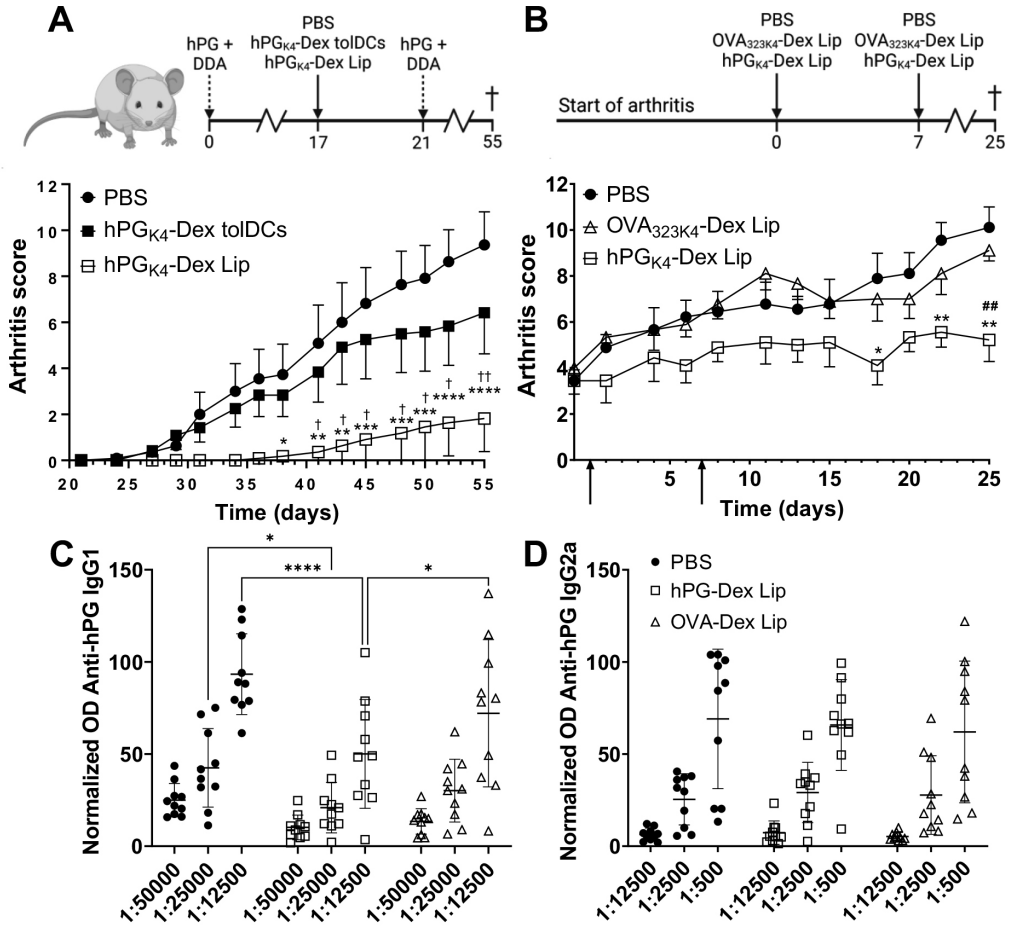


Figure 3. hPG^{K4}-Dex Liposomes inhibit the development of arthritis in mice. Female Balb/cAnNCrl mice were injected i.p. on days 0 and 21 with a mixture of 2 mg DDA and 250 µg human proteoglycan to induce arthritis. (A) In the preventative model mice were treated on day 17 via intravenous injection of PBS, hPG_{K4}-Dex tolDCs (20 nmol hPG_{K4}-Dex), or hPG_{K4}-Dex liposomes (2 nmol hPG_{K4}-Dex). (B) In the curative model, mice were enrolled after arthritis was established based on visual scoring and treated on days 0 and 7 after arthritis induction via intravenous injection of PBS, hPG_{K4}-Dex liposomes (2 nmol hPG_{K4}-Dex), or OVA_{323K4}-Dex liposomes (2 nmol OVA_{323K4}-Dex). Means ± SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to PBS group, and † $p < 0.05$, †† $p < 0.01$ compared to hPG_{K4}-Dex tolDCs, and ## $p < 0.01$ compared to OVA_{323K4}-Dex liposomes as determined by two-way ANOVA and Bonferroni's multiple comparisons test. (C) Anti-hPG IgG1 and (D) IgG2a antibodies were measured in the serum of mice 25 days after the first injection of the treatment by ELISA. OD values per plate were normalized based on a calibration curve. The serum dilutions are shown on the x-axis. Means + SD, * $p < 0.05$, **** $p < 0.0001$, as determined by one-way ANOVA and Bonferroni's multiple comparisons tests.

Further analysis of the spleens of mice sacrificed on day 25 after the first injection of liposomes or control revealed that, in the mice that received the hPG_{K4}-Dex liposomes, the %$CD11c^+CD86^+$ DCs were reduced compared to the other groups (*Figure 4A*), while PD-L1 was unchanged (*Figure 4B*). This coincided with an increase in $CD25^+Foxp3^+$ and PD-1^+ regulatory T cells (*Figure 4C and 4D*) within the $CD4^+$ T cell population (*Figure S5*). $ROR\gamma T^+$ and $Tbet^+$ populations were unchanged (*Figure 4E and 4F*). Interestingly, in the paws of mice, we observed a non-significant reduction in MPO and IL1B (*Figure 4G and H*) while the expression of IL10 was enhanced (*Figure 4I*).

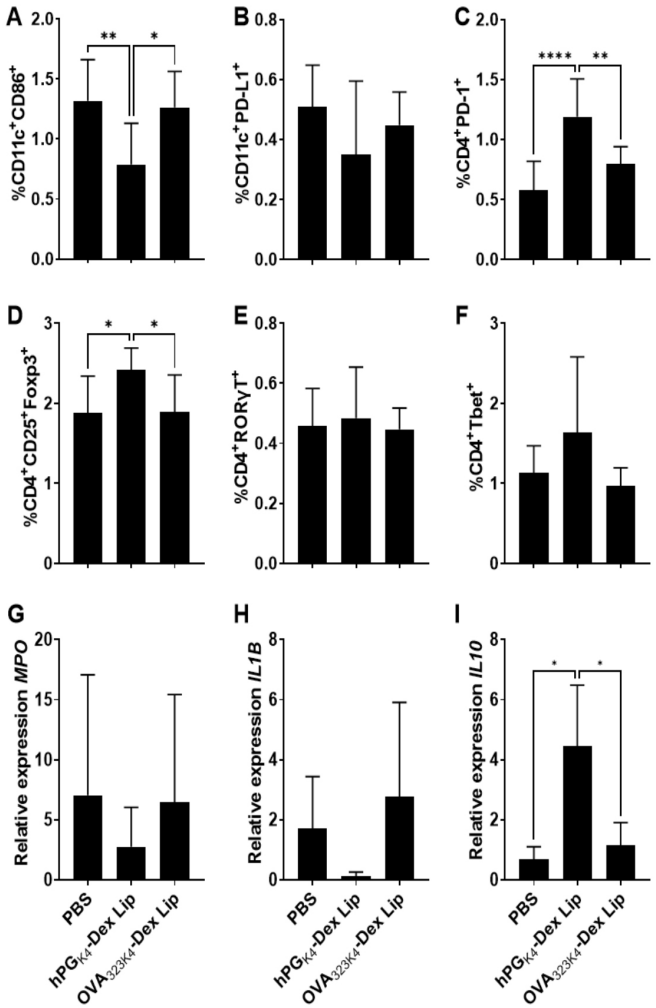


Figure 4. hPG_{K4}-Dex liposomes enhance tolerogenic responses in arthritic mice. Female Balb/cAnNCrl mice were injected i.p. on days 0 and 21 with a mixture of 2 mg DDA and 250 µg human proteoglycan to induce arthritis. Mice were enrolled after arthritis was established and treated on days 0 and 7 via intravenous injection of PBS, hPG_{K4}-Dex liposomes (2 nmol hPG_{K4}-Dex), or OVA_{323K4}-Dex liposomes (2 nmol OVA_{323K4}-Dex). Mice were sacrificed on day 25 and organs were isolated for analysis. (A) $CD11c^+CD86^+$ DCs, (B) $CD11c^+$PD-$L1^+$ DCs, (C) $CD4^+$PD-1^+ T cells, (D) $CD4^+CD25^+Foxp3^+$ Tregs, (E) $CD4^+ROR\gamma T^+$ Th17, and (F) $CD4^+Tbet^+$ Th1 cells, % of all live cells in the spleen, measured by flow cytometry. (G) MPO, (H) IL1B, and (I) IL10 expression in the paws of mice, normalized based on HPRT expression using the Pfaffl method, measured by qPCR. Means $^+$ SD, * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ as determined by one-way ANOVA and Bonferroni's multiple comparisons test.

Discussion

Restoring immune tolerance is essential for the treatment of autoimmune and chronic inflammatory diseases. Current clinical trials are making use of DCs pulsed with immunomodulators and disease-relevant antigens to achieve this[19–22]. Despite encouraging results, the production of these tolDCs requires specialized research centers and is costly, making them less accessible to the large groups of patients that need treatment. Accordingly, there's a need for a strategy that overcomes these limitations. Nanoparticles, including liposomes, are promising drug-delivery vehicles that can surpass the need for *ex vivo* culturing of tolDCs[23–26]. Here, we prepared anionic DSPG-containing liposomes for the delivery of novel antigen-Dex conjugates to induce antigen-specific immune tolerance *in vitro, in vivo*, and in a pre-clinical model for RA. Dex was chosen because of its potent immune-suppressing properties and proven ability to induce tolDCs[34–36]. However, it is known that Dex can induce side effects, especially at high doses[37]. Therefore, we coupled Dex to our antigens of interest with the aim of preventing the uptake of Dex by APCs in a non-antigen-specific context, thereby minimizing non-antigen-specific effects. In addition, we hypothesized that the liposomes facilitated more efficient uptake of antigen-Dex conjugates by APCs than free antigen-Dex conjugates, allowing us to greatly reduce the required dose, further minimizing side effects. To optimize the liposomal preparation, we reasoned that adding lysine tetramer to antigen-Dex conjugates could enable electrostatic complexation with DSPG, which resulted in a marked enhancement of LE (*Figure S1*).

BMDCs and moDCs exhibit a tolerogenic phenotype upon stimulation with free hPG_{K4}-Dex (*Figure 1*). This is in line with previous research showing the effect of Dex on DCs, using similar assays and Dex concentrations[43,44]. This indicates that the linking of Dex to an antigen does not hinder the functionality of Dex. Encapsulation of hPG_{K4}-Dex into liposomes crucially does not impede the immunomodulatory effects of Dex (*Figure 1 and Figure 2*). On the contrary, stimulation of DCs with hPG_{K4}-Dex liposomes increased gene expression of IDO (*Figure 1B*), the release of IL-10 (*Figure 1C*), and protein expression of LAP (*Figure 1F*) compared to free hPG_{K4}-Dex. Dex is a GR ligand that must cross the host cell membrane and bind to GRs present in the cell cytoplasm in order to exert its effects[45,46]. Accordingly, enhanced intracellular delivery of Dex by liposomes could increase interactions with GRs, possibly explaining the enhanced effects observed after hPG_{K4}-Dex liposome treatment. The DSPG-liposomes themselves have tolerogenic properties, as we have shown before[29], but the addition of Dex enhanced some of these effects (*Figure 1*). Dex stimulates uptake of liposomes, possibly due to an increase in the phagocytic receptor MerTK (*Figure S3*). This increased uptake of the inherently tolerogenic liposomes, together with the tolerogenic properties of Dex, could explain why the Dex-containing liposomes show a more favorable tolDC phenotype.

Our studies using TCR-specific transgenic $CD4^+$ T cells indicate that the coupling of Dex to an antigen via the lysine tetramer does not prevent MHC presentation by DCs and subsequent TCR recognition by antigen-specific T cells as we observe potent T cell responses. The most striking result is that hPG_{K4}-Dex liposome-treated BMDCs increase $CD49b^+LAG\text{-}3^+$ Tr1 numbers in *in vitro* co-culture experiments (*Figure 2B*) , while free Dex and liposomes without Dex do not induce Tr1 cells. In a naïve adoptive transfer model, OVA_{323K4}-Dex liposomes were able to expand antigen-specific $CD25^+Foxp3^+$ Tregs to the same extent as free OVA_{323K4}-Dex (*Figure S4*), albeit at a much lower dose (1 nmol vs 85 nmol). Kim et al. reported that poly (lactic-co-glycolic acid) (PLGA)-nanoparticles co-encapsulating OVA and Dex, using a comparable dose of Dex (administered orally) as described in this paper, resulted in antigen-specific Treg induction compared to nanoparticles with OVA alone in an adoptive transfer model[30]. In an inflammatory adoptive transfer model, hPG_{K4}-Dex liposome induced antigen-specific $CD49b^+LAG\text{-}3^+$ Tr1 cells (*Figure 3E*). Antigen-specific $CD25^+Foxp3^+$ Tregs were increased in the inflammatory adoptive transfer

model in mice that received hPG_{K4} liposomes, but not hPG_{K4}-Dex liposomes (*Figure 3D*). This indicates that, in an inflammatory environment, the hPG_{K4}-Dex liposomes lose the ability to induce or maintain antigen-specific Tregs, while they can still enhance Tr1 cells. This difference between the skewing of T cell subsets between Dex-free and Dex-liposomes could be explained by the fact that the Dex liposomes inhibited costimulatory molecules in DCs[47], and increased IL-10, LAP[48], IDO[49,50], and TLR2[51,52] compared to Dex-free liposomes (*Figure 1*). Furthermore, Dex has previously been shown to upregulate glucocorticoid-induced tumor necrosis factor-receptor family-related gene (GITR) expression in a variety of T cells[53], and the interaction between GITR and its ligand (GITRL) has been previously shown to be responsible for an increase in Tr1 cells[54]. However, it should be stated that the consequences of GITR activation are complex and can be dependent on the activation state of T cells and the host environment.

Since the hPG_{K4}-Dex liposomes induced potent Tr1 responses, and these responses are important for protection against arthritis[52], we decided to test these liposomes in a murine PGIA disease model for arthritis. hPG_{K4}-Dex liposomes significantly reduced arthritis development compared to mice that received hPG_{K4}-Dex tolDCs or PBS (*Figure 3A*). It should be stated that antigen-loaded dexamethasone-induced tolDCs are a potent therapy against rheumatoid arthritis, and the lack of effect in the current study is likely due to the suboptimal dose of hPG_{K4}-Dex[11,55]. More importantly, the liposomes could prevent the further progression of arthritis in mice that had established diseases (*Figure 3B*). The lack of arthritis development in mice that received hPG_{K4}-Dex liposomes is more pronounced than in previous research using arthritis-related agent-pulsed tolDC therapy in a mouse model[7,11]. It was previously shown in an experimental autoimmune encephalomyelitis (EAE) model that co-encapsulation of MOG with Dex in Dextran nanoparticles was essential to treat disease; MOG + Dex nanoparticles performed significantly better than PBS, empty nanoparticles, Dex nanoparticles, MOG nanoparticles, or free Dex + MOG[31]. Mechanistically, we show that mice treated with hPG_{K4}-Dex liposomes had a significantly lower proportion of $\%CD11c^+CD86^+$ DCs compared to the PBS group (*Figure 4A*). At the same time, there was a greater amount of $CD4^+CD25^+Foxp3^+$ and $CD4^+PD\text{-}1^+$ T cells in the spleens of these mice (*Figure 4C and D*). This suggests that there were some long-lasting systemic effects of the therapy. However, it is unknown whether these cells contributed to protection against arthritis or are the result of reduced inflammation in mice. The effect does seem to be antigen-specific since it was not observed in the mice receiving OVA_{323K4}-Dex liposomes. Further studies should be performed to unravel this. Nguyen et al. also observed a decrease in $CD86^+$ APCs and an increase in $Foxp3^+CD4^+$ T cells in the spleens of mice after nanoparticle treatment, which coincided with protection against EAE[56]. Interestingly, we saw a significant increase in the expression of *IL10* in the paws of mice treated with hPG_{K4}-Dex liposomes (*Figure 4J*), which likely explains why these mice had a lower arthritis score than controls[57,58].

Liposomes have been used as delivery vehicles to target Dex towards the inflamed joints, thereby reducing arthritis symptoms through the broad inflammation-inhibiting properties of Dex. To test the antigen-specificity of our treatment, and the effect of delivering liposomal Dex in arthritis mice, we treated mice with OVA_{323K4} -Dex liposomes, OVA_{323} being a disease-irrelevant MHC-II antigen. We observed no changes in any assays between OVA_{323K4}-Dex liposomes compared to PBS (*Figure 3 and 4*). This was expected, since to obtain an accumulation of liposomes in inflamed joints through the leaky vasculature that is associated with inflammation[59], it is necessary to functionalize the liposomes through e.g., PEGylation[60], peptides[61], or other small molecules[62] on the surface of the liposomes. Furthermore, the dose of Dex reported in studies that deliver Dex to the site of inflammation via nanoparticles is 5 to 30 times higher than the dose used in this study (0.1-1.2 mg/kg vs 0.02 mg/kg),

and often requires more than 2 injections. Therefore, we can conclude that the observed effect in our study is not due to the accumulation of Dex liposomes in the joints that affect the inflamed tissue, but rather due to antigen-specific skewing of T cells toward protective responses against arthritis.

Since the hPG_{K4}-Dex conjugates are amphipathic molecules, they could potentially self-assemble into micelles or nanoparticles during the fabrication process of the liposomes. During the formulation and characterization process, we did not observe such particles forming, most likely due to the low concentration of hPG_{K4}-Dex conjugates used, although we do not know what the critical micellar concentration of the conjugates is. Further studies are required to assure that the purification through ultracentrifugation would eliminate the self-assembled particles and their potential *in vivo* effects.

More research is required for translation to human studies. However, the liposomes are similar to a formulation that is already approved in humans[63] and dexamethasone is a commonly prescribed drug[64]. Furthermore, we propose that the liposomes result in a large dose-reduction of dexamethasone, which would suggest that the therapy is safe to use in humans. The antigen chosen in this study was hPG since it is used to induce arthritis in the mouse model, however, in human patients, we would likely require a different antigen, such as (citrullinated) peptides derived from fibrinogen, vimentin and collagen, or heat shock protein (HSP)70[65]. The only clinical trial carried out so far with a similar treatment strategy for rheumatoid arthritis showed that the liposomes were well-tolerated and induced T cells with tolerogenic TCR signaling and exhaustion profiles[66].

The liposomes in combination with our novel antigen-Dex conjugates provide a promising strategy to inhibit arthritis development. The results presented here highlight the therapeutic potential of antigen-Dex-loaded DSPG-containing liposomes in immune therapy against autoimmune diseases.

Acknowledgements

The authors would like to thank Dr. I. S. Ludwig and Dr. A. J. Stoppelenburg for helpful discussions and help during the animal studies. Graphics in the graphical abstract and *Figure 3* were created in Biorender.com.

Declaration of funding sources

This work was funded in part by the DC4Balance collaboration project. which is co-funded by the PPP Allowance made available by Health~Holland, Top Sector Life Sciences and Health, to the Dutch Cooperation of Health Foundations (SGF) and the Dutch Arthritis Foundation.

Conflict of interest statement

The authors declare no conflict of interest related to this publication.

Author contributions

Contribution	Author
Conceptualization	**Daniëlle ter Braake**, Naomi Benne, Femke Broere
Formal Analysis	**Daniëlle ter Braake**, Naomi Benne
Funding Acquisition	Femke Broere, Enrico Mastrobattista
Investigation	**Daniëlle ter Braake**, Naomi Benne, Deja Porenta, Chun Yin Jerry Lau
Methodology	**Daniëlle ter Braake**, Naomi Benne, Chun Yin Jerry Lau
Project Administration	**Daniëlle ter Braake**, Naomi Benne
Resources	Femke Broere, Enrico Mastrobattista
Supervision	Naomi Benne, Femke Broere, Enrico Mastrobattista
Validation	**Daniëlle ter Braake**, Naomi Benne, Deja Porenta
Visualization	**Daniëlle ter Braake**, Naomi Benne
Writing – Original Draft Preparation	**Daniëlle ter Braake**, Naomi Benne
Writing – Review & Editing	**Daniëlle ter Braake**, Naomi Benne, Deja Porenta, Chun Yin Jerry Lau, Enrico Mastrobattista, Femke Broere

References

1. Almutairi, K., Nossent, J., Preen, D., Keen, H. & Inderjeeth, C. The global prevalence of rheumatoid arthritis: a meta-analysis based on a systematic review. *Rheumatol. Int.* 41, 863–877 (2021).
2. Smolen, J. S. et al. Rheumatoid arthritis. *Nat. Rev. Dis. Prim.* 4, 18001 (2018).
3. Han, J. Y. & Reynolds, M. A. Effect of anti-rheumatic agents on periodontal parameters and bio markers of inflammation: A systematic review and meta-analysis. *J. Periodontal Implant Sci.* 42, 3–12 (2012).
4. Shams, S. *et al.* The Therapeutic Landscape of Rheumatoid Arthritis: Current State and Future Directions. *Frontiers in Pharmacology vol.* 12 at https://doi.org/10.3389/fphar.2021.680043 (2021).
5. Her, M. & Kavanaugh, A. Alterations in immune function with biologic therapies for autoimmune disease. *Journal of Allergy and Clinical Immunology* vol. 137 19–27 at https://doi.org/10.1016/j.jaci.2015.10.023 (2016).
6. Passeri, L., Marta, F., Bassi, V. & Gregori, S. Tolerogenic dendritic cell-based approaches in autoimmunity. *International Journal of Molecular Sciences* vol. 22 at https://doi.org/10.3390/ijms22168415 (2021).
7. Hilkens, C. M. U. & Isaacs, J. D. Tolerogenic dendritic cell therapy for rheumatoid arthritis: Where are we now? *Clin. Exp. Immunol.* 172, 148–157 (2013).
8. Page, A., Fusil, F. & Cosset, F. L. Antigen-specific tolerance approach for rheumatoid arthritis: Past, present and future. *Joint Bone Spine* vol. 88 at https://doi.org/10.1016/j.jbspin.2021.105164 (2021).
9. Domogalla, M. P., Rostan, P. V., Raker, V. K. & Steinbrink, K. Tolerance through education: How tolerogenic dendritic cells shape immunity. *Frontiers in Immunology* vol. 8 at https://doi.org/10.3389/fimmu.2017.01764 (2017).
10. Steinbrink, K., Graulich, E., Kubsch, S., Knop, J. & Enk, A. H. CD4+ and CD8+ anergic T cells induced by interleukin-10-treated human dendritic cells display antigen-specific suppressor activity. *Blood* 99, 2468–2476 (2002).
11. Jansen, M. A. A. et al. Matured tolerogenic dendritic cells effectively inhibit autoantigen specific CD4+ T cells in a murine arthritis model. *Front. Immunol.* 10, (2019).
12. Kretschmer, K. et al. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat. Immunol.* 6, 1219–1227 (2005).
13. Apostolou, I. & Von Boehmer, H. In vivo instruction of suppressor commitment in naive T cells. *J. Exp. Med.* 199, 1401–1408 (2004).
14. Harris, N. L. & Ronchese, F. The role of B7 costimulation in T-cell immunity. *Immunology and Cell Biology* vol. 77 304–311 at https://doi.org/10.1046/j.1440-1711.1999.00835.x (1999).
15. Vogel, I. et al. Foxp3+ regulatory T cells are activated in spite of B7-CD28 and CD40-CD40L blockade. *Eur. J. Immunol.* 43, 1013–1023 (2013).
16. García-González, P. A. et al. Dexamethasone and monophosphoryl lipid a induce a distinctive profile on monocyte-derived dendritic cells through transcriptional modulation of genes associated with essential processes of the immune response. *Front. Immunol.* 8, (2017).
17. Navarro-Barriuso, J. et al. Comparative transcriptomic profile of tolerogenic dendritic cells differentiated with vitamin D3, dexamethasone and rapamycin. *Sci. Rep.* 8, (2018).
18. Braake, D. Ter, Benne, N., Lau, C. Y. J., Mastrobattista, E. & Broere, F. Retinoic acid-containing liposomes for the induction of antigen-specific regulatory T cells as a treatment for autoimmune diseases. *Pharmaceutics* 13, (2021).
19. Willekens, B. et al. Tolerogenic dendritic cell-based treatment for multiple sclerosis (MS): A harmonised study protocol for two phase i clinical trials comparing intradermal and intranodal cell administration. *BMJ Open* 9, (2019).

20. Bell, G. M. et al. Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis. *Ann. Rheum. Dis.* 76, 227–234 (2017).
21. Phillips, B. E., Garciafigueroa, Y., Engman, C., Trucco, M. & Giannoukakis, N. Tolerogenic dendritic cells and T-regulatory cells at the clinical trials crossroad for the treatment of autoimmune disease; emphasis on type 1 diabetes therapy. *Frontiers in Immunology* vol. 10 at https://doi.org/10.3389/fimmu.2019.00148 (2019).
22. Phillips, B. E., Garciafigueroa, Y., Trucco, M. & Giannoukakis, N. Clinical tolerogenic dendritic cells: xploring therapeutic impact on human autoimmune disease. *Frontiers in Immunology* vol. 8 at https://doi.org/10.3389/fimmu.2017.01279 (2017).
23. Benne, N., ter Braake, D., Stoppelenburg, A. J. & Broere, F. Nanoparticles for Inducing Antigen-Specific T Cell Tolerance in Autoimmune Diseases. *Frontiers in Immunology* vol. 13 at https://doi.org/10.3389/fimmu.2022.864403 (2022).
24. Kishimoto, T. K. & Maldonado, R. A. Nanoparticles for the induction of antigen-specific immunological tolerance. *Frontiers in Immunology* vol. 9 at https://doi.org/10.3389/fimmu.2018.00230 (2018).
25. Krienke, C. et al. A noninflammatory mRNA vaccine for treatment of experimental autoimmune encephalomyelitis. *Science (80-.).* 371, 145–153 (2021).
26. Schmidt, S. T., Foged, C., Korsholm, K. S., Rades, T. & Christensen, D. Liposome-based adjuvants for subunit vaccines: Formulation strategies for subunit antigens and immunostimulators. *Pharmaceutics* vol. 8 at https://doi.org/10.3390/pharmaceutics8010007 (2016).
27. Pattni, B. S., Chupin, V. V. & Torchilin, V. P. New Developments in Liposomal Drug Delivery. *Chemical Reviews* vol. 115 10938–10966 at https://doi.org/10.1021/acs.chemrev.5b00046 (2015).
28. Guimarães, D., Cavaco-Paulo, A. & Nogueira, E. Design of liposomes as drug delivery system for therapeutic applications. *International Journal of Pharmaceutics* vol. 601 at https://doi.org/10.1016/j.ijpharm.2021.120571 (2021).
29. Benne, N. et al. Anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) liposomes induce antigen-specific regulatory T cells and prevent atherosclerosis in mice. *J. Control. Release* 291, 135–146 (2018).
30. Kim, S. H. et al. Induction of antigen-specific immune tolerance using biodegradable nanoparticles containing antigen and dexamethasone. *Int. J. Nanomedicine* 14, 5229–5242 (2019).
31. Peine, K. J. et al. Treatment of experimental autoimmune encephalomyelitis by codelivery of disease associated peptide and dexamethasone in acetalated dextran microparticles. *Mol. Pharm.* **11**, 828–835 (2014).
32. Xia, C. Q., Peng, R., Beato, F. & Clare-Salzler, M. J. Dexamethasone induces IL-10-producing monocyte-derived dendritic cells with durable immaturity. *Scand. J. Immunol.* 62, 45–54 (2005).
33. Visser, J. et al. Differential regulation of interleukin-10 (IL-10) and IL-12 by glucocorticoids in vitro. *Blood* 91, 4255–4264 (1998).
34. Rhen, T. & Cidlowski, J. A. Antiinflammatory Action of Glucocorticoids — New Mechanisms for Old Drugs. *N. Engl. J. Med.* 353, 1711–1723 (2005).
35. Tsotas, V. A., Mourtas, S. & Antimisiaris, S. G. Dexamethasone incorporating liposomes: Effect of lipid composition on drug trapping efficiency and vesicle stability. *Drug Deliv.* 14, (2007).
36. Bhardwaj, U. & Burgess, D. J. Physicochemical properties of extruded and non-extruded liposomes containing the hydrophobic drug dexamethasone. *Int. J. Pharm.* 388, (2010).
37. Madamsetty, V. S. et al. Dexamethasone: Insights into Pharmacological Aspects, Therapeutic Mechanisms, and Delivery Systems. *ACS Biomater. Sci. Eng.* (2022) doi:10.1021/acsbiomaterials.2c00026.

38. Lau, C. Y. J. et al. Modulating albumin-mediated transport of peptide-drug conjugates for antigen-specific Treg induction. *J. Control. Release* 348, 938–950 (2022).
39. Xue, L., Wang, D., Zhang, X., Xu, S. & Zhang, N. Targeted and triple therapy-based liposomes for enhanced treatment of rheumatoid arthritis. *Int. J. Pharm.* 586, (2020).
40. Berlo, S. E. et al. Naive transgenic T cells expressing cartilage proteoglycan-specific TCR induce arthritis upon in vivo activation. *J. Autoimmun.* 25, 172–180 (2005).
41. Van Eenige, R. et al. RandoMice, a novel, user-friendly randomization tool in animal research. *PLoS One* 15, (2020).
42. Hanyecz, A. et al. Achievement of a Synergistic Adjuvant Effect on Arthritis Induction by Activation of Innate Immunity and Forcing the Immune Response Toward the Th1 Phenotype. *Arthritis Rheum.* 50, 1665–1676 (2004).
43. Piemonti, L. et al. Glucocorticoids Affect Human Dendritic Cell Differentiation and Maturation. *J. Immunol.* 162, 6473–6481 (1999).
44. Rozkova, D., Horvath, R., Bartunkova, J. & Spisek, R. Glucocorticoids severely impair differentiati on and antigen presenting function of dendritic cells despite upregulation of Toll-like receptors. *Clin. Immunol.* 120, 260–271 (2006).
45. Weikum, E. R., Knuesel, M. T., Ortlund, E. A. & Yamamoto, K. R. Glucocorticoid receptor control of transcription: Precision and plasticity via allostery. *Nature Reviews Molecular Cell Biology* vol. 18 159–174 at https://doi.org/10.1038/nrm.2016.152 (2017).
46. Kadmiel, M. & Cidlowski, J. A. Glucocorticoid receptor signaling in health and disease. *Trends in Pharmacological Sciences* vol. 34 518–530 at https://doi.org/10.1016/j.tips.2013.07.003 (2013).
47. Wakkach, A. et al. Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. *Immunity* 18, 605–617 (2003).
48. Levings, M. K., Bacchetta, R., Schulz, U. & Roncarolo, M. G. The role of IL-10 and TGF-beta in the differentiation and effector function of T regulatory cells. *International archives of allergy and immunology* vol. 129 263–276 at https://doi.org/10.1159/000067596 (2002).
49. Rothhammer, V. & Quintana, F. J. The aryl hydrocarbon receptor: an environmental sensor integrating immune responses in health and disease. *Nature Reviews Immunology* vol. 19 184–197 at https://doi.org/10.1038/s41577-019-0125-8 (2019).
50. Mellor, A. L. & Munn, D. H. IDO expression by dendritic cells: Tolerance and tryptophan catabolism. *Nature Reviews Immunology* vol. 4 762–774 at https://doi.org/10.1038/nri1457 (2004).
51. Jeon, S. G. et al. Probiotic Bifidobacterium breve induces IL-10-producing Tr1 cells in the colon. *PLoS Pathog.* 8, (2012).
52. Volz, T. et al. Nonpathogenic bacteria alleviating atopic dermatitis inflammation induce IL-10-producing dendritic cells and regulatory Tr1 cells. *J. Invest. Dermatol.* 134, 96–104 (2014).
53. Nocentini, G. et al. A new member of the tumor necrosis factor/nerve growth factor receptor family inhibits T cell receptor-induced apoptosis. *Proc. Natl. Acad. Sci. U. S. A.* 94, 6216–6221 (1997).
54. Carrier, Y. et al. Enhanced GITR/GITRL interactions augment IL-27 expression and induce IL-10-producing Tr-1 like cells. *Eur. J. Immunol.* 42, 1393–1404 (2012).
55. Anderson, A. E. et al. Tolerogenic dendritic cells generated with dexamethasone and vitamin D3 regulate rheumatoid arthritis CD4+ T cells partly via transforming growth factor-β1. *Clin. Exp. Immunol.* 187, 113–123 (2017).
56. Nguyen, T. L. et al. Immunosuppressive biomaterial-based therapeutic vaccine to treat multiple sclerosis via re-establishing immune tolerance. *Nat. Commun.* 13, (2022).

57. Whalen, J. D. et al. Adenoviral Transfer of the Viral IL-10 Gene Periarticularly to Mouse Paws Sup presses Development of Collagen-Induced Arthritis in Both Injected and Uninjected Paws. *J. Immunol.* 162, 3625–3632 (1999).
58. Finnegan, A. et al. Collagen-induced arthritis is exacerbated in IL-10-deficient mice. *Arthritis Res. Ther.* **5**, (2003).
59. Mitragotri, S. & Yoo, J. W. Designing micro- and nano-particles for treating rheumatoid arthritis. *Archives of Pharmacal Research* vol. 34 1887–1897 at https://doi.org/10.1007/s12272-011-1109-9 (2011).
60. Lorscheider, M. et al. Dexamethasone palmitate nanoparticles: An efficient treatment for rheumatoid arthritis. *J. Control. Release* 296, 179–189 (2019).
61. Meka, R. R., Venkatesha, S. H., Acharya, B. & Moudgil, K. D. Peptide-targeted liposomal delivery of dexamethasone for arthritis therapy. *Nanomedicine* 14, 1455–1469 (2019).
62. Yan, F. et al. Exosome-based biomimetic nanoparticles targeted to inflamed joints for enhanced treatment of rheumatoid arthritis. *J. Nanobiotechnology* 18, (2020).
63. Stone, N. R. H., Bicanic, T., Salim, R. & Hope, W. Liposomal Amphotericin B (AmBisome®): A Review of the Pharmacokinetics, Pharmacodynamics, Clinical Experience and Future Directions. *Drugs* vol. 76 485–500 at https://doi.org/10.1007/s40265-016-0538-7 (2016).
64. World Health Organization. The selection and use of essential medicines 23rd List, 2023. *World Health Organ. Tech. Rep. Ser.* (2023).
65. Mansilla, M. J., Hilkens, C. M. U. & Martínez-Cáceres, E. M. Challenges in tolerogenic dendritic cell therapy for autoimmune diseases: the route of administration. *Immunotherapy Advances* vol. 3 at https://doi.org/10.1093/immadv/ltad012 (2023).
66. Sonigra, A. et al. Randomized phase I trial of antigen-specific tolerizing immunotherapy with peptide/calcitriol liposomes in ACPA+ rheumatoid arthritis. *JCI Insight* 7, (2022).

Supplementary Materials & Methods

Mice for adoptive transfer assay using liposome-encapsulated OVA_{323K4}-Dex

8-week old female C57BL/6-Ly5.1 and C57BL/6-Tg(TcraTcrb)425Cbn/Crl (OTII) mice used for adoptive transfer experiments were purchased from Charles River laboratories.

Adoptive transfer in vivo using liposome-encapsulated OVA_{323K4}-Dex

$CD4^+$ T cells were purified from OT-II transgenic mice using a $CD4^+$ T cell enrichment kit according to the manufacturer's instructions (Miltenyi, Netherlands). On day -1, all $CD45.1^+$ Ly5.1 mice received 500,000 $CD4^+$ T cells intravenously via the tail vein. On day 0, mice were immunized subcutaneously by injection into the left and right flanks (50 µL each side) of 85 nmol OVA_{323K4}, 85 nmol OVA_{323K4}-Dex, or 1 nmol OVA_{323K4}-Dex encapsulated in liposomes. Seven days after immunization, mice were sacrificed and spleens were removed and processed as described above. For FACS analysis, 2 x 10^6 splenocytes were plated out in 96-well U-bottom plates. Cells were blocked for 15 min with Fc Block (2.4G2, in-house produced). $CD4^+$ T cells were stained with a monoclonal antibody mix of CD4-BV785 (RM4-5, BioLegend, USA), CD45.2-PerCP-Cy5.5 (104, eBioscience), and CD25-BV650 (PC61, Biolegend), and ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA) in FACS Buffer (1X PBS supplemented with 2% FCS and 2 mM EDTA). After 30 min incubation at 4°C in the dark, cells were washed with PBS, and fixed and permeabilized using the FoxP3 transcription factor staining set (eBioscience, San Diego, CA, USA). Subsequently, cells were stained intracellularly according to the manufacturer's instructions with FoxP-eFluor450 (FJK-16s, eBioscience, Thermo Fisher Scientific). Finally, cells were washed and resuspended in 100 µL PBS for measurement. To ensure correct analysis, relevant single-stain, and fluorescence minus one (FMO) controls were used. Samples were measured on a Beckman Coulter Cytoflex LX at the Flow Cytometry and Cell Sorting Facility at the Faculty of Veterinary Medicine at Utrecht University. Acquired data were analyzed using FlowJo Software v.10.7 (FlowJo LLC, Ashland, OR, USA).

Supplementary Results

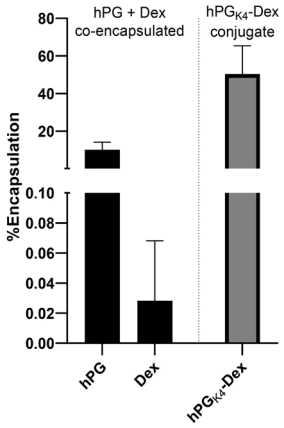


Figure S1. hPG_{K4}-Dex conjugates increase the encapsulation of hPG and Dex. Liposomes were prepared as described above, either with hPG_{K4}-Dex conjugates, or the equivalent amount of free hPG and free Dex. Encapsulation was measured by UPLC.

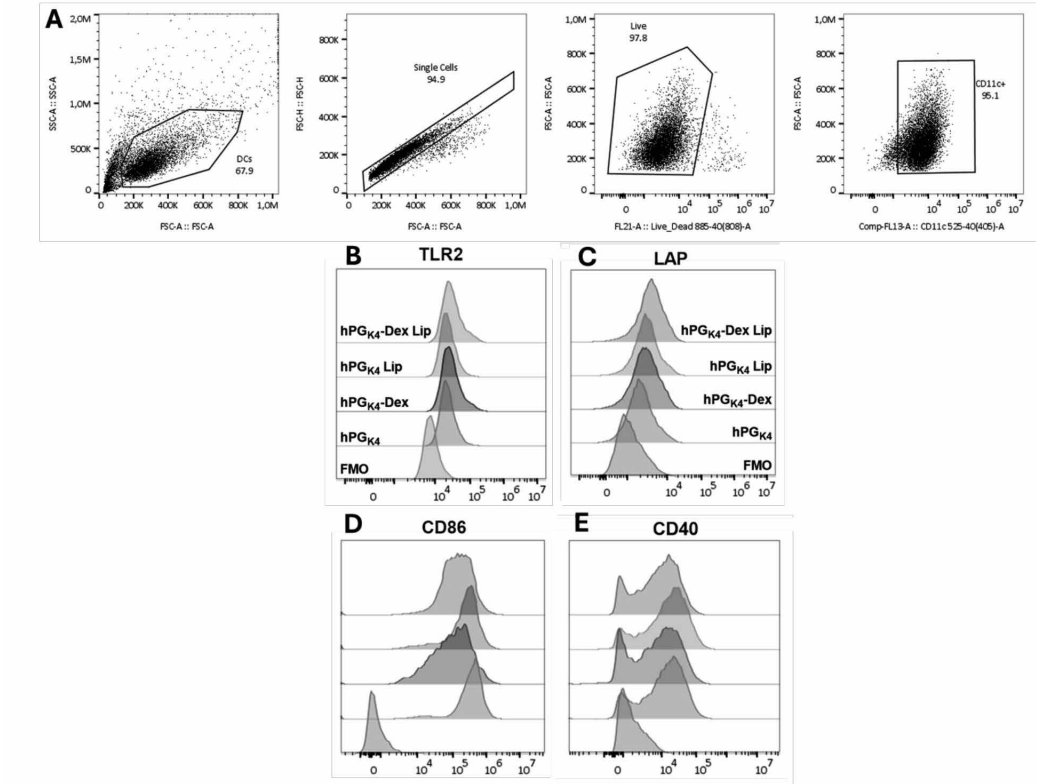


Figure S2. Gating strategy and representative flow cytometry histograms of BMDCs incubated with LPS and hPG_{K4}, hPG_{K4}-Dex, hPG_{K4} liposomes or hPG_{K4}-Dex liposomes. (A) Cells were gated based on forward and side scatter, single cells were selected based on forward scatter area and height, live cells were selected based on the staining of Viakrome, and DCs were selected based on the expression of CD11c. Representative histograms of (B) TLR2, (C) LAP, (D) CD86, and (E) CD40 staining, including fluorescence minus one (FMO) controls. fluorescence minus one (FMO) controls.

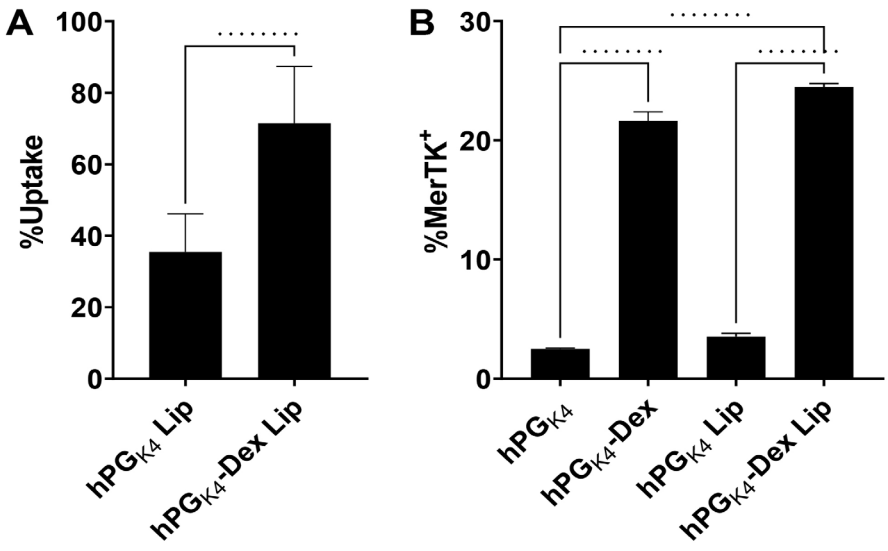


Figure S3. Dexamethasone increases the uptake of liposomes by BMDCs. (A) Liposomes were prepared as described above, with the addition of 0.02 mol% 1,1-dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine. Immature BMDCs were stimulated overnight with LPS and fluorescently labelled hPG_{K4} Liposomes (Lip) or hPG_{K4}-Dex Lip. The % of $CD11c^+$ that were positive for the fluorescent label in the liposomes was measured using flow cytometry. (B) Immature BMDCs were stimulated overnight with LPS and hPG_{K4}, hPG_{K4}-Dex, hPG_{K4} Lip, or hPG_{K4}-Dex Lip. %MerTK of live $CD11c^+$ cells were measured using flow cytometry. Means + SD **** $p < 0.0001$ as determined by t-test or one-way ANOVA and Tukey's multiple comparisons test.

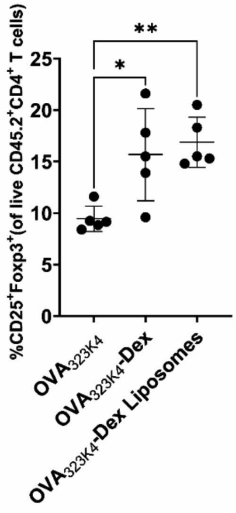


Figure S4. Free and encapsulated OVA_{323K4}-Dex induces antigen-specific $CD25^+FoxP3^+$ Tregs *in vivo*. $CD45.1^+$ Bl6 mice were injected intravenously with 500,000 OT-II $CD45.2^+CD4^+$ T cells. 1 day later, mice were injected subcutaneously with 85 nmol OVA_{323K4}, 85 nmol OVA_{323K4}-Dex, or 1 nmol OVA_{323K4}-Dex encapsulated in liposomes. 7 days later, mice were sacrificed and spleens were isolated for flow cytometry. Cells were stained for Viakrome, CD45.2, CD4, CD25, and FoxP3 and measured with flow cytometry. Means + SD, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, as determined by one-way ANOVA and Bonferroni's multiple comparisons tests.

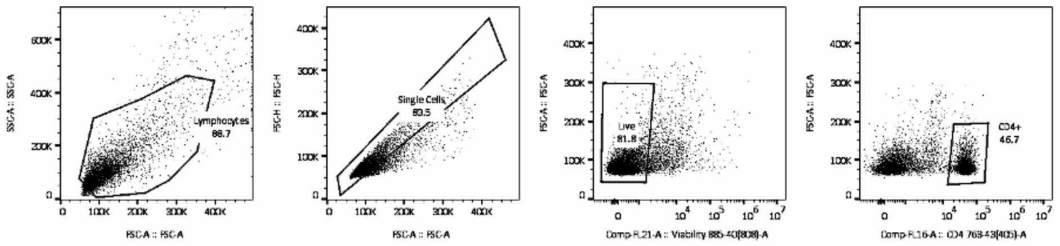


Figure S5. Gating strategy for CD4⁺ T cells in the spleens of mice with PGIA. Fixed and permeabilized cells were gated on forward side scatter, single cells were selected based on forward scatter area and height, live cells were selected based on the staining of Viakrome, and CD4⁺ T cells were selected based on the expression of CD4.

Table S1. Additional data PGIA mouse studies

Preventative study	**PBS**	**hPG$_{K4}$-Dex Lip**	**hPG$_{K4}$-Dex tolDCs**
% Mice with arthritis[a]	100	27	75
Day of onset	35 ± 7	47 ± 9	33 ± 9
AUC arthritis score	143.7 ± 114.9	1.1 ± 2.3	106.8 ± 110.4
Curative study	**PBS**	**hPG$_{K4}$-Dex Lip**	**OVA$_{K4}$-Dex Lip**
Δ Arthritis score vs day of treatment[a]	6.7 ± 3.3	1.8 ± 3.0	5.1 ± 2.0
% Mice with improved arthritis[a]	0	33	0
AUC arthritis score	90.8 ± 50.4	32.3 ± 28.0	74.5 ± 57.5

All data shown as mean or mean ± SD.
[a] at the end of the study.

Dexamethasone-Phosphate and Protein-Carrying Liposomes for the Induction of Antigen-Specific Tolerance to Cas9

Dexamethasone-Phosphate and Protein-Carrying Liposomes for the Induction of Antigen-Specific Tolerance to Cas9

Daniëlle ter Braake[1*], Johanna Walther[2*], Deja Porenta[1,2], Vasilis Papamoschou[1,2], Bonne Keijer[1,2], Cornelis Seinen[3], Naomi Benne[1], Femke Broere[1], Enrico Mastrobattista[2]

1 Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, The Netherlands
2 Department of Pharmaceutics, Utrecht Institute of Pharmaceutical Sciences (UIPS), Utrecht University, The Netherlands
3 Gemeenschappelijk Dierenlaboratorium, Faculty of Veterinary Medicine, Utrecht University, The Netherlands

* Both authors contributed equally

Abstract

CRISPR-Cas9 technology is considered one of the most promising gene editing tools currently available for application as therapy for several genetic disorders, as the technique allows for introducing specific double-stranded breaks at targeted sites within the genome. However, the bacterial origin of SpCas9 may hinder the efficiency in patients since pre-existing immunity towards the SpCas9 protein could result in the systemic clearance of the complex resulting in subsequent elimination of its gene editing features or clearance of cells modified with the CRISPR-Cas9 components. To avoid such adaptive immune responses against SpCas9 protein, it is vital to induce tolerance towards the bacterial components. To achieve this, anionic DPPC:DPPG:Cholesterol liposomes were formulated for the co-delivery of the SpCas9 protein alongside the immunomodulator prodrug dexamethasone disodium phosphate (DexPhos). Immunomodulators, such as DexPhos, induce a tolerogenic state in dendritic cells. Tolerogenic dendritic cells play a major role in the establishment of T cell tolerance by e.g. T cell anergy and stimulation of suppressive regulatory T cells. *In vitro*, we show that our DexPhos liposomes are efficiently taken up by DCs, leading to a tolerogenic phenotype, also when co-encapsulating SpCas9. *In vivo*, these DexPhos-SpCas9 liposomes migrate to the spleen and liver and can slightly decrease the formation of SpCas9-specific antibodies.

Introduction

Clustered Regularly Interspaced Palindromic Sequences (CRISPR)-Cas9 is a bacterial defense mechanism that has been reprogrammed as a tool for specific gene editing and correction. The CRISPR-Cas9 system is a complex between an endonuclease and a short synthetic guide RNA, which directs the protein to a specific location within the DNA where it introduces a double-strand break[1]. Subsequent DNA-repair mechanisms can repair the double-strand breaks by various mechanisms, amongst them homology-directed repair where disrupted genes can be specifically repaired in the presence of a homologous DNA template[2]. Therefore, ongoing efforts are investigating how to deliver the CRISPR-Cas9 components using non-viral particles such as exosomes or lipid nanoparticles for clinical application *in vivo*[3–5].

A major drawback is, however, that the CRISPR-Cas9 components originate from bacteria. The Cas9 protein ortholog, SpCas9, originates from *S. pyogenes* which is a known human commensal that can also be pathogenic. Therefore, due to the abundance of bacteria within the human population, such as *S.pyogenes*, it has been reported that humans are routinely exposed to SpCas9 and have generated SpCas9-specific antibodies. Charlesworth et al. and Simhadri et al. reported seropositivity for SpCas9 and another ortholog, SaCas9 from *S. aureus*[6,7]. While direct delivery via non-viral vectors such as lipid nanoparticles can overcome the risk of neutralizing antibodies to SpCas9, the additional finding of pre-existing effector T cells specific for SpCas9 is cause for more concern. The presence of the nuclease in correctly targeted cells would be expected to result in presentation on major histocompatibility complex (MHC) molecules, specifically MHC-I, potentially attracting the attention of cytotoxic T cells[6,8]. Therefore, the presence of preexisting immunity may counteract the efficacious use of CRISPR-Cas9 or cause systemic inflammatory reactions when treating patients with the CRISPR-Cas9 system. Hence, an approach to overcome neutralization or clearance of CRISPR-Cas9 is to exploit the principles of immune tolerance and actively accommodate the foreign gene editing components[9,10].

Dendritic cells (DCs) are antigen-presenting cells (APCs) with a variety of functions in the immune system, both adaptive and innate immune responses, and are pivotal regulators of immunity as well as tolerance[11,12]. The function of DCs is dependent on their maturation stage and subtype[13]. DCs can be converted to a tolerogenic state using dexamethasone disodium phosphate (DexPhos), a prodrug of the immunomodulator and anti-inflammatory drug dexamethasone[14]. Tolerogenic DCs (tolDCs) are characterized by a semi-mature state with reduced expression of MHCII, CD40 and CD86, decreased secretion of inflammatory cytokines such as IL-1, IL-6, and IFN-γ and instead an increased release of anti-inflammatory cytokines such as IL-10 and transforming growth factor ß (TGF-β) in comparison to their mature state[15]. Due to this change in DC state, these cells can present antigens whilst inducing regulatory T cells (Tregs) and inhibiting the proliferation of effector T cells[16,17]. Tregs can be subdivided into different subtypes, including the conventional $CD4^+CD25^+FoxP3^+$ Tregs and $CD4^+Foxp3^-CD49b^+Lag3^+$ Type 1 Regulatory cells (Tr1s).

To utilize immunomodulators for induction of specific tolerance towards biologic drugs or self-antigens, ongoing efforts focus on polymeric or lipid nanoparticles as tolerogenic nanoparticles (tolNP)[18–20]. Kim et al. report that poly(lactic-co-glycolic-acid) polymeric particles co-delivering dexamethasone and ovalbumin protein result in suppression of Ova-specific IgG and cytotoxic T cells, while Tregs were induced[21]. Liposomes entrapping rapamycin with CD22-ligand and ovalbumin conjugated to PEGylated lipid-reduced antibodies targeting ovalbumin *in vivo* after intravenous injections[22]. Furthermore, liposomes have been reported to be delivered to the liver and spleen *in vivo*, key organs in immune responses and tolerance[23].

However, to our knowledge no studies have yet investigated using tolNP as adjunctive therapy for gene therapy with CRISPR-Cas9.

In this study, we therefore hypothesize that DPPC:DPPG:cholesterol liposomes encapsulating the SpCas9 protein and DexPhos is a potent delivery system for induction of SpCas9 tolerance *in vivo*. We investigate the physical characteristics and stability of the liposomes and study the induction of tolerance both *in vitro* and *in vivo*.

Materials & Methods

Reagents

All reagents and chemicals used in this study were acquired from Sigma-Aldrich (Zwijndrecht, The Netherlands) unless mentioned differently. sgRNA (sequence: 5'-GCUGAAGCACUGCACGCCGU-3') was purchased from Sigma-Aldrich (Haverhill, UK). The lipids 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-sn-glycero-3-phospho-(1'- rac-glycerol) (sodium salt) (DPPG) were acquired from Lipoid (Steinhausen, Switzerland. 1,10-((2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl) piperazine-1-yl)ethyl)azanediyl) bis(dodecane-2-ol) (C12-200) was bought from CordonPharma (Plankstadt, Germany), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) from Lipoid, Cholesterol and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethyleneglycol-2000 (PEG-DMG) from Sigma-Aldrich and 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) from Merck (Darmstadt, Germany).

Formulation of liposomes encapsulating dexamethasone phosphate and SpCas9 protein

Liposomes were produced via the thin film lipid hydration method. DPPC, DPPG, and cholesterol were weighed and dissolved at a molar ratio of 47.4/0.6/52 and a final total molar concentration of 50 mM in 3 mL chloroform:methanol (v/v 9:1). The lipid film was obtained by evaporation of the organic solvents at full rotation speed and 60 °C for 5 minutes with a rotavapor and then dried further under mild nitrogen flow for 10 minutes. Afterwards, the lipid film was rehydrated with 500 µl of 50 mg/mL dexamethasone phosphate (DexPhos; Duchefa Biochemie, Haarlem, The Netherlands) resuspended in formulation buffer (5 mM HEPES, 15 mM NaCl, 5% w/v d-Glucose, pH 7.4) while rotating at 60 °C for 15 minutes. In the case of formulating empty liposomes or only loading with SpCas9, the lipid film was rehydrated with 500 µl formulation buffer solely. Subsequently, the liposomes were extruded with the mini hand extruder (Avanti Polar Lipids, Inc, Alabaster, AL, USA) at 60 °C through two drain disks (Whatman 10MM PE 230300) and two 200 nm membranes (Whatman Nucleopore Track-Etch Membrane PC MB 19MM 0.2µm) 15 times. To encapsulate SpCas9 protein (recombinantly produced in Clearcoli in-house) 3.6 mg/ml SpCas9 was added to the formulation (v/v 1:9) and then freeze-thawed three times[24]. Finally, the formulation was ultracentrifuged twice at 40,000 rpm and 4 °C using a Beckman Coulter Optima L-90K Ultracentrifuge with a 70.1Ti rotor to remove any free dexamethasone phosphate or SpCas9. The liposome pellet was resuspended in 500 µL of formulation buffer and stored at 4 °C.

To follow the biodistribution of tolerogenic liposomes *in vitro* and *in vivo*, empty liposomes were formulated as described above. However, before making the lipid film 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DiD) (Invitrogen Thermo Fischer Scientific) was added to the lipids dissolved in chloroform:MeOH at a mol% of 0.02 of the total lipids, resulting in a final molar concentration of 0.01 mM.

For the formulation of liposomes for *in vivo* studies, a commercially available endotoxin-free SpCas9, Alt-R SpCas9 nuclease V3 (IDT-DNA, Leuven, Belgium) was used. Additionally, a formulation encapsulating DexPhos and ovalbumin (v/v 1:9 ovalbumin:lipids) instead of SpCas9 were formulated. Each formulation was diluted 16.6 times to maintain < 1.44 mg total lipids (tolerated amount determined from previous intravenous injections in mice at animal facility in Utrecht) for safety precautions when injecting in mice.

Formulation of lipid nanoparticles complexating SpCas9-ribonucleoprotein complex (RNP)

Lipid nanoparticles (LNPs) complexating SpCas9-RNP were formulated as previously described[24]. Briefly, RNP complexation occurred in a 1:1 ratio of sgRNA to SpCas9 in nuclease-free water and incubated for 15 minutes at RT. Meanwhile, a lipid mixture of 40x more total lipid weight than sgRNA with the following lipids was prepared: C12-200, DOPE, cholesterol, PEG-DMG, and DOTAP (molar ratio: 35:16:46.5:2.5:0.25). Then, the RNP was mixed in a volume ratio of 3:1 with the lipid mixture and incubated for 15 minutes at RT. LNPs were formulated to ensure a final amount of 2 µg of SpCas9 in 200 µl LNPs.

Physical characterization of liposomes and lipid nanoparticles

For the determination of the average size and polydispersity index (PDI) of the nanoparticles dynamic light scattering on a Zetasizer nano-s (Malvern ALV CGS-3, Malvern, UK) was used. To determine the ζ-potential, the formulations were diluted 1:100 in 10 mM HEPES buffer pH 7.4 and measured using a dip cell cuvette on the zetasizer nano-z (Malvern ALV CGS-3).

Determining encapsulation of dexamethasone phosphate and SpCas9 in liposomes (and SpCas9 in lipid nanoparticles)

Encapsulation of DexPhos in DPPC:DPPG:cholesterol liposomes was determined via reversed-phase high-performance liquid chromatography (HPLC) analysis. Samples were run over an XBridge protein BEH C4 300 Å column (3.5 µm, 4.6 mm X 150 mm, serial no 0166312161884, Waters Alliance e2695, Milford, MA, USA) attached to an XBridge protein BEH C4 300 Å sentry guard cartridge (Waters Alliance, 3.5 µm, 4.6 mm X 20 mm, 2/pk) and with a linear acetonitrile gradient from 5% to 100% in 5 min and back again in 1 min. Starting conditions were then equilibrated for another 4 minutes before injection of the next sample. The mobile phase additionally contained 0.1% trifluoroacetic acid. UV-Vis detection was set to 214 and 280 nm (2pts/s). Before injection samples were treated with 1% triton X-100 and injected with a volume of 50 µl at a flow rate of 1 ml/min. A calibration curve of dexamethasone phosphate diluted to a concentration range of 0-400 µg/ml in formulation buffer was prepared to determine the concentration of encapsulated DexPhos. Encapsulation efficiency was then determined by dividing the concentration of encapsulated DexPhos in ultracentrifuged liposomes by the concentration of the total amount of DexPhos in non-ultracentrifuged liposomes via the EMPOWER software. Verification of encapsulation of SpCas9 in lipid nanoparticles complexating the SpCas9-RNP was performed as above with slight differences and described in a previous publication[24]. Briefly, SpCas9-RNP loaded LNPs were injected onto the same column and same mobile phase as above. However, detection was with fluorescence signal and the detector was set at excitation 280 nm, emission 350 nm. Prior to injection were treated with 2% triton X-100. Entrapment of SpCas9 in LNPs was calculated by dividing the concentration of encapsulated SpCas9 in dialyzed (MWCO 300kDa) LNPs by the concentration of total amount of SpCas9 in non-dialyzed LNPs.

Cryo-TEM liposome imaging

For Cryo-TEM imaging, 10 µl of nanoparticles in suspension were added to freshly glow-discharged Quantifoils and incubated for at least 10 minutes in a humidified environment and then vitrified using an FEI Mark IV Vitrobot (Fei, Hillsboro OR, USA). After vitrification samples were stored in liquid nitrogen until imaging. Samples were imaged on an FEI Tecnai G2 20 TWIN 200kV transmission electron microscope. Vitrified Quantifoils were loaded in a Gatan 70° tilt cryo-transfer system which was pre-cooled using liquid nitrogen and inserted in the microscope. Samples were imaged at a magnification of 29k and images were acquired by the bottom-mounted FEI High-Sensitive (HS) 4k x 4k Eagle CCD Camera System.

Release assay of dexamethasone phosphate at 37 °C and 4 °C

Liposomes encapsulating dexamethasone phosphate and SpCas9 protein were formulated as described above. The formulation was stored in aliquots of 50 µl in the fridge at 4 °C or in an incubator at 37 °C for different time points (0, 1h, 4h, 7h, 24h, 48h, 120h, 168h, 240h, 336h, 504h, 672h). Per timepoint, 2 aliquots were assigned at both 4 °C and 37 °C. At each given time, one of the aliquots was diluted in formulation buffer to a final volume of 12 ml and ultracentrifuged for 1 hour at 40,000 x g at 4 °C. The supernatant was removed and the liposome pellet was resuspended overnight in 50 µl of fresh formulation buffer. Resuspended samples were then stored in the fridge until all time points were collected. Subsequently, the samples were treated with a volume ratio 10% triton-X100 (v/v 10:1). In parallel, the other aliquot was not ultracentrifuged and instead immediately treated with 10% triton-X100 (v/v 10:1) as a control for the total amount of DexPhos (released and still entrapped). All samples were analyzed with reversed-phase high-performance liquid chromatography as described above. Afterwards, the peak area of the DexPhos peaks on the chromatograms was integrated into the EMPOWER software and a release ratio was calculated by dividing the peak area of samples that were centrifuged (equivalent to entrapped DexPhos) by the mean peak area of non-centrifugated samples of all timepoints (equivalent to total DexPhos).

$$\text{release ratio} = \frac{A(DexPhos)_t}{A(\text{mean}((\text{total DexPhos})(_{t1\text{-}t0})))}$$

DexPhos = entrapped DexPhos (centrifuged sample)
Total DexPhos = non-centrifuged sample
t = timepoint x
t_1 = 0 hours
t_0 = 672 hours

Mice

Wildtype Balbc/cANCrl mice (female) at 8 weeks old were purchased from Charles River laboratories for use in *in vitro* bone marrow-derived dendritic cell cultures. For the *in vivo* study, 18 Balb/cAnNCrl mice were purchased from Charles River Laboratory to be 6 weeks old at the start of the study. Mice were given one week of acclimatization and housed under standard conditions at the animal facility facility (standard chow and water ad libitum). Mice were randomized into experimental groups based on weight using RandoMice. All experiments were approved by the Animal Experiment Committee of Utrecht University (AVD10800202115687 *in vitro* (experiments) & AVD10800202115026 *in vivo* (experiments)). Humane end points considered for immediate euthanasia were: no food intake for 24 hours (result in 10% weight loss), stop of normal activity or inability to stand up or walk, and clear evidence of discomfort such as piloerection.

Induction of tolerogenic dendritic cells on bone marrow-derived dendritic cells (BMDCs)

Murine femurs and tibias were flushed with a 21G needle. Bone marrow was homogenized and seeded in 6-well plates at a cell density of 450,000 cells/mL in 2 mL IMDM (Gibco, Thermo Fisher Scientific, Landsmeer, The Netherlands), supplemented with 10% FCS (Bodinco, Alkmaar, The Netherlands), 100 units/mL of penicillin (Gibco, ThermoFisher Scientific) 100 µg/mL of streptomycin (Gibco, Thermo-Fisher Scientific and 0.5 µM β-mercaptoethanol (Gibco, ThermoFisher Scientific). Cells were cultured at 37°C and 5% CO2 in the presence of 20 ng/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF, in-house produced) for 7 days. On the second day, 2 mL of complete IMDM and 20ng/mL

GM-CSF were added to the wells. Extra GM-CSF (20ng/mL) was supplemented on day 5. Cells were matured by 10 ng/mL lipopolysaccharide (LPS, O111:B4) and simultaneously treated with 1µM free Dex (D4902; Sigma Aldrich), 1µM free DexPhos, 12.5nM SpCas9 or differentially generated liposomes on day 7. After 16 h, DCs were harvested for phenotypic characterization by flow cytometry.

Flow Cytometry of BMDCs in vitro

For all flow cytometry experiments, cells were resuspended in a 96-well round bottom plate (Corning) at a concentration of 200,000 cells/mL in 200 µl FACS buffer. The suspensions were first blocked for 15 minutes with 10µg/mL Fc Block (clone 2.4G2, in-house produced) to prevent non-specific antibody binding. Extracellular staining was performed using a cocktail of antibodies, consisting of CD11c-APC (N418, eBioscience, Thermo Fisher Scientific), MHCII-eFluor450 (M5/114.15.2, eBioscience, Thermo Fisher Scientific), CD40-PE (3/23, BD Biosciences, Franklin Lakes, NJ, USA), CD86-FITC (GL-1, BD Biosciences) and ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA) in FACS Buffer (1X PBS supplemented with 2% FCS). For all flow cytometric analyses, appropriate single-stain and fluorescence minus one controls were taken along. Flow cytometry was performed using the Beckman Coulter Cytoflex LX at the Flow Cytometry and Cell Sorting Facility located at the Faculty of Veterinary Medicine at Utrecht University. Acquired data were analyzed using FlowJo Software v.10.7 (FlowJo LLC, Ashland, OR, USA).

In vitro uptake studies of DiD-labelled liposomes

DC2.4 cells (ATCC) were cultured at 80% cell density in RPMI-1640 medium supplemented with 10% FCS, 10 µM β-mercaptoethanol, 1x HEPES, 1x Glutamax, and 1x MEM non-essential amino acids at 37 °C and 5% CO2. Twelve microliters of DiD-labelled empty liposomes were added to LPS-matured DC2.4 cells (LPS; E. coli O127:B8; 20 µg/mL LPS for 30 minutes) seeded on 24-well plate at a 50000 cells/well density for 1h, 7h, and 24h before analysis and incubated at 37 °C and 5% CO2. Cells were harvested and transferred onto a BD FALCON U-bottom 96-well plate (Becton Dickinson, Franklin Lakes, NJ, USA) for flow cytometry. Cells were washed 3 times with PBS. After washing the cells with PBS again, the cells were fixated in 1% PFA before measuring the DiD signal on the FACS CANTO II. Additionally, 10.000 cells per well were harvested and transferred to a black 96-well imaging plate for confocal microscopy. Cells were washed with PBS and then the cell pellet was resuspended in 100 µl of 2 µg/ml Hoechst 33342 and incubated for 10 minutes at 37 °C and 5% CO2. Then, the cells were imaged on the Confocal spinning disc microscope Cell Voyager 70005 (Yokogawa, Yokogawa Corporation, Tokyo, Japan), whereby Hoechst was excited by the 405 nm laser and DiD was excited with the 640 nm laser.

In vivo assessment of tolerance induction by tolNPs

Eighteen female naïve Balb/c mice were randomly assigned to the three experimental groups (6 mice per group) according to weight. On day 0, each mouse of each experimental group was treated with 200 µl of liposome formulations (group 1 – DexPhos-SpCas9 liposomes, group 2 – DexPhos-Ova liposomes, group 3 – SpCas9 liposomes) with a final amount of 46.8 µg DexPhos and 2 µg SpCas9. Seven days later, the mice were injected with the same formulations and same concentrations dependent on their assigned experimental group. On day 15, blood samples were collected for each mouse via cheek punctures into z-serum separation tubes (Greiner Bio-One, Kremsmünster, Austria). Serum was separated from red blood cells by centrifuging the blood samples at 10.000 x g for 5 minutes at 4 °C and collected into separate tubes and stored at – 20 °C. On day 17, all mice were challenged with SpCas9 via lipid nanoparticles complexating SpCas9-RNP at a final amount of 2 µg SpCas9 and a volume of 200 µl per injection. The challenge was repeated one week later on day 24. Before the second challenge, blood samples were collected and serum stored as described above.

One week later, on day 31, blood samples were collected again via cheek punctures and serum was stored, and the mice were sacrificed. Spleens were collected from each mouse for flow cytometry analysis.

Biodistribution of DiD-labelled liposomes in vivo

DiD-labelled empty liposomes were injected in three selected mice (one of each experimental group) 1h before sacrifice. After sacrificing the mouse, liver, spleens, lungs, kidneys, heart, ovaries and bones were harvested from the mice injected with DiD-labelled liposomes and three control mice (again one from each experimental group). The organs were weighed and then snap-frozen in liquid nitrogen and stored at -80 °C until imaging. The organs were imaged on the Odyssey scanner (LI-COR Biosciences, Lincoln, NE, USA) with the following settings to determine uptake of DiD-labelled empty liposomes: channel 700 nm, resolution 169 µm, intensity 1, quality high. The mean fluorescent intensity of each organ area on the image was determined with the Image Studio software. The mean fluorescent intensity was divided by the weight of the organ to determine the MFI/weight of each organ.

Determination of SpCas9-antibody levels in mouse serum

The ELISA protocol described by Charlesworth et al. was used in this study[6]. 96-well ELISA well plates were coated with 1 µg/well SpCas9 in 100 µl 1x coating solution (ELITech, Group B.V., Spankeren, The Netherlands) overnight at 4°C. The following day, the wells were washed three times with 100 µl 1X wash buffer for 5 minutes at 200 rpm at RT and then blocked with 100 µl 1% BSA blocking solution (ELITech Group B.V.) for 2 hours at RT. Meanwhile, the serum samples were prepared in serial dilutions ranging from 1:500 – 1:1.000.000 (diluted in 1% BSA blocking solution). Additionally, commercial antibodies against SpCas9 (Sanbio B.V., Uden, The Netherlands) and ovalbumin (Merck) were serial diluted 1:1.000 – 1:10.000.000 in 1% BSA blocking solution. After blocking the wells, the serum samples (100 µl) and commercial antibodies (100 µl) were added to the wells and incubated for 5 hours at 4 °C and shaking at 200 rpm. Then, the wells were washed again three times with 100 µl 1x wash buffer and shaken at 200 rpm for 5 minutes. Next, the wells were incubated with 100 µl HRP-labelled goat anti-mouse IgG1 (BD Biosciences, Vianen, The Netherlands) (1:1.000 diluted in 1% BSA blocking solution) for 1 hour at RT and then washed four times. The wells were then treated with 100 µl of ABTS ELISA HRP (Abcam, Amsterdam, The Netherlands) substrate for 15 minutes. The reaction was stopped with 1% SDS and then measured on the SpectraMax for absorbance at 405 nm.

Flow cytometry analysis of splenocytes at t=0 after in vivo study

Spleens were collected from each mouse, mashed through a 70 µm filter (Falcon, Corning, New York, USA) and erythrocytes were lysed using Ammonium–Chloride–Potassium (ACK) lysis buffer (0.15 M NH4Cl, 1 mM KHCO3, 0.1 mM Na2EDTA; pH 7.3). Cells were seeded in a round bottom 96-well plate (Falcon, Corning, New York, USA) at 1,000,000 cells/well. Before staining, cell suspensions were blocked for 15 min with Fc Block (2.4G2, in-house produced). Cells were stained at t=0 with a monoclonal antibody mix of CD4-BV785 (RM4-5, BioLegend, USA), Lag3-PE (C9B7W, eBioscience, Thermo Fisher Scientific, USA), CD49b-APC (DX5, Biolegend, USA) and CD25-PerCPCy5.5 (PC61.5, eBioscience, Thermo Fisher Scientific, USA) and ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA) in FACS Buffer. After 30 min incubation at 4°C in the dark, cells were washed with PBS, fixed, and permeabilized using the FoxP3 transcription factor staining set (eBioscience, San Diego, CA, USA). Subsequently, cells were stained intracellularly according to the manufacturer's instructions with FoxP3-eFluor450 (FJK-16s, eBioscience, San Diego, CA, USA). Finally, cells were washed and resuspended in 100 µL PBS for measurement. To ensure correct analysis, relevant single-stain and fluorescence minus one (FMO) controls were used. Samples were measured on a Beckman Coulter

Cytoflex LX at the Flow Cytometry and Cell Sorting Facility at the Faculty of Veterinary Medicine at Utrecht University. Acquired data were analyzed using FlowJo Software v.10.7 (FlowJo LLC, Ashland, OR, USA).

Restimulation of splenocytes for cytokine measurement after the in vivo study

Splenocytes were seeded in a 96-well round bottom plate (Falcon, Corning, USA) at 1.000,000 cells/well and restimulated with medium, SpCas9 (20 µg/mL), Ova protein (20 µg/mL) and PMA (50ng/mL) / Ionomycin (1 mL) for 6 hours at 37°C and 5% CO2. After 2 hours, cells were supplemented with 1 µg/mL Brefaldin A. Cells were stained with CD4-BV785 (RM4-5, BioLegend, USA), LAP-PE (TW7-16B4, eBioscience, San Diego, CA, USA), ViaKrome808 (Beckman Coulter, Indianapolis, IN, USA), IL-10-APC (JES5-16E3, BD Biosciences, Franklin Lakes, NJ, USA), FoxP3-eFluor450 (FJK-16s, eBioscience, San Diego, CA, USA) and IFNy-FITC (XMG1.2, BD Biosciences, Franklin Lakes, NJ, USA) using the protocol described above and the FoxP3 transcription factor staining set (eBioscience, San Diego, CA, USA). For all experiments, cells were washed and resuspended in 100 µL PBS for measurement. To ensure correct analysis, relevant single-stain and fluorescence minus one (FMO) controls were used. Samples were measured on a Beckman Coulter Cytoflex LX at the Flow Cytometry and Cell Sorting Facility at the Faculty of Veterinary Medicine at Utrecht University. Acquired data were analyzed using FlowJo Software v.10.7 (FlowJo LLC, Ashland, OR, USA).

Restimulation of splenocytes for immune cell analysis

Splenocytes were labeled with carboxyfluorescein succinimidyl ester (CFSE, 0.5 nM) according to the manufacturer's protocol (ThermoFisher) and seeded in a 96-well flat bottom plate (Falcon, Corning, USA) at 1.000.000 cells/well and restimulated with medium, SpCas9 (20 µg/mL), Ova protein (20 µg/mL), Ova peptide (323-339; 20 µg/mL) and ConA (10 µg/mL) for 3 days at 37°C and 5% CO2. Cells were transferred to a 96-well round bottom plate, blocked with FcBlock (2.4G2, in-house produced), and stained with CD4-BV785 (RM4-5, BioLegend, USA) and Viakrome808 (Beckman Coulter, Indianapolis, IN, USA) using the protocol described above. To ensure correct analysis, relevant single-stain and FMO controls were used. Samples were measured on a Beckman Coulter Cytoflex LX at the Flow Cytometry and Cell Sorting Facility at the Faculty of Veterinary Medicine at Utrecht University. Acquired data were analyzed using FlowJo Software v.10.7 (FlowJo LLC, Ashland, OR, USA).

Results

Physical characterizations of DexPhos-SpCas9 liposomes

Liposomes that are either empty, loaded with either DexPhos or SpCas9, or encapsulating DexPhos-SpCas9 were produced via thin film lipid hydration. All formulations were on average approximately 200 nm in size with a variation of less than 10% (*Figure 1A*). The formulation containing both SpCas9 and DexPhos had a higher polydispersity index. The determined ζ-potential ranged from -4.2 mV for SpCas9-loaded liposomes to -8.3 mV for SpCas9 & DexPhos-loaded liposomes.

The formulations remained stable (defined as only slight fluctuations in size, PDI and charge) for over one month. Interestingly, after one week a slight decrease in particle size for all formulations was found which however then remained stable over time, as shown in *Figure 1D*. Furthermore, the four different formulations were characterized to be stable in the presence of 25% human plasma up to 24 hours incubation at 37 °C (*Supplementary Figure 2*). Cryo-TEM images show clear spherical bilayer membrane structures, typical for liposomes. However, the cryo-TEM images additionally indicate that multilamellar liposomes and spherical particles were formed during the formulation (*Figure 1B*). The encapsulation efficiency of DexPhos and SpCas9 was determined to be 10% and 55% via HPLC and SDS Page analysis, respectively, shown in *Figure 1A*, and HPLC chromatograms and SDS PAGEs are depicted in *Supplementary Figure 1*. It was additionally found through a release study that the ratio of entrapped DexPhos to total DexPhos remained rather consistent over the time span of one month during storage at 4 °C or 37 °C (*Figure 1C*).

Uptake of DiD-labelled liposomes in vitro in DC2.4 cells

DC2.4 cells treated with DiD-labelled empty liposomes show uptake of liposomes after 1 hour (MFI = 4000) already and significantly higher uptake after 24 hours (MFI 20000) as confirmed by microscopy and flow cytometry (*Figure 2A,B*).

Induction of tolerogenic BMDCs after treatment with DexPhos-SpCas9 liposomes

To determine whether tolNPs induce tolerogenic dendritic cells *in vitro*, BMDCs were treated with PBS or LPS alone and in combination with free Dex, free DexPhos, free SpCas9, or differentially generated liposomes and analyzed for the expression of co-stimulatory molecules CD40 and CD86 on their cell surface. A significant lack of expression of CD40 (*Figure 2C*) and CD86 (*Figure 2D*) in comparison to mature BMDCs was observed in cells treated with free Dex or DexPhos and in cells treated with formulations encapsulating DexPhos at a final concentration of 1 µM (DexPhos-SpCas9- and DexPhos-loaded liposomes). BMDCs treated with empty or SpCas9-loaded liposomes did not induce a change in CD40 and CD86 surface marker expression. Expression levels of surface marker MHC-II did decrease in the presence of free or encapsulated DexPhos, however maturation with LPS did not upregulate MHC-II (*Supplementary Figure 4*).

A

formulation	lipid composition	encapsulation efficiency (%)	concentration of component [mg/ml]	size (nm)	PDI	ζ-potential (mV)
empty	DPPC:DPPG:cholesterol	n.a.	n.a.	205 ± 3	0.08 ± 0.01	-8 ± 0.3
SpCas9	DPPC:DPPG:cholesterol	55	0.006	211 ± 2	0.1 ± 0.01	-4 ± 2
DexPhos	DPPC:DPPG:cholesterol	10	3.9	195 ± 3	0.06 ± 0.02	-8 ± 0.2
DexPhos & SpCas9	DPPC:DPPG:cholesterol	10 (DexPhos) & 55 (SpCas9)	500 (DexPhos) & 360 (SpCas9)	217 ± 4	0.2 ± 0.01	-8 ± 0.3

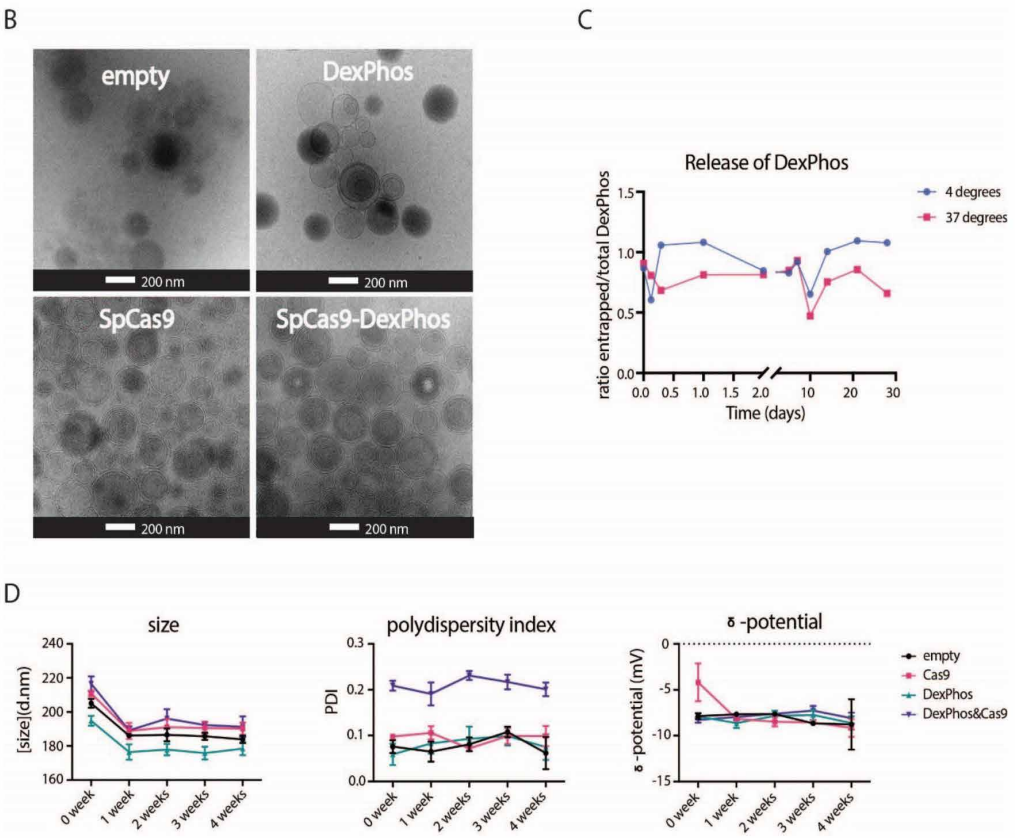


Figure 1. Physical characterizations of empty and various cargo-loaded DPPC:DPPG:cholesterol liposomes. A) An overview table that shows the encapsulation efficiencies of the different cargos, size, polydispersity index, and ζ-potential of the formulations. B) Cryo-TEM images at 29k magnification of empty, SpCas9-loaded, DexPhos-loaded, and SpCas9-DexPhos-loaded liposomes. Depicted scale bar represents 200 nm. C) Release of DexPhos at 4 °C and 37 °C for 1 month from SpCas9-DexPhos-loaded liposomes (same batch, one-time experiment) given as the ratio of DexPhos entrapped in particle vs mean total DexPhos. Mean total DexPhos is the mean peak area of total DexPhos at each time point: 4°C: 901146 ± 228854, Pearson correlation $r^2 = 0.2$; 37°C: 1247651 ± 873731, Pearson correlation $r^2 = 0.08$. D) Stability study of empty, SpCas9-loaded, DexPhos-loaded and SpCas9-DexPhos-loaded liposomes over 4 weeks. The left graph depicts the size, the middle graph depicts the polydispersity index, and the right graph depicts the ζ-potential of the formulations. Data depicts 3 replicates within each DLS and zeta-potential measurement.

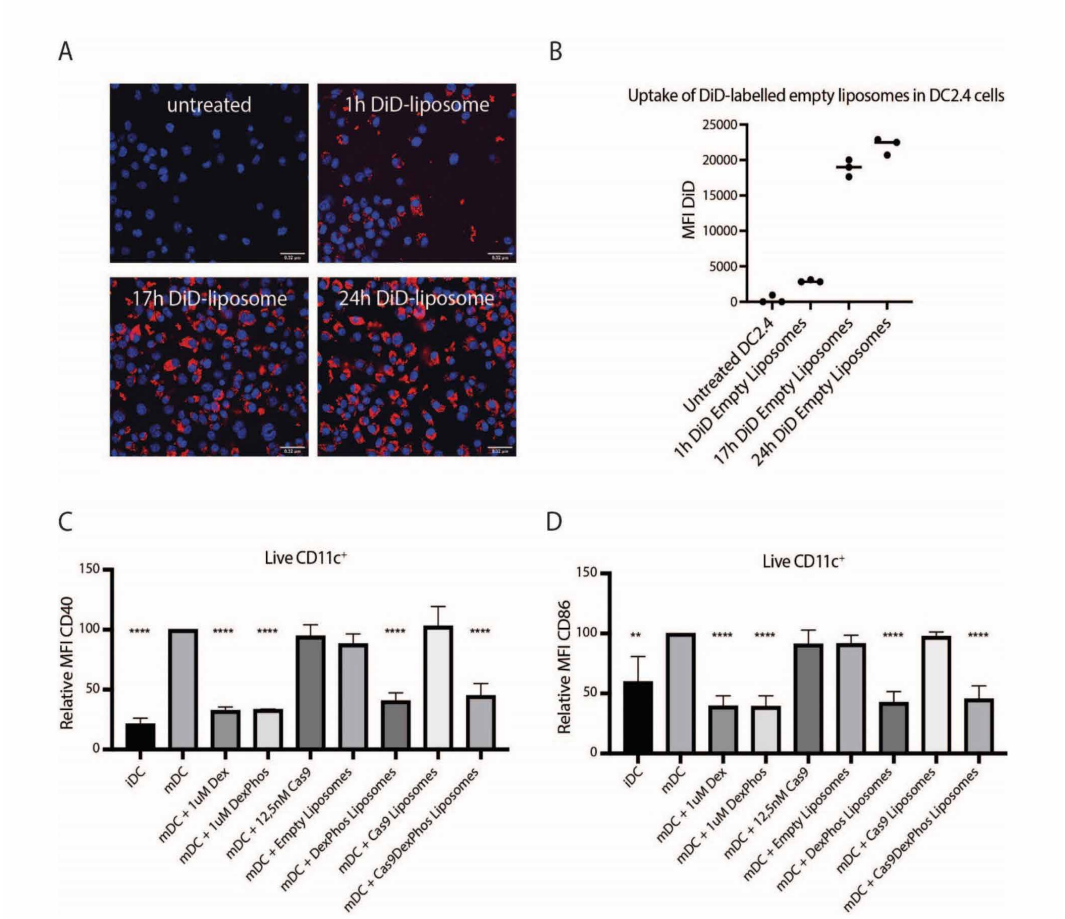


Figure 2. ***In vitro*** **characterizations of DPPC:DPPG:cholesterol liposomes.** A) Confocal microscopy of DC2.4 cells treated with DiD-labelled empty liposomes (red signal) for 1h, 17h, and 24h. Before microscopy cells were treated with 2 µg/ml Hoechst 33342 to stain the nuclei (blue signal). Scale bars indicate 0.22 µm. Experiment was performed in triplicate. B) Quantification of MFI of DiD in DC2.4 cells via flow cytometry. C, D) Expression of surface markers depicted in relative MFI compared to mDC (%) of the antibody staining CD40 and CD86, respectively (n=3). BMDCs were matured with 10 ng/ml LPS (derived from O111:B4 E.coli) and then treated with 1 µM free dexamethasone and DexPhos or with liposomes. Gating strategies are shown in *Supplementary Figure 3*.

DiD-labelled liposomes are observed in the liver and spleen of Balb/c mice

Next, to analyze the uptake of DiD-labelled liposomes in DC2.4 cells *in vitro*, the DiD-labelled empty liposomes were found back mainly in the spleen and liver of Balb/c female mice after intravenous injection, as shown by the significantly higher weight-corrected MFI in comparison to untreated mice). (*Figure 3; Supplementary Figure 6*).

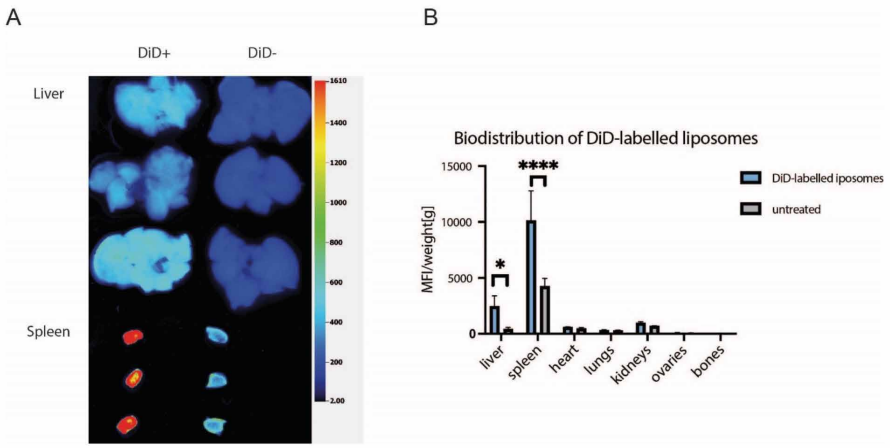


Figure 3. Biodistribution of DiD-labelled empty liposomes in Balb/c mice. A) Images of livers and segments of spleen treated with and without DiD-labelled liposomes. Images were obtained on the Odyssey scanner (channel 700 nm, resolution 169 µm, intensity level) to depict the fluorescent signal of DiD. The scale bar on the right indicates that dark blue represents the lowest signal intensities (2.00) and red represents the highest signal intensities (1610). Images of all other organs (heart, lungs, kidneys, ovaries, bones) are in *Supplementary Figure 5*. B) Mean fluorescent intensity of DiD per weight of liver, spleen, heart, lungs, kidneys, ovaries, and bones of 3 mice treated with and 3 mice treated without DiD-labelled empty liposomes 1h before sacrifice. A 2-way ANOVA via GraphPhad determined significant effects: * - p = 0.02, **** - p = < 0.0001. The scanned images of the heart, lungs, kidneys, ovaries, and bones are shown in the supplementary information.

Induction of SpCas9-specific tolerance in Balb/c mice

To study whether the described SpCas9-DexPhos liposomes would induce SpCas9-specific tolerance *in vivo*, female Balb/c mice were tolerized with SpCas9-DexPhos-, Ova-DexPhos- or SpCas9-liposomes, and then challenged with SpCas9-LNPs (*Figure 4A*). Subsequently, SpCas9-specific IgG levels were measured in serum as well as levels of $CD4^+FoxP3^+CD25^+$ Tregs was measured in the spleens of mice (*Figure 4C*). Mice tolerized with SpCas9-DexPhos liposomes show a tendency of slightly (non-significant) lower SpCas9-specific IgG titer after challenge with SpCas9-RNP loaded LNP, in comparison to mice treated with a non-specific antigen (Ova-DexPhos liposomes), as depicted in *Figure 4B* and additionally supported by the calculated area under the absorbance curve resulting in 8.5 ± 1.2 , 7.8 ± 0.7 , and 8.3 ± 0.6 for Ova-DexPhos, SpCas9-DexPhos and SpCas9 treated groups, respectively (*Supplementary Figure 6B*). Mean IgG titer in mice treated with SpCas9-liposomes did not change value before or after challenge (*Figure 4B*). Through sinusoidal fitting of the standard antibody titer curve (*Supplementary Figure 7C*), the concentration of the SpCas9-specific IgG in mice tolerized with tolNPs and after challenge with SpCas9-RNP LNPs was interpolated to be 0.03 ± 0.03 µg/ml at a 1:2000 serum dilution shown in *supplementary Figure 6D*. In comparison, though again non-significantly, the concentration of SpCas9-specific IgG for mice treated with a-specific antigen (Ova-DexPhos liposomes) and mice treated with SpCas9-liposomes resulted in 0.08 ± 0.13 µg/ml and 0.04 ± 0.04 µg/ml, respectively (*Supplementary Figure 6D*). Directly after tolerization, mice treated with SpCas9-liposomes have started developing more antibodies than mice treated with SpCas9-DexPhos tolNP or liposomes encapsulating Ovalbumin and DexPhos (*Supplementary Figure 6D*). Understandably, mice treated with the Ova-DexPhos formulation do not have any SpCas9-specific IgG present in their serum, but those levels noteworthily increase over time to the overall highest concentration of antibodies of all experimental groups (*Supplementary Figure 6D*).

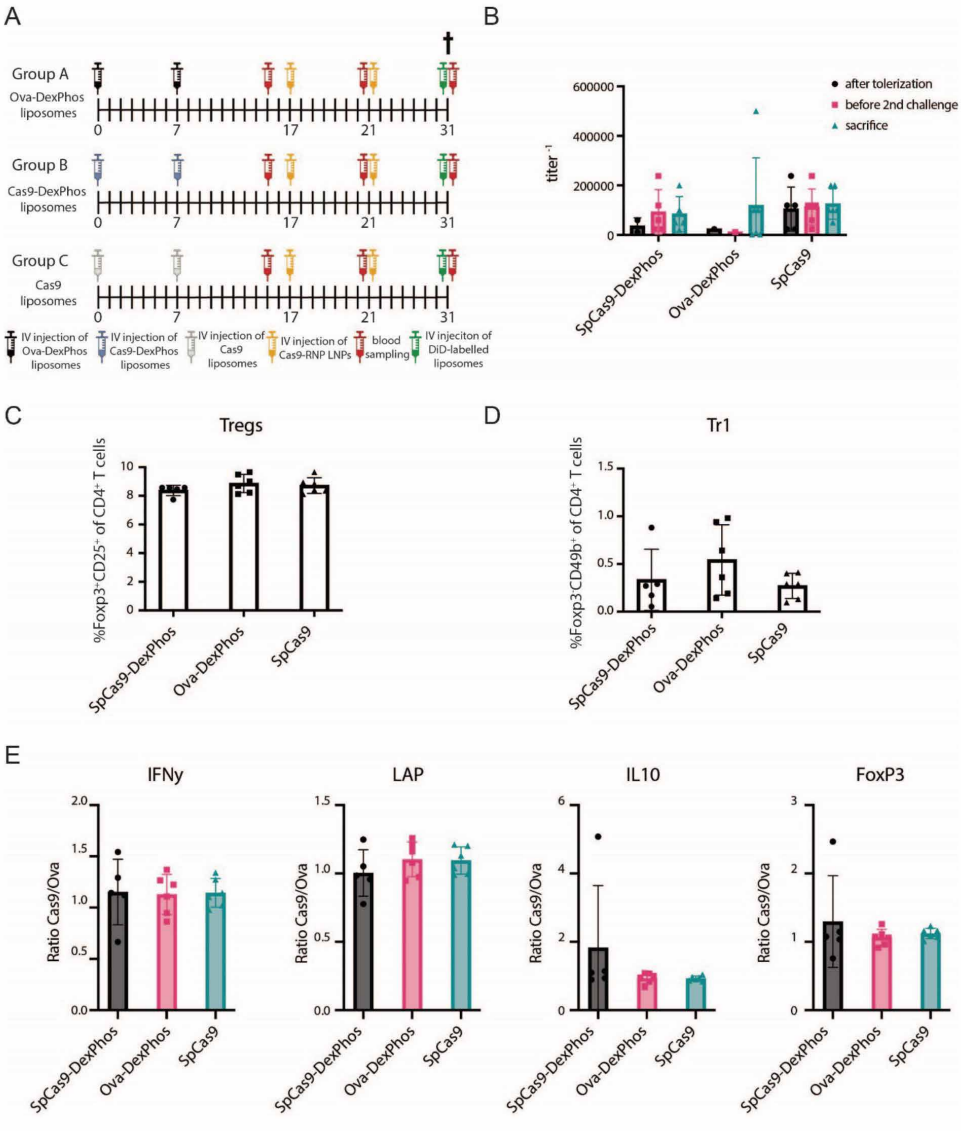


Figure 4. Induction of tolerance towards SpCas9 protein *in vivo*. A) Schematic representation of the *in vivo* study showing the three different experimental groups and the time points of tolerization and challenge and serum collection. B) SpCas9-specific IgG titers in mouse serum. Antibody titers were determined by selecting serum dilution at which absorbance of ABTS HRP substrate was at least 2-fold higher than the background. C, D) Mice were sacrificed and spleens were processed, stained, and analyzed using flow cytometry for $CD4^+FoxP3^+CD25^+$Tregs and $CD4^+CD49b^+Lag3^+$Tr1s, respectively. E) Splenocytes were restimulated with SpCas9 and Ova and incubated for 6 hours, of which 4 were in the presence of Brefeldin A. Cells were gated for live $CD4^+$ single cells.

It was confirmed that the ELISA was specific for SpCas9 by primary treatment with commercially available anti-SpCas9 antibody and anti-Ova antibody (*Supplementary Figure 7A*). Furthermore, Balb/c mice were confirmed to have no prior existing antibodies towards SpCas9 (*Supplementary Figure 7A*).

Tolerizing mice with SpCas9-DexPhos liposomes does not affect CD4⁺ regulatory T cells in the spleen

Using flow cytometry to assess the spleens of liposome-treated mice for the presence of conventional CD4⁺FoxP3⁺CD25⁺Tregs and CD4⁺CD49b⁺Lag3⁺Tr1s revealed no significant differences in non-antigen specific regulatory T cells in the spleen (*Figure 4C&D*).

Tolerizing mice with SpCas9-DexPhos liposomes does not affect cytokine production in the spleen

To assess antigen-specific T-cell responses, splenocytes were isolated and restimulated with SpCas9 and Ova. Intracellular cytokines were measured after 6 hours. The final 4h of incubation were in the presence of the Golgi-stop Brefeldin A. No significant differences were found in cytokine production between the different restimulations (*Figure 4E*).

Restimulation of splenocytes from tolerized mice with SpCas9 for three days does not induce a CD4⁺ T cell response

Next, we hypothesized that the restimulation time might be too short or that the whole protein restimulations might be affecting the efficiency of our restimulation experiments. Here, splenocytes obtained from untreated, SpCas9-liposome or Ova-DexPhos-liposome treated mice were restimulated for 3 days with medium, SpCas9 (protein), Ova (protein & peptide) or ConA. Restimulation with ConA resulted in an average of 40 % T cell proliferation, characterized by a lack of CFSE signal, in all splenocyte groups (*Figure 5*), however, splenocytes restimulated with SpCas9 protein, Ova protein, or Ova peptide did not show changes in proliferation, suggesting a lack of CD4⁺ T cell responses to both SpCas9 and Ova antigens.

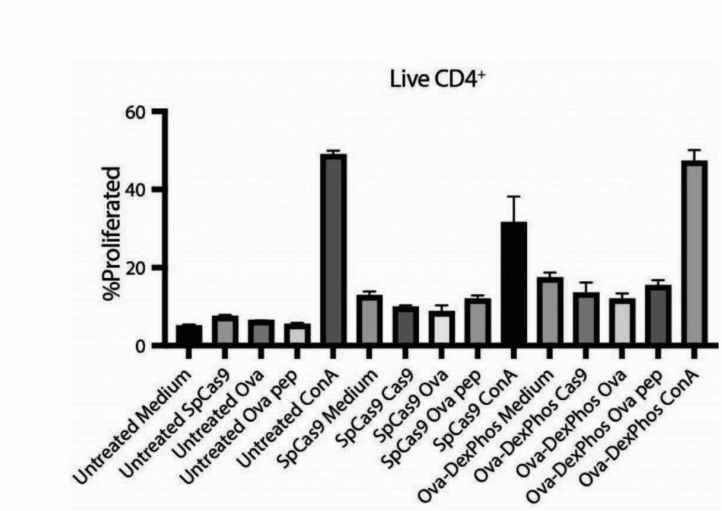


Figure 5. Three-day restimulation of splenocytes with SpCas9 does not change CD4⁺ T cell proliferation. Untreated, SpCas9-liposome-treated or Ova-DexPhos-liposome-treated splenocytes were each restimulated with medium, SpCas9, Ova (protein or peptide (indicated with pep) or ConA and incubated for 3 days as shown in the overview plot. Restimulation with proteins SpCas9 and Ova did not affect CD4⁺ T cell proliferation, assessed by a lack of CFSE signal, in untreated, SpCas9-liposome-treated or Ova-DexPhos-liposome-treated mice.

Discussion

Inducing immune tolerance at the site of SpCas9 release is essential to avoid elimination of CRISPR-Cas9-edited cells *in vivo*. Currently, very little is known about SpCas9-encapsulating nanoparticles for the tolerization towards components of CRISPR-Cas9 gene therapy. Here we show that tolerogenic nanoparticles encapsulating both DexPhos and SpCas9 efficiently reduce a pro-inflammatory phenotype *in vitro* (*Figure 2*) and are taken up in the spleen and liver *in vivo* (*Figure 3*), organs generally associated with tolerance induction.

The liposomal formulations described in this study are stable in size, monodispersity, and charge (*Figure 1D*) and were even found to remain stable in the presence of human plasma (*Supplementary Figure 2*). Using these liposomes in an *in vitro* culture of matured BMDCs, we observed reduction in the expression of costimulatory surface markers CD40 (*Figure 2A*) and CD86 (*Figure 2B*). Liposomes solely encapsulating SpCas9 did not reduce the expression levels of CD86 and CD40 on matured BMDCs, highlighting the maturation-inhibiting effect of DexPhos encapsulation (*Figure 2*). Fluorescently labeling these liposomes with DiD shows that they are efficiently taken up by DC2.4 cells (*Figure 2C & D*), indicating they might be good delivery vehicles for SpCas9 and DexPhos. Previous reports show that particles larger or equal to 200 nm (*Figure 1*) are mainly taken up via phagocytosis by APCs, commonly mediated through targeting of scavenger receptors which are predominantly expressed by phagocytes[18,25,26].

The observed *in vitro* efficacy of the liposomes lead us to study the liposomes *in vivo* for SpCas9 tolerization. However, our hypothesis was not met and SpCas9-specific tolerance could not be measured *in vivo*. Mice tolerized with DexPhos-SpCas9-loaded nanoparticles and then challenged with SpCas9-RNP LNPs only had slightly lower, but non-significant levels in SpCas9-specific IgG than the DexPhos-Ova- or SpCas9-liposome controls (*Figure 4*). The high immunogenicity of the SpCas9 protein that we observed has been reported before, as 2 injections of 2 µg of SpCas9 resulted in a high amount of SpCas9-specific IgG (*Supplementary Figure 6D*)[27]. Furthermore, the described liposomes did not induce differences in conventional $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs and $CD4^+$ $CD49b^+$ $Lag3^+$ Tr1s in the spleen, nor differences in levels of inflammatory and non-inflammatory cytokines between treatment groups (*Figure 4 C, 4D & 4E*). Based on *in vitro* studies on DCs showing transformation to tolDCs after treatment with tolNPs (*Figure 2*), we expected tolDCs to obtain a tolerogenic phenotype, characterized by reduced expression of CD40 and CD86 on their cell surface, and to secrete anti-inflammatory cytokines, enabling them to stimulate $CD4^+$ Tregs. Further analysis of antigen-specific immune responses by restimulating splenocytes with SpCas9 for 3 days revealed again no differences in immune responses to SpCas9 between the groups (*Figure 5*). A reason for this could be that the APCs in the spleen have only seen limited amounts of SpCas9, for example, due to a lack of liposome uptake or protein presentation, or that T cells in the spleen do not have sufficient T cell receptors (TCRs) to bring about a measurable response to SpCas9. Despite the uptake of liposomes in DC2.4 cells *in vitro, in vivo* uptake by APCs might have been less efficient. Incorporating targeting ligands, such as DC-Sign, that allow liposomes to be specifically taken up by dendritic cells might be able to ensure DC uptake[28]. Further studies on the release profile of DexPhos should be conducted. Though release was barely detected in liposomes over time *in vitro* (*Figure 1C*), no conclusions can be drawn regarding the premature release of DexPhos *in vivo*. It needs to be noted that one mouse died during i.v. injection of DexPhos-Cas9 liposomes. However, no specific conclusions could be drawn that that was related to formulation and most likely response to intravenous injections or shock.

Another strategy would be to explore transient immune suppression using glucocorticoids or rapamycin[29,30], where patients are treated with the immunomodulator on the same day as SpCas9-LNP administration. The rationale behind using the co-encapsulation of DexPhos and SpCas9 in liposomes laid out in this manuscript is based on a multitude of factors. Firstly, the described approach would avoid dexamethasone-mediated systemic immune suppression, assuming dexamethasone does not leak from liposomes[31]. Secondly, incorporating dexamethasone in liposomes has been shown to increase its half-life[32]. SpCas9 has been determined to have a half-life of 24 hours in cells[33]. The increased half-life of liposomal encapsulated dexamethasone would ensure immune suppression in liposome-targeted organs and cells during exposure to SpCas9. Inducing long-lasting antigen-specific tolerance towards SpCas9 would allow for the potential need of multiple injections of SpCas9-LNPs to sustain gene editing efficiency. The use of LNPs removes the added risk of immune activation observed when viral vectors, such as AAV vectors, are used[34] rendering them safe for repeated administration for cumulative gene editing. Pre-tolerization, mediated through liposomes co-encapsulating DexPhos and SpCas9, in patients selected for CRISPR-Cas9 gene therapy reduces the need for repeated dexamethasone treatments and thereby circumvents the unwanted side-effects observed with systemic dexamethasone treatment[35].

Conclusively, we show that liposomes encapsulating DexPhos with or without co-delivery of the antigen SpCas9 can reduce the upregulation of the costimulatory molecules CD40 and CD86 on DCs and thereby convert them into tolDCs *in vitro*. *In vivo*, these liposomes are localized in the liver and spleen and if even at all only minorly decrease the amount of anti-SpCas9 antibodies formulated after challenging mice with SpCas9-RNP loaded lipid nanoparticles, whilst not affecting $CD4^+$ T cell responses. Despite the data indicating the need for further optimizations of the liposomes, this study nonetheless provides leads on a strategy to induce SpCas9-specific tolerance for better applicability of the gene editing tool for gene therapy, a currently unexplored frontier.

Acknowledgements

Thank you Antoinette van Dikkenberg Teixeira for performing the AF4 studies. We would like to thank Dr. Irati Beltran Hernandez for her help with the Odyssey scanner to follow the biodistribution of DiD-labelled liposomes *in vivo*. This research was funded by the Netherlands Organization for Scientific Research (NWO) Talent Program VICI, grant number 865.17.005. Furthermore, we'd like to thank the Flow Cytometry and Cell Sorting Facility of the Faculty of Veterinary Medicine at Utrecht University for support.

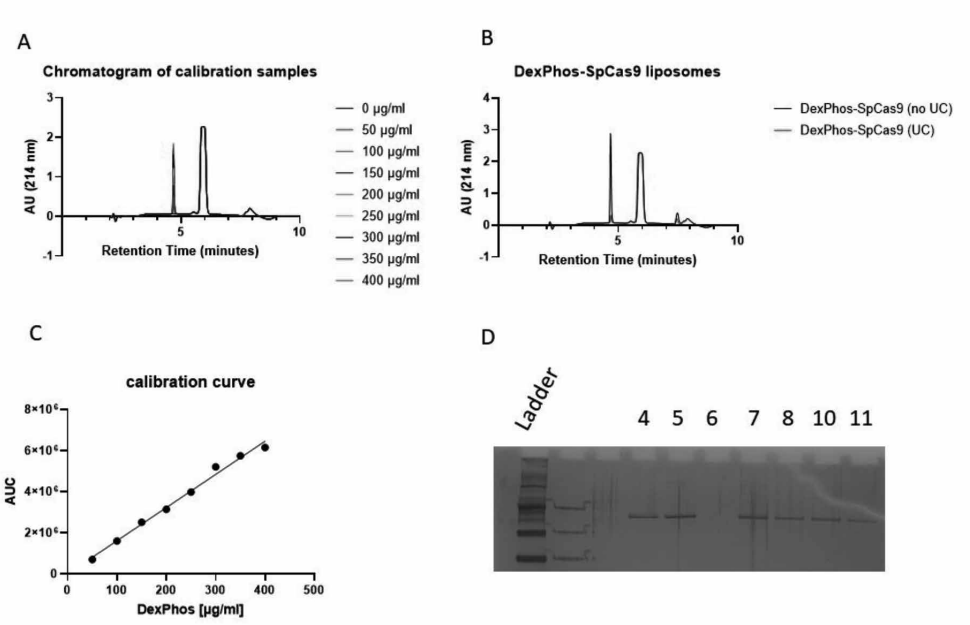
Author Contributions

Contribution	Author
Conceptualization	**Daniëlle ter Braake**, Johanna Walther, Naomi Benne, Femke Broere, Enrico Mastrobattista
Formal Analysis	**Daniëlle ter Braake**, Johanna Walther
Funding Acquisition	Femke Broere, Enrico Mastrobattista
Investigation	**Daniëlle ter Braake**, Johanna Walther, Deja Porenta, Vasilis Papamoschou, Bonne Keijer, Cornelis Seinen, Naomi Benne
Methodology	**Daniëlle ter Braake**, Johanna Walther, Deja Porenta, Naomi Benne
Project Administration	**Daniëlle ter Braake**, Johanna Walther
Resources	Femke Broere, Enrico Mastrobattista
Supervision	Naomi Benne, Femke Broere, Enrico Mastrobattista
Validation	**Daniëlle ter Braake**, Johanna Walther, Deja Porenta, Vasilis Papamoschou, Bonne Keijer, Naomi Benne
Visualization	**Daniëlle ter Braake**, Johanna Walther
Writing – Original Draft Preparation	**Daniëlle ter Braake**, Johanna Walther
Writing – Review & Editing	**Daniëlle ter Braake**, Johanna Walther, Deja Porenta, Naomi Benne, Femke Broere, Enrico Mastrobattista

References

1. Zhang, F., Wen, Y. & Guo, X. CRISPR/Cas9 for genome editing: Progress, implications and challenges. *Hum. Mol. Genet.* 23, 40–46 (2014).
2. Xue, C. & Greene, E. C. DNA Repair Pathway Choices in CRISPR-Cas9-Mediated Genome Editing. *Trends Genet.* 37, 639–656 (2021).
3. Gee, P. et al. Extracellular nanovesicles for packaging of CRISPR-Cas9 protein and sgRNA to induce therapeutic exon skipping. *Nat. Commun.* 11, 1334 (2020).
4. Wei, T., Cheng, Q., Min, Y. L., Olson, E. N. & Siegwart, D. J. Systemic nanoparticle delivery of CRISPR-Cas9 ribonucleoproteins for effective tissue specific genome editing. *Nat. Commun.* 11, 1–12 (2020).
5. Duan, L. et al. Nanoparticle Delivery of CRISPR/Cas9 for Genome Editing. *Front. Genet.* 12, (2021).
6. Charlesworth, C. T. et al. Identification of preexisting adaptive immunity to Cas9 proteins in humans. *Nat. Med.* 25, 249–254 (2019).
7. Simhadri, V. L. *et al*. Prevalence of Pre-existing Antibodies to CRISPR-Associated Nuclease Cas9 in the USA Population. *Mol. Ther. - Methods Clin. Dev.* 10, 105–112 (2018).
8. Wagner, D. L. et al. High prevalence of Streptococcus pyogenes Cas9-reactive T cells within the adult human population. *Nat. Med.* 25, 242–248 (2019).
9. Crudele, J. M. & Chamberlain, J. S. Cas9 immunity creates challenges for CRISPR gene editing therapies. *Nat. Commun.* 9, 9–11 (2018).
10. Wignakumar, T. & Fairchild, P. J. Evasion of Pre-Existing Immunity to Cas9: a Prerequisite for Successful Genome Editing In Vivo? *Curr. Transplant. Reports* 6, 127–133 (2019).
11. Audiger, C., Rahman, M. J., Yun, T. J., Tarbell, K. V. & Lesage, S. The Importance of Dendritic Cells in Maintaining Immune Tolerance. *J. Immunol.* 198, 2223–2231 (2017).
12. Banchereau, J. et al. Immunobiology of Dendritic Cells. *Annu. Rev. Immunol.* 18, 767–811 (2000).
13. Castenmiller, C., Keumatio-Doungtsop, B.-C., van Ree, R., de Jong, E. C. & van Kooyk, Y. Tolerogenic Immunotherapy: Targeting DC Surface Receptors to Induce Antigen-Specific Tolerance. *Front. Immunol.* 12, (2021).
14. Cauwels, A. & Tavernier, J. Tolerizing Strategies for the Treatment of Autoimmune Diseases: From ex vivo to in vivo Strategies. *Front. Immunol.* 11, (2020).
15. Iberg, C. A. & Hawiger, D. Natural and Induced Tolerogenic Dendritic Cells. *J. Immunol.* 204, 733–744 (2020).
16. Sakaguchi, S., Yamaguchi, T., Nomura, T. & Ono, M. Regulatory T Cells and Immune Tolerance. *Cell* 133, 775–787 (2008).
17. Keijzer, C., Van der Zee, R., Van Eden, W. & Broere, F. Treg inducing adjuvants for therapeutic vaccination against chronic inflammatory diseases. *Front. Immunol.* 4, 1–10 (2013).
18. Dangkoub, F., Sankian, M., Tafaghodi, M., Jaafari, M. R. & Badiee, A. The impact of nanocarriers in the induction of antigen-specific immunotolerance in autoimmune diseases. *J. Control. Release* 339, 274–283 (2021).
19. Li, H., Yang, Y. G. & Sun, T. Nanoparticle-Based Drug Delivery Systems for Induction of Tolerance and Treatment of Autoimmune Diseases. *Front. Bioeng. Biotechnol.* 10, 1–14 (2022).
20. Kishimoto, T. K. et al. Improving the efficacy and safety of biologic drugs with tolerogenic nanoparticles. *Nat. Nanotechnol.* 11, 890–899 (2016).
21. Kim, S. H. et al. Induction of antigen-specific immune tolerance using biodegradable nanoparticles containing antigen and dexamethasone. *Int. J. Nanomedicine* 14, 5229–5242 (2019).
22. Pang, L., Macauley, M. S., Arlian, B. M., Nycholat, C. M. & Paulson, J. C. Encapsulating an Immunosuppressant Enhances Tolerance Induction by Siglec-Engaging Tolerogenic Liposomes. *ChemBioChem* 18, 1226–1233 (2017).

23. Bozzuto, G. & Molinari, A. Liposomes as nanomedical devices. *Int. J. Nanomedicine* 10, 975–999 (2015).
24. Walther, J. et al. Impact of Formulation Conditions on Lipid Nanoparticle Characteristics and Functional Delivery of CRISPR RNP for Gene Knock-Out and Correction. *Pharmaceutics* 14, (2022).
25. Kishimoto, T. K. & Maldonado, R. A. Nanoparticles for the induction of antigen-specific immunological tolerance. *Front. Immunol.* 9, (2018).
26. Benne, N., ter Braake, D., Stoppelenburg, A. J. & Broere, F. Nanoparticles for Inducing Antigen-Specific T Cell Tolerance in Autoimmune Diseases. *Front. Immunol.* 13, 1–15 (2022).
27. Toral, M. A. et al. Investigation of Cas9 antibodies in the human eye. *Nat. Commun.* 13, 1–9 (2022).
28. Du, J., Zhang, Y. S., Hobson, D. & Hydbring, P. Nanoparticles for immune system targeting. *Drug Discov. Today* 22, 1295–1301 (2017).
29. Seregin, S. S. et al. Transient pretreatment with glucocorticoid ablates innate toxicity of systemically delivered adenoviral vectors without reducing efficacy. *Mol. Ther.* 17, 685–696 (2009).
30. Xiang, Z. et al. The Effect of Rapamycin and Ibrutinib on Antibody Responses to Adeno-Associated Virus Vector-Mediated Gene Transfer. *Hum. Gene Ther.* 33, 614–624 (2022).
31. Giles, A. J. et al. Dexamethasone-induced immunosuppression: Mechanisms and implications for immunotherapy. *J. Immunother. Cancer* 6, 1–13 (2018).
32. Metselaar, J. et al. A phase I first-in-man study to investigate the pharmacokinetics and safety of liposomal dexamethasone in patients with progressive multiple myeloma. *Drug Deliv. Transl. Res.* 13, 915–923 (2023).
33. Tu, Z. et al. Promoting Cas9 degradation reduces mosaic mutations in non-human primate embryos. *Sci. Rep.* 7, 1–11 (2017).
34. Wilbie, D., Walther, J. & Mastrobattista, E. Delivery Aspects of CRISPR/Cas for in Vivo Genome Editing. *Acc. Chem. Res.* 52, 1555–1564 (2019).
35. Madamsetty, V. S. et al. Dexamethasone: Insights into Pharmacological Aspects, Therapeutic Mechanisms, and Delivery Systems. *ACS Biomater. Sci. Eng.* (2022). doi:10.1021/acsbiomaterials.2c00026
36. Gagnon, R. C. & Peterson, J. J. Estimation of confidence intervals for area under the curve from destructively obtained pharmacokinetic data. *J. Pharmacokinet. Biopharm.* 26, 87–102 (1998).

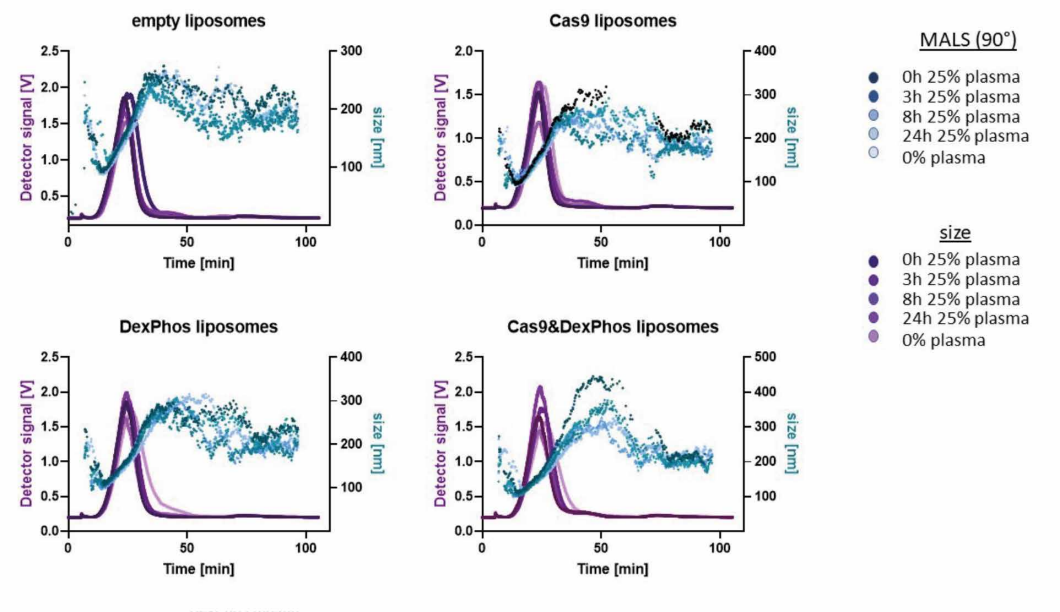


Supplementary Figure 1. Determination of encapsulation efficiency of DexPhos and SpCas9 in DPPC:DPPG:cholesterol liposomes. A) Chromatogram (UV detector 214 nm) of the calibration samples (DexPhos resuspended at different concentrations in formulation buffer) run on Xbridge protein BEH C4 300Å column. B) Chromatogram of DexPhos-SpCas9 liposomes were free DexPhos and SpCas9 were removed by ultracentrifugation compared to the chromatogram of liposomes not centrifuged (=total amount of DexPhos). C) Calibration curve determined with EMPOWER software (linear fit equation). D) SDS-PAGE of SpCas9 and DexPhos-SpCas9 samples to determine encapsulation efficiency of SpCas9 via gel densitometry. Lane 4 – SpCas9, lane 5 – SpCas9 treated with 1% triton-X100, lane 6 – empty liposomes, lane 7 - DexPhos-SpCas9 liposomes (total amount), lane 8 – DexPhos-SpCas9 liposome ultracentrifuged to show encapsulated SpCas9, lane 9&10 – sterile-filtered DexPhos-SpCas9 liposomes non-centrifuged and ultracentrifuged, respectively. Encapsulation efficiency via gel densitometry was estimated to be 55%.

Stability of liposomes in the presence of 25% human plasma via asymmetric flow field flow fractionation

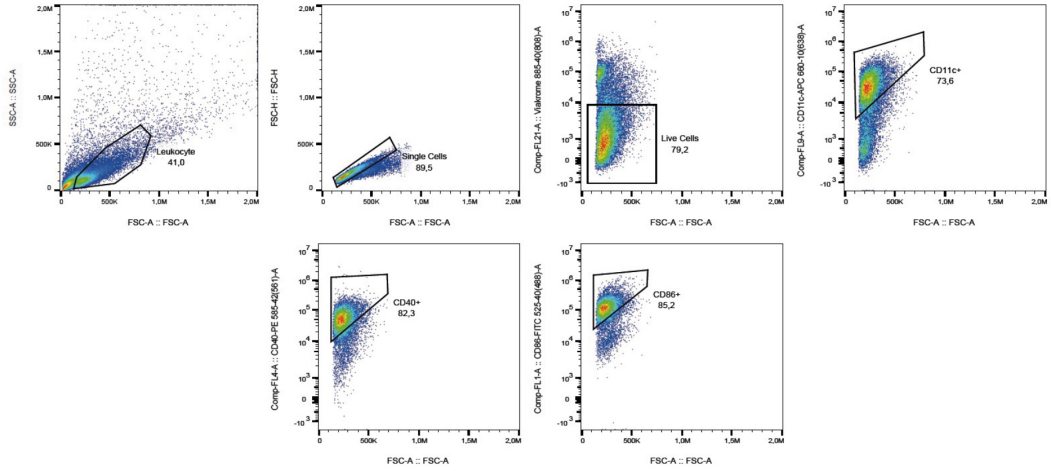
The stability of liposomes in the presence of human plasma was measured by asymmetric flow field flow fractionation (AF4) using the AF2000 separation system (Postnova Analytics, Landsberg, Germany). The system is equipped with a degasser, isocratic pumps, auto samples, an in-line MALS detector (), and an in-line DLS detector (Zeta Nano ZS, Malvern Instruments, Malvern, UK). A FFF channel with 350 µm spacer and regenerated cellulose membrane with molecular weight cut-off of 10 kDa was used for particle separation. The mobile phase was PBS.

Liposomes were incubated with 25% human plasma at 37 °C and 300 rpm for 0, 3, 8, and 24 hours. Then, particles were diluted 20x in a HEPES buffer with the same concentration as the formulation (5 mM HEPES, 15 mM NaCl) and injected at a flow rate of 0.2 ml/min, and focused for 4 minutes with a cross-flow of 2 ml/min and a focused flow of 2.30 ml/min. Then, over 60 minutes the cross flow was decreased with an exponential decay of 0.03 and then kept consistent at 0 ml/min for another 40 minutes. There, the detector flow rate was set to 0.5 ml/min throughout the entire run.

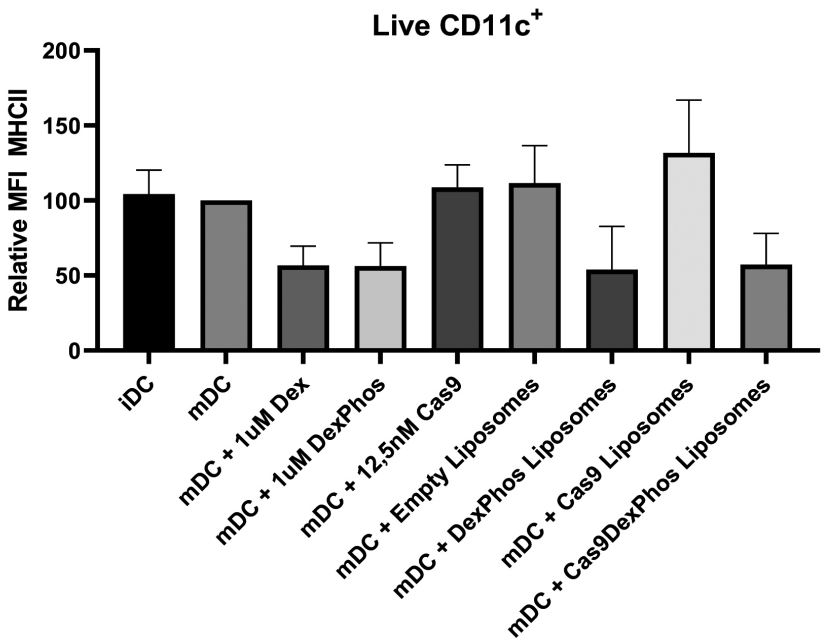


Supplementary Figure 2. Stability of liposomes in the presence of 25% human plasma via AF4. Liposomes were incubated at 37 °C and 300 rpm for 0h, 3h, 8h, and 24h and then characterized via AF4 studies on size stability and light scattering at an angle of 90°. AF4 fractograms recorded by the MALS detector and DLS detector show the light scattering signal (purple) and particle size (blue) for empty (top left), SpCas9-loaded (top right), DexPhos-loaded (bottom left), and SpCas9-DexPhos-loaded (bottom right) liposomes.

Gating strategy for bone marrow-derived dendritic cells



Supplementary Figure 3. Gating strategy BMDCs. Balb/c BMDCs were cultured in the presence of GM-CSF for 7 days. LPS and free Dex, free DexPhos, free SpCas9, or liposomes were added and cells were incubated for another 16 hours. Cells were gated for leukocytes, single cells, and live cells. Subsequently, in the $CD11c^+$ population, cells were analyzed for expression of CD40 and CD86.



Supplementary Figure 4. Expression of surface markers depicted in MFI of the antibody staining MHC-II. BMDCs were matured with 10 ng/ml LPS (derived from O111:B4 E.coli) and then treated with 1 µM free dexamethasone and DexPhos or with liposomes.

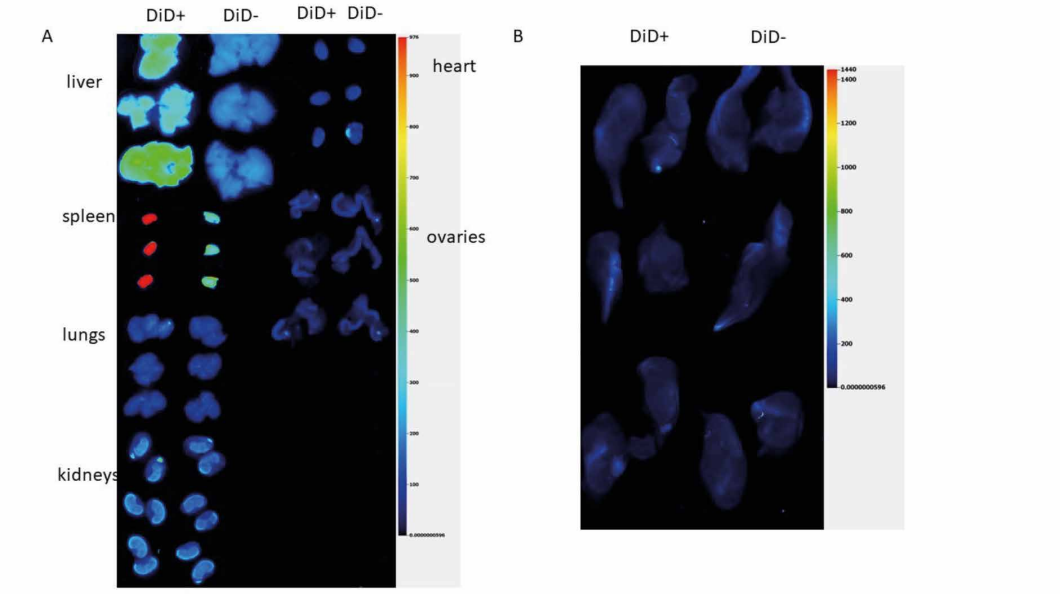
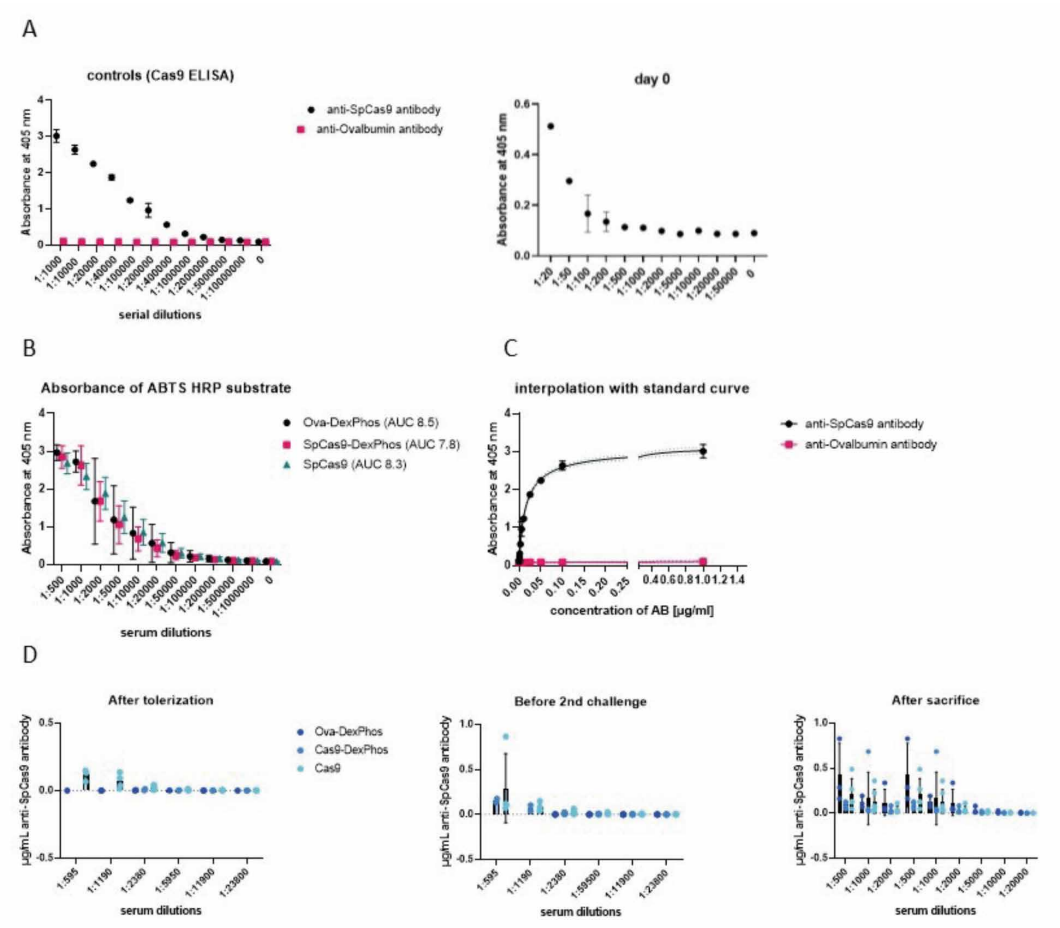


Figure 5. Images of all organs studied in the biodistribution study of DiD-labelled liposomes *in vivo*. A) Images of liver, spleen, lungs, kidneys, heart, and ovaries harvested from DiD-labelled liposome treated mice (DiD+) and control mice (DiD-). B) Bones of hindlegs of mice treated with DiD-labelled liposomes (DiD+) and control mice (DiD-).



Supplementary Figure 6. Additional graphs from the ELISA study to determine antibody levels in mouse serum.
A) Absorbance levels of HRP ABTS substrate after primary treatment of well coated with SpCas9 with commercial anti-SpCas9 antibody and anti-Ova antibody as control of SpCas9 (left) and absorbance levels of HRP ABTS substrate. Collective serum sample of all mice before the start of *in vivo* study (right). B) Absorbance values of HRP substrate after treatment of SpCas9-coated ELISA plate with serial serum dilution of all mice serum (n=6, except for experimental group SpCas9-DexPhos n=5) for each experimental group. The area under the curve (AUC) was calculated based on the mean and SD at each dilution for each experimental group via GraphPad[36]. The resulting confidence intervals are: Ova-DexPhos – 8.5 ± 1.2; SpCas9-DexPhos – 7.8 ± 0.7; SpCas9 – 8.3 ± 0.6. C) Calibration curve of absorbance at 405 nm of HRP ABTS substrate in correlation to the antibody concentration of the commercial anti-SpCas9 antibody.
D) Concentration of SpCas9-specific IgG for each experimental group after tolerization (left), before 2nd challenge (middle), and after the *in vivo* study (right). Not all absorbances could be interpolated into concentration of antibodies as they laid outside of the calibration curve.

General Discussion

General Discussion

In recent years, the landscape of treatment strategies for autoimmune diseases has witnessed significant advancements. In **Chapter 1**, the important mechanisms and cells underlying the autoimmune response are extensively introduced. This information provides context for the cutting-edge developments in immunotherapy involving tolerogenic dendritic cells (tolDCs) and theutilization of nanoparticles, such as liposomes, for the induction of antigen-specific immune tolerance as described in future chapters. In **Chapter 2**, different strategies for nanoparticle-mediated antigen-specific immune tolerance for the treatment of autoimmune diseases are summarized. As demonstrated in **Chapter 3**, liposomes encapsulating an immunomodulator and a disease-relevant antigen are just as effective at inducing antigen-specific immune tolerance as similarly pulsed tolDCs. Furthermore, this co-encapsulation of an antigen and immunomodulator in liposomes minimizes off-target and systemic side effects. This prompted further research into the co-encapsulation of antigen and immunomodulator. In **Chapter 4** and **Chapter 5**, RA was substituted with dexamethasone (dex) as the immunomodulator of choice. Dex is a potent inducer of tolDCs *in vitro*[1] and *in vivo*[2] that has previously been investigated in the context of rheumatoid arthritis[3,4], making the synthetic glucocorticoid a promising candidate for encapsulation into nanoparticles. A previous study using polymeric PLGA nanoparticles that co-encapsulated dex and ovalbumin (Ova) reported efficient tolDC induction *in vitro* and this nanoparticle treatment efficiently resulted in the generation of Ova-specific FoxP3$^+$ regulatory T cells (Tregs) whilst Ova-specific cytotoxic T cells were suppressed[5].

The systemic use of dexamethasone can result in severe side effects depending on the dose and dosing scheme[6], and the presence of cholesterol in liposomes combined with the hydrophobic nature of dex[7] can make retention in liposomes complicated[8]. To overcome these challenges, in **Chapter 4** dex was linked to the antigens using a cationic lysine tetramer, resulting in the formation of easily encapsulated antigen-dex conjugates (hPG$_{K4}$-Dex). The cationic charge of the tetramer[9] facilitates the encapsulation of the complex. The addition of the lysine tetramer significantly increased the encapsulation of the antigen. For large proteins such as SpCas9, as used in **Chapter 5**, this type of linker is more difficult to control, as the protein provides more potential binding sites. To improve encapsulation of this larger protein, dexamethasone sodium phosphate (DexPhos) was used instead of Dex. DexPhos is derived from dex, but the added sodium phosphate group increases the water-solubility[10] of the compound, facilitating the encapsulation of DexPhos in the aqueous core of lipid nanoparticles. As described in **Chapter 3**, cellular uptake of RA-containing liposomes was relatively low, reducing the antigen-specificity of DCs that take up these particles. Therefore, the increased phagocytotic abilities of hPG$_{K4}$-Dex liposome-treated BMDCs is a convenient, alternate way to approach this issue.

One of the main aims of our research is to induce antigen-specific Tregs. As described in **Chapter 1**, there are different types of regulatory T cells, including CD25$^+$FoxP3$^+$ Tregs and CD49b$^+$Lag3$^+$ Type 1 regulatory T (Tr1) cells. To make sure we fully assessed the tolerance-inducing potential of the hPG$_{K4}$-Dex liposomes and hPG$_{K4}$-Dex liposomes-pulsed tolDCs, both regulatory T cell types were assessed in all experiments in **Chapter 4**. In an *in vivo* adoptive transfer experiment we found that hPG$_{K4}$-Dex containing liposomes were able to induce antigen-specific Tr1 cells, but not FoxP3$^+$ Tregs, highlighting the importance of investigating different regulatory cell subsets. The Tr1 subset was only investigated in **Chapter 4**, not in **Chapter 3**, however, another study that looked into the effect of RA as an adjuvant for the model antigen Keyhole limpet hemocyanin (KLH) in mice and found that immunization of mice with KLH and RA promoted the induction of CD49b$^+$Lag3$^+$ Tr1 cells[11]. Interestingly, the frequency of FoxP3$^+$ Tregs was not increased, suggesting that *in vivo* RA-mediated tolerance induction is mainly Tr1-driven.

The use of (auto)antigen and immunomodulator-loaded liposomes as described in **Chapters 3** and **4** proved to be a promising strategy for the prevention and inhibition of arthritis development. This led to the consideration of new fields for the application of this technique. Broadening the scope for applications of this new tool resulted in interest from the field of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) gene editing. This revolutionary technology allows for precise gene manipulation and therefore holds great promise for application in fundamental research and herapeutic interventions. **Chapter 5** focuses on the efficient co-delivery of SpCas9 and DexPhos using liposomes *in vivo*. As mentioned before, liposomes can be formulated with a plethora of different lipids, and in **Chapter 5** it was decided to use DPPC:DPPG:CHOL liposomes. Compared to the previously used DSPC:DSPG:CHOL liposomes as described in **Chapters 3** and **4**, preliminary experiments showed that DPPC:DPPG:CHOL liposomes were more suitable for the encapsulation of the SpCas9 protein. Currently, knowledge on SpCas9-encapsulating nanoparticles for *in vivo* gene editing is scarce, and although *in vivo* utilization of the liposomes presented in **Chapter 5** requires optimization, the *in vitro* data is a promising new lead to improve the applicability of CRISPR-SpCas9 gene editing for the treatment of a variety of diseases, including autoimmune diseases[12] and cancer[13].

Perspectives

TolDCs allow for the induction of antigen-specific Tregs, and hold great potential for the treatment of autoimmune diseases, as demonstrated by their use in newfangled clinical trials focused on inducing *in vivo* immune tolerance to disease-related antigens. The generation of tolDCs involves manipulating DCs *ex vivo* using one or more immunomodulators and an autoantigen, to program them to induce antigen-specific immune tolerance in *vivo*.

One of the considerations for current tolDC therapies is the location and method of administration[14]. TolDCs need to be introduced into the body whilst maintaining their viability and function, which might require specialized procedures, such as intranodal injections, or advanced delivery methods. Studies describing tolDCs in a preclinical and clinical setting generally aim for the restoration of immune tolerance by restoring the immune balance[3,4,15–19]. This can be done through the induction of Tregs, but also the inhibition of effector T cells [4,14,20]. In all preclinical tolDC experimental autoimmune encephalomyelitis (EAE) studies, MS-relevant antigen-pulsed tolDCs were i.v. injected[21–30]. For murine studies of rheumatoid arthritis, the collagen-induced arthritis (CIA) model is a commonly described, Th17-dependent model[31,32]. A beneficial clinical effect has been observed with i.v., intraperitoneal (i.p.), and subcutaneously (s.c.) injected tolDCs in the CIA model[19,33–42]. Notably, a singular i.v. injection of 1 million tolDCs pulsed with the immunosuppressant tacrolimus (10^{-8}M) was sufficient to reach therapeutic benefits in mice with established arthritis in the CIA model[38]. Surprisingly, three i.v. injections of these tolDCs did not have the same positive therapeutic effects. On the other hand, for the EAE model, multiple i.v. tolDC injections are required to induce long-lasting clinical effects[29,30]. It should be noted here, however, that Dex-VitaminD3-pulsed tolDCs required three injections in the CIA model[19], emphasizing the importance of the immunomodulator used. Not only this, but a study using Dex-VitaminD3-pulsed tolDCs also showed that shifting the route of injection from i.v. to i.p. negated the beneficial effect of the therapy. In the Th1-dependent PGIA model[43] described in **Chapter 4**, previous research has shown the efficiency of i.p.[44,45] and i.v.[4] injection of tolDCs. As the tolDCs used for i.v. injection in a previous study[4] were closest to the tolDCs generated in **Chapters 3** and **4**, tolDCs and liposomes were administered i.v. in the described studies. It is important to investigate the optimal method of tolDC delivery *in vivo*, as tolDC properties, injection site and amount of injections can severely impact therapeutic efficacy and safety.

Currently, the safety and tolerance-inducing abilities of tolDCs for the treatment of rheumatoid arthritis have been investigated in different phase I clinical trials[15–17]. In the Rheumavax trial, patients received autologous DCs that were modified with an NF-κB inhibitor and loaded with a citrullinated peptide pool via intradermal (i.d.) injection. This administration route was safe and efficient, as a reduction in effector T cells and pro-inflammatory cytokines was observed in patients[16]. In another phase I clinical trial, AuToDeCra, patients received a singular administration of autologous synovial-fluid-pulsed Dex-VitD3-tolDCs in the knee joint via intraarticular (i.a.) injection[15]. This strategy was also proven to be safe and well-tolerated by patients. The CreaVax-Rheumatoid Arthritis trial analyzed s.c. administration of autologous tolDCs, and reported that five administrations were safe and reduced antigen-specific autoantibodies[17]. The currently ongoing phase I/II TOLERANT trial provides patients with two inguinal lymph node intranodal (i.n.) injections of autologous B29-loaded Dex-VitaminD3-tolDCs. The developments in the field of tolDC therapy are incredibly promising, and new findings on the safety and efficiency of different tolDCs and their injection routes and frequencies will provide new insights into the future of this therapeutic strategy.

Despite promising results with tolDCs for the induction of immune tolerance *in vitro* and *in vivo* the generation of autologous tolDCs is a time-consuming, costly, and complex process, requiring specialized protocols and expertise. Furthermore, the quality and potency of tolDCs can vary among individuals, affecting treatment consistency and possibly effectiveness[18,46]. The development and administration of tolDC therapies can therefore be cost-intensive, possibly limiting their availability to a broader patient population and increasing the burden on the health care system.

In **Chapter 2** the use of nanoparticles for the induction of antigen-specific immune tolerance is explored. These nanoparticles yield significant benefits over the use of tolDCs, as they are relatively easy to formulate and can be produced on a large scale, and do not require any cell culturing protocols, thereby offering a more cost-effective solution to tolDC therapy. Although tolDC clinical trials already report promising and favorable findings, the induction of antigen-specific immune tolerance *in vivo* can be improved by targeting antigens and immunomodulators to immune cells in specific tissues. This can be accomplished through the use of antibody-antigen or glycan-antigen conjugates[47,48], or nanoparticles, such as liposomes. The physiochemical properties of liposomes determine their immunogenicity[49], and as liposomes are highly flexible and can easily be modified[50], they are interesting for immunotherapy approaches.

Although the use of nanoparticles for immunotherapy is very promising, caution is warranted regarding the efficiency of nanoparticle administration, as described in **Chapter 2**. In **Chapters 3** and **4**, the aim was to induce antigen-specific immune tolerance in a PGIA model. Multiple studies reported the efficiency of localized injections, with several studies reporting the safety and efficacy of i.a. injections of empty and antigen-containing liposomes[51–54] in animal models for osteoarthritis and inflammatory arthritis. Although this is an interesting strategy, another study suggests efficient suppression of rheumatoid arthritis severity using s.c. injected liposomes encapsulating vitamin D3 and antigenic peptide $OVA_{323-339}$[55]. The efficiency of s.c. injected liposomes are confirmed in a recently completed randomized phase I clinical trial using s.c. injections of anionic egg-PC:egg-PG liposomes that encapsulated both a self-peptide and calcitriol. In this clinical trial, the liposome-treated patients showed improved rheumatoid arthritis disease activity and subsequent single-cell sequencing experiments identified T cell transcripts associated with tolerogenic TCRs[56]. In **Chapter 3** it is shown that i.v. injection of hPG and RA encapsulating liposomes can induce Tregs *in vivo*, underscoring a previous finding by Benne et al., who showed that i.v. injected liposomes with a similar formulation but

which carried the atherosclerosis-relevant antigen were also able to do so[57]. In the preventative arthritis study in the PGIA model described in **Chapter 4**, a singular i.v. injection of hPG_{K4}-Dex liposomes was enough to significantly inhibit arthritis development, and in mice with active arthritis, two i.v. injections resulted in the inhibition of arthritis development for 18 days after the second injection. The localization of liposomes upon *in vivo* injection greatly affects their subsequent immune effects. Therefore, analysis of the biodistribution of liposomes could contribute to better understanding of immune responses elicited by liposomes. Tracking fluorescently labeled liposomes throughout the body could offer new insights, and could especially be helpful for the identification of APC subsets that take up liposomes to further understand the *in vivo* mechanisms that are responsible for antigen-specific tolerance induction.

The liver and spleen are two organs that are generally associated with immune tolerance induction. In **Chapter 4** we showed that i.v. administration of hPG_{K4}-Dex liposomes results in a reduction in $CD11c^+CD86^+$ cells in the spleen, indicating a potential shift of DCs to a tolerogenic phenotype. This was accompanied by an observed increase in $CD4^+PD\text{-}1^+$ and $CD4^+CD25^+FoxP3^+$ Tregs in the hPG_{K4}-Dex liposome-treated mice compared to the PBS and Ova_{323K4}-Dex liposome groups. Even though it is unknown whether these cells are the result of reduced inflammation or actively contributed to protection against arthritis, the localization in the spleen does indicate the presence of antigen-specific systemic effects 18 days after the last administration of liposomes. This hypothesis is also supported by the work of Nguyen et al., who observed a decrease in $CD86^+$ APCs and an increase in $CD4^+FoxP3^+$ Tregs after i.v. injection with multiple sclerosis (MS)-relevant $MOG_{35\text{-}55}$-antigen-loaded mesoporous silica nanoparticles (MSN) in a mouse model for MS (experimental autoimmune encephalomyelitis (EAE))[58].

The use of SpCas9 mRNA could yield significant benefits over whole-protein encapsulation. Encapsulation of mRNA enables in situ antigen protein synthesis. This is beneficial, as it eliminates the need for protein purification and long-term stabilization[59], as would be needed for the method described in **Chapter 5**. The encapsulation of SpCas9 mRNA would allow for short-term expression, and eventually, complete removal of the nuclease from the body. A recent study has shown that the injection of lipid nanoparticles (LNPs) co-encapsulating Cas9 mRNA and sgRNAs was not only safe but also conferred the desired immunomodulatory effects[60]. The efficiency and safety of Cas9 mRNA delivery using LNPs have been corroborated by several other studies[61–63]. Although LNPs and liposomes both are lipid nano-formulations, they are different in composition. LNPs generally have a single phospholipid outer layer that encapsulates the interior[64]. This interior may be non-aqueous. Liposomes, on the other hand, consist of one or more lipid bilayers surrounding an aqueous core[65]. Despite these differences, we do hypothesize that encapsulation of SpCas9 mRNA in LNPs could be favorable for the induction of *in vivo* immune tolerance to SpCas9 compared to SpCas9 whole-protein delivery, as described in **Chapter 5**.

The application of mRNA for the treatment of rheumatoid arthritis, as in **Chapter 4**, is slightly more challenging, especially in humans as the autoantigens are largely unknown. A possible target is the mB29a peptide, as mentioned earlier. The B29 peptide is derived from heat shock protein 70 (HSP70) which, in rheumatoid arthritis, has been described to have a multitude of roles[66]. A previous study showed that the levels of anti-HSP70 autoantibodies are significantly higher in sera of rheumatoid arthritis patients compared to healthy controls[67]. Furthermore, extracellular HSP70 has been shown to downregulate proinflammatory processes in preclinical arthritis models through the modulation of a variety of signaling pathways, including the NF-κB pathway, resulting in a decrease in several

pro-inflammatory cytokines[68]. In a murine DNA vaccination study in a model for adjuvant-induced arthritis, HSP70 has been shown to direct T cells to Tregs rather than to Th1 cells[69], emphasizing its anti-inflammatory function in arthritis. The B29 peptide was first described by van Herwijnen et al. and can induce antigen-specific Tregs *in vivo*[70,71]. Therefore, this antigen is not only an interesting target for mRNA-based nanoparticle vaccines but linking this peptide to Dex using a polylysine linker as described in **Chapter 4**, could be an interesting strategy to confer long-term antigen-specific immune tolerance *in vivo*.

The discovery in **Chapter 4** that hPG_{K4}-Dex-containing liposomes can induce antigen-specific Tregs *in vivo* and can suppress arthritis development and progression is incredibly promising. However, for translation to humans, it is important to take into account each patient's medical profile. Between individuals, their immune systems, disease profiles, and medical histories are unique, and these factors can all influence the efficacy of human liposomal vaccination. The administration of tolDC therapies raises similar concerns, as developing patient-specific tolDC therapies demands a tailored approach for each individual, resulting in resource-intensive therapy. Current treatments for rheumatoid arthritis focus on symptom management and increasing the patient's quality of life. A commonly described treatment modality for the autoimmune disease is disease-modifying antirheumatic drugs (DMARDs). Not only has DMARD usage been associated with more severe outcomes of respiratory infections, such as influenza and SARS-Cov-272–74, but ongoing DMARD therapy interfered with vaccination efficiency[75]. This finding could have implications for the use of the liposomes described in **Chapter 4** in humans, as many patients are already on a treatment plan. Additionally, the blood of rheumatoid arthritis patients might contain different levels and quantities of proteins compared to healthy blood, which could affect the formation of the protein corona, possibly interfering with the therapeutic efficiency of liposomes *in vivo*[76–80]. This highlights the importance of further analyzing the effect of a patient's medication profile and blood composition before administering the liposomes described in **Chapters 3**, **4**, and **5** in a human system.

The liposomal encapsulation of a disease-relevant antigen and immunomodulator is a promising strategy for the treatment of rheumatoid arthritis in mice, as shown in **Chapters 3** and **4**. This raises the question of the broader applicability of the encapsulated $antigen_{K4}$-dex conjugate for the treatment of other autoimmune diseases. Liposomes are suitable nanocarriers for these different antigens, as previous studies using phosphatidylserine (PS)-containing liposomes have demonstrated the ability of these liposomes to be adapted to other autoimmune diseases, including type 1 diabetes and MS[81–83]. Furthermore, anionic DSPG-containing liposomes, as used in **Chapters 3** and **4**, loaded with an atherosclerosis-relevant antigen ApoB100-derived antigen have previously been described to be able to induce antigen-specific Tregs in mice[57]. Linking this peptide to the K4-dex construct could further enhance the *in vivo* efficiency of these liposomes.

Conclusion

This thesis provides exciting new insights into the use of liposomes for the treatment of autoimmune diseases and for the facilitation of *in vivo* CRISPR-gene editing.

References

1. Raverdeau M, Christofi M, Malara A, et al. Retinoic acid-induced autoantigen-specific type 1 regulatory T cells suppress autoimmunity. *EMBO Rep*. 2019;20(5). doi:10.15252/embr.201847121
2. García-González P, Morales R, Hoyos L, et al. A short protocol using dexamethasone and monophosphoryl lipid A generates tolerogenic dendritic cells that display a potent migratory capacity to lymphoid chemokines. *J Transl Med.* 2013;11(1). doi:10.1186/1479-5876-11-128
3. Anderson AE, Swan DJ, Wong OY, et al. Tolerogenic dendritic cells generated with dexamethasone and vitamin D3 regulate rheumatoid arthritis CD4+ T cells partly via transforming growth factor-β1. *Clin Exp Immunol.* 2017;187(1):113-123. doi:10.1111/cei.12870
4. Jansen MAA, Spiering R, Ludwig IS, van Eden W, Hilkens CMU, Broere F. Matured tolerogenic dendritic cells effectively inhibit autoantigen specific CD4+ T cells in a murine arthritis model. *Front Immunol.* 2019;10(AUG). doi:10.3389/fimmu.2019.02068
5. Kim SH, Moon JH, Jeong SU, et al. Induction of antigen-specific immune tolerance using biodegradable nanoparticles containing antigen and dexamethasone. *Int J Nanomedicine.* 2019;14:5229-5242. doi:10.2147/IJN.S210546
6. Noreen S, Maqbool I, Madni A. Dexamethasone: Therapeutic potential, risks, and future projection during COVID-19 pandemic. *Eur J Pharmacol.* 2021;894. doi:10.1016/j.ejphar.2021.173854
7. Kim DH, Martin DC. Sustained release of dexamethasone from hydrophilic matrices using PLGA nanoparticles for neural drug delivery. *Biomaterials.* 2006;27(15):3031-3037. doi:10.1016/j.biomaterials.2005.12.021
8. Briuglia ML, Rotella C, McFarlane A, Lamprou DA. Influence of cholesterol on liposome stability and on in vitro drug release. *Drug Deliv Transl Res.* 2015;5(3):231-242. doi:10.1007/s13346-015-0220-8
9. Lau CYJ, Benne N, Lou B, et al. Modulating albumin-mediated transport of peptide-drug conjugates for antigen-specific Treg induction. *Journal of Controlled Release.* 2022;348:938-950. doi:10.1016/j.jconrel.2022.06.025
10. Salt AN, Hartsock JJ, Piu F, Hou J. Dexamethasone and dexamethasone phosphate entry into perilymph compared for middle ear applications in Guinea Pigs. *Audiology and Neurotology.* 2018;23(4):245-257. doi:10.1159/000493846
11. Raverdeau M, Christofi M, Malara A, et al. Retinoic acid-induced autoantigen-specific type 1 regulatory T cells suppress autoimmunity. *EMBO Rep.* 2019;20(5). doi:10.15252/embr.201847121
12. Lee MH, Shin J Il, Yang JW, et al. Genome Editing Using CRISPR-Cas9 and Autoimmune Diseases: A Comprehensive Review. *Int J Mol Sci.* 2022;23(3). doi:10.3390/ijms23031337
13. Akram F, Ikram ul Haq, Ahmed Z, Khan H, Ali MS. CRISPR-Cas9, A Promising Therapeutic Tool for Cancer Therapy: A Review. *Protein Pept Lett.* 2020;27(10). doi:10.2174/0929866527666200407112432
14. Mansilla MJ, Hilkens CMU, Martínez-Cáceres EM. Challenges in tolerogenic dendritic cell therapy for autoimmune diseases: the route of administration. *Immunotherapy Advances*. 2023;3(1). doi:10.1093/immadv/ltad012
15. Bell GM, Anderson AE, Diboll J, et al. Autologous tolerogenic dendritic cells for rheumatoid and inflammatory arthritis. *Ann Rheum Dis.* 2017;76(1):227-234. doi:10.1136/annrheumdis-2015-208456
16. Benham H, Nel HJ, Law SC, et al. Citrullinated peptide dendritic cell immunotherapy in HLA risk genotype–positive rheumatoid arthritis patients. *Sci Transl Med.* 2015;7(290). doi:10.1126/scitranslmed.aaa9301

17. Joo Y Bin, Park JE, Choi CB, et al. Phase 1 Study of Immunotherapy Using Autoantigen-Loaded Dendritic Cells in Patients with Anti-Citrullinated Peptide Antigen Positive Rheumatoid Arthritis. *ACR/ARHP Annual Meeting.* Published online 2014. Accessed October 27, 2023. https://acrabstracts.org/abstract/phase-1-study-of-immunotherapy-using-autoantigen-loaded-dendritic-cells-in-patients-with-anti-citrullinated-peptide-antigen-positive-rheumatoid-arthritis/
18. Hilkens CMU, Isaacs JD. Tolerogenic dendritic cell therapy for rheumatoid arthritis: Where are we now? *Clin Exp Immunol.* 2013;172(2):148-157. doi:10.1111/cei.12038
19. Stoop JN, Harry RA, von Delwig A, Isaacs JD, Robinson JH, Hilkens CMU. Therapeutic effect of tolerogenic dendritic cells in established collagen-induced arthritis is associated with a reduction in Th17 responses. *Arthritis Rheum.* 2010;62(12). doi:10.1002/art.27756
20. Cifuentes-Rius A, Desai A, Yuen D, Johnston APR, Voelcker NH. Inducing immune tolerance with dendritic cell-targeting nanomedicines. *Nat Nanotechnol.* 2021;16(1):37-46. doi:10.1038/s41565-020-00810-2
21. Zhou F, Zhang GX, Rostami A. LPS-treated bone marrow-derived dendritic cells induce immune tolerance through modulating differentiation of CD4+ regulatory T cell subpopulations mediated by 3G11 and CD127. *Immunol Res.* 2017;65(3):630-638. doi:10.1007/s12026-016-8881-z
22. Zhou F, Zhang GX, Rostami A. Apoptotic cell-treated dendritic cells induce immune tolerance by specifically inhibiting development of CD4+ effector memory T cells. *Immunol Res.* 2016;64(1):73-81. doi:10.1007/s12026-015-8676-7
23. Quirant-Sánchez B, Mansilla MJ, Navarro-Barriuso J, et al. Combined therapy of vitamin d3-tolerogenic dendritic cells and interferon-β in a preclinical model of multiple sclerosis. *Biomedicines.* 2021;9(12). doi:10.3390/biomedicines9121758
24. Zhou Y, Leng X, Li H, et al. Tolerogenic dendritic cells induced by BD750 ameliorate proinflammatory T cell responses and experimental autoimmune encephalitis in mice. *Molecular Medicine.* 2017;23:204-214. doi:10.2119/molmed.2016.00110
25. Zhou Y, Leng X, Luo S, et al. Tolerogenic dendritic cells generated with tofacitinib ameliorate e xperimental autoimmune encephalomyelitis through modulation of Th17/Treg balance. *J Immunol Res.* 2016;2016. doi:10.1155/2016/5021537
26. Derdelinckx J, Mansilla MJ, De Laere M, et al. Clinical and immunological control of experimental autoimmune encephalomyelitis by tolerogenic dendritic cells loaded with MOG-encoding mRNA. *J Neuroinflammation.* 2019;16(1). doi:10.1186/s12974-019-1541-1
27. Zhou Y, Leng X, Luo X, et al. Regulatory dendritic cells induced by K313 display anti-inflammatory properties and ameliorate experimental autoimmune encephalitis in mice. *Front Pharmacol.* 2020;10. doi:10.3389/fphar.2019.01579
28. Xie Z, Chen J, Zheng C, et al. 1,25-dihydroxyvitamin D3-induced dendritic cells suppress experimental autoimmune encephalomyelitis by increasing proportions of the regulatory lymphocytes and reducing T helper type 1 and type 17 cells. *Immunology*. 2017;152(3):414-424. doi:10.1111/imm.12776
29. Mansilla MJ, Contreras-Cardone R, Navarro-Barriuso J, et al. Cryopreserved vitamin D3-tolerogenic dendritic cells pulsed with autoantigens as a potential therapy for multiple sclerosis patients. J *Neuroinflammation.* 2016;13(1). doi:10.1186/s12974-016-0584-9
30. Mansilla MJ, Sellès-Moreno C, Fàbregas-Puig S, et al. Beneficial Effect of Tolerogenic Dendritic Cells Pulsed with MOG Autoantigen in Experimental Autoimmune Encephalomyelitis. *CNS Neurosci Ther.* 2015;21(3):222-230. doi:10.1111/cns.12342

31. Chiang EY, Kolumam GA, Yu X, et al. Targeted depletion of lymphotoxin-α-expressing T H 1 and T H 17 cells inhibits autoimmune disease. *Nat Med.* 2009;15(7):766-773. doi:10.1038/nm.1984
32. Stoop JN, Tibbitt CA, van Eden W, Robinson JH, Hilkens CMU. The choice of adjuvant determines the cytokine profile of T cells in proteoglycan-induced arthritis but does not influence disease severity. *Immunology*. 2013;138(1):68-75. doi:10.1111/imm.12019
33. Byun SH, Lee JH, Jung NC, et al. Rosiglitazone-mediated dendritic cells ameliorate collagen-induced arthritis in mice. *Biochem Pharmacol.* 2016;115:85-93. doi:10.1016/j.bcp.2016.05.009
34. Chorny A, Gonzalez-Rey E, Fernandez-Martin A, Pozo D, Ganea D, Delgado M. Vasoactive intestinal peptide induces regulatory dendritic cells with therapeutic effects on autoimmune disorders. *PNAS.* Published online 2005. www.pnas.orgcgidoi10.1073pnas.0504484102
35. Kim SH, Kim S, Evans CH, Ghivizzani SC, Oligino T, Robbins PD. Effective Treatment of Established Murine Collagen-Induced Arthritis by Systemic Administration of Dendritic Cells Genetically Modified to Express IL-4. *The Journal of Immunology.* 2001;166(5):3499-3505. doi:10.4049/jimmunol.166.5.3499
36. Kim SH, Kim S, Oligino TJ, Robbins PD. Effective Treatment of Established Mouse Collagen-Induced Arthritis by Systemic Administration of Dendritic Cells Genetically Modified to Express FasL. *Molecular Therapy.* 2002;6(5):584-590. doi:10.1006/mthe.2002.0712
37. Zhao Y, Sun X, Yang X, et al. Tolerogenic Dendritic Cells Generated by BAFF Silencing Ameliorate Collagen-Induced Arthritis by Modulating the Th17/Regulatory T Cell Balance. *The Journal of Immunology.* 2020;204(3):518-530. doi:10.4049/jimmunol.1900552
38. Ren Y, Yang Y, Yang J, Xie R, Fan H. Tolerogenic dendritic cells modified by tacrolimus suppress CD4+ T-cell proliferation and inhibit collagen-induced arthritis in mice. *Int Immunopharmacol.* 2014;21(1):247-254. doi:10.1016/j.intimp.2014.05.004
39. Martin E, Capini C, Duggan E, et al. Antigen-specific suppression of established arthritis in mice by dendritic cells deficient in NF-κB. *Arthritis Rheum.* 2007;56(7). doi:10.1002/art.22655
40. Salazar L, Aravena O, Abello P, et al. Modulation of established murine collagen-induced arthritis by a single inoculation of short-term lipopolysaccharide-stimulated dendritic cells. *Ann Rheum Dis.* 2007;67(9). doi:10.1136/ard.2007.072199
41. Oh JS, Hwang SU, Noh KE, et al. Synthetic TGF-β Signaling Agonist-Treated Dendritic Cells Induce Tolerogenicity and Antirheumatic Effects. *Curr Issues Mol Biol.* 2022;44(9):3809-3821. doi:10.3390/cimb44090261
42. Wu H, Shen J, Liu L, Lu X, Xue J. Vasoactive intestinal peptide-induced tolerogenic dendritic cells attenuated arthritis in experimental collagen-induced arthritic mice. *Int J Rheum Dis.* 2019;22(7). doi:10.1111/1756-185X.13578
43. Hanyecz A, Berlo SE, Szántó S, Broeren CPM, Mikecz K, Glant TT. Achievement of a synergistic adjuvant effect on arthritis induction by activation of innate immunity and forcing the immune response toward the Th1 phenotype. *Arthritis Rheum.* 2004;50(5). doi:10.1002/art.20180
44. Spiering R, Van Der Zee R, Wagenaar J, Van Eden W, Broere F. Mycobacterial and mouse HSP70 have immuno-modulatory effects on dendritic cells. *Cell Stress Chaperones.* 2013;18(4):439-446. doi:10.1007/s12192-012-0397-4
45. Spiering R, Margry B, Keijzer C, et al. DEC205+ Dendritic Cell–Targeted Tolerogenic Vaccination Promotes Immune Tolerance in Experimental Autoimmune Arthritis. *The Journal of Immunology.* 2015;194(10):4804-4813. doi:10.4049/jimmunol.1400986
46. Passeri L, Marta F, Bassi V, Gregori S. Tolerogenic dendritic cell-based approaches in autoimmunity. *Int J Mol Sci.* 2021;22(16). doi:10.3390/ijms22168415
47. Hossain MK, Wall KA. Use of dendritic cell receptors as targets for enhancing anti-cancer immune responses. *Cancers (Basel).* 2019;11(3). doi:10.3390/cancers11030418

48. Castenmiller C, Keumatio-Doungtsop BC, van Ree R, de Jong EC, van Kooyk Y. Tolerogenic Immunotherapy: Targeting DC Surface Receptors to Induce Antigen-Specific Tolerance. *Front Immunol.* 2021;12. doi:10.3389/fimmu.2021.643240
49. Benne N, van Duijn J, Kuiper J, Jiskoot W, Slütter B. Orchestrating immune responses: How size, shape and rigidity affect the immunogenicity of particulate vaccines. *Journal of Controlled Release.* 2016;234:124-134. doi:10.1016/j.jconrel.2016.05.033
50. Akbarzadeh A, Rezaei-Sadabady R, Davaran S, et al. Liposome: Classification, preparation, and applications. *Nanoscale Res Lett.* 2013;8(1). doi:10.1186/1556-276X-8-102
51. Vanniasinghe AS, Bender V, Manolios N. The Potential of Liposomal Drug Delivery for the Treatment of Inflammatory Arthritis. *Semin Arthritis Rheum.* 2009;39(3):182-196. doi:10.1016/j.semarthrit.2008.08.004
52. Dong J, Jiang D, Wang Z, Wu G, Miao L, Huang L. Intra-articular delivery of liposomal celecoxib-hyaluronate combination for the treatment of osteoarthritis in rabbit model. *Int J Pharm.* 2013;441(1-2):285-290. doi:10.1016/j.ijpharm.2012.11.031
53. Cho H, Stuart JM, Magid R, et al. Theranostic immunoliposomes for osteoarthritis. *Nanomedicine.* 2014;10(3):619-627. doi:10.1016/j.nano.2013.09.004
54. Cipollaro L, Trucillo P, Bragazzi NL, Porta G Della, Reverchon E, Maffulli N. Liposomes for intra-articular analgesic drug delivery in orthopedics: State-of-art and future perspectives. insights from a systematic mini-review of the literature. *Medicina (Lithuania).* 2020;56(9):1-17. doi:10.3390/medicina56090423
55. Galea R, Nel HJ, Talekar M, et al. PD-L1– and calcitriol-dependent liposomal antigen-specific regulation of systemic inflammatory autoimmune disease. *JCI Insight.* 2019;4(18). doi:10.1172/jci.insight.126025
56. Sonigra A, Nel HJ, Wehr P, et al. Randomized phase I trial of antigen-specific tolerizing immunotherapy with peptide/calcitriol liposomes in ACPA + rheumatoid arthritis. *JCI Insight.* Published online 2022. doi:10.1172/jci
57. Benne N, van Duijn J, Lozano Vigario F, et al. Anionic 1,2-distearoyl-sn-glycero-3-phosphoglycerol (DSPG) liposomes induce antigen-specific regulatory T cells and prevent atherosclerosis in mice. *Journal of Controlled Release.* 2018;291:135-146. doi:10.1016/j.jconrel.2018.10.028
58. Nguyen TL, Choi Y, Im J, et al. Immunosuppressive biomaterial-based therapeutic vaccine to treat multiple sclerosis via re-establishing immune tolerance. *Nat Commun.* 2022;13(1). doi:10.1038/s41467-022-35263-9
59. Zeng C, Zhang C, Walker PG, Dong Y. Formulation and Delivery Technologies for mRNA Vaccines. In: *Current Topics in Microbiology and Immunology.* Vol 440. Springer Science and Business Media Deutschland GmbH; 2022:71-110. doi:10.1007/82_2020_217
60. Yi J, Lei X, Guo F, et al. Co-delivery of Cas9 mRNA and guide RNAs edits hepatitis B virus episomal and integration DNA in mouse and tree shrew models. *Antiviral Res.* 2023;215. doi:10.1016/j.antiviral.2023.105618
61. Qiu M, Glass Z, Chen J, et al. Lipid nanoparticle-mediated codelivery of Cas9 mRNA and single-guide RNA achieves liver-specific in vivo genome editing of Angptl3. *PNAS.* 2021;118(10). doi:10.1073/pnas.2020401118/-/DCSupplemental
62. Pil Han J, Kim M, Seok Choi B, et al. *In Vivo Delivery of CRISPR-Cas9 Using Lipid Nanoparticles Enables Antithrombin Gene Editing for Sustainable Hemophilia A and B Therapy.* Vol 8.; 2022.
63. Miller JB, Zhang S, Kos P, et al. Non-viral CRISPR/Cas gene editing in vitro and in vivo enabled by synthetic nanoparticle co-delivery of Cas9 mRNA and sgRNA HHS Public Access. *Angew Chem Int Ed Engl.* 2017;56(4):1059-1063. doi:10.1002/anie.20X

64. Hald Albertsen C, Kulkarni JA, Witzigmann D, Lind M, Petersson K, Simonsen JB. The role of lipid components in lipid nanoparticles for vaccines and gene therapy. *Adv Drug Deliv* Rev. 2022;188. doi:10.1016/j.addr.2022.114416
65. Large DE, Abdelmessih RG, Fink EA, Auguste DT. Liposome composition in drug delivery design, synthesis, characterization, and clinical application. *Adv Drug Deliv Rev.* 2021;176. doi:10.1016/j.addr.2021.113851
66. Fouani M, Basset CA, Mangano GD, et al. Heat Shock Proteins Alterations in Rheumatoid Arthritis. *Int J Mol Sci.* 2022;23(5). doi:10.3390/ijms23052806
67. Mantej J, Polasik K, Piotrowska E, Tukaj S. Autoantibodies to heat shock proteins 60, 70, and 90 in patients with rheumatoid arthritis. *Cell Stress Chaperones.* 2019;24(1):283-287. doi:10.1007/s12192-018-0951-9
68. Luo X, Zuo X, Zhou Y, et al. Extracellular heat shock protein 70 inhibits tumour necrosis factor-α induced proinflammatory mediator production in fibroblast-like synoviocytes. *Arthritis Res Ther.* 2008;10(2). doi:10.1186/ar2399
69. Quintana FJ, Carmi P, Mor F, Cohen IR. Inhibition of adjuvant-induced arthritis by DNA vaccination with the 70-kd or the 90-kd human heat-shock protein: Immune cross-regulation with the 60-kd heat-shock protein. *Arthritis Rheum.* 2004;50(11):3712-3720. doi:10.1002/art.20635
70. Van Herwijnen MJC, Wieten L, Van Der Zee R, et al. Regulatory T cells that recognize a ubiquitous stress-inducible self-antigen are long-lived suppressors of autoimmune arthritis. *Proc Natl Acad Sci U S A.* 2012;109(35):14134-14139. doi:10.1073/pnas.1206803109
71. Van Herwijnen MJC, Van Der Zee R, Van Eden W, Broere F. In vivo induction of functionally suppressive induced regulatory T cells from CD4+CD25- T cells using an Hsp70 peptide. *PLoS One*. 2015;10(6). doi:10.1371/journal.pone.0128373
72. Conway R, Grimshaw AA, Konig MF, et al. SARS–CoV-2 Infection and COVID-19 Outcomes in Rheumatic Diseases: A Systematic Literature Review and Meta-Analysis. *Arthritis and Rheumatology.* 2022;74(5):766-775. doi:10.1002/art.42030
73. George MD, Baker JF, Winthrop K, et al. Risk for Serious Infection With Low-Dose Glucocorticoids in Patients With Rheumatoid Arthritis : A Cohort Study. *Ann Intern Med.* 2020;173(11):870-878. doi:10.7326/M20-1594
74. Kroon FPB, Najm A, Alunno A, et al. Risk and prognosis of SARS-CoV-2 infection and vaccinati on against SARS-CoV-2 in rheumatic and musculoskeletal diseases: a systematic literature review to inform EULAR recommendations. *Ann Rheum Dis.* 2022;81(3):422-432. doi:10.1136/annrheumdis-2021-221575
75. van Sleen Y, van der Geest KSM, Huckriede ALW, van Baarle D, Brouwer E. Effect of DMARDs on the immunogenicity of vaccines. *Nat Rev Rheumatol.* 2023;19(9):560-575. doi:10.1038/s41584-023-00992-8
76. Walkey CD, Chan WCW. Understanding and controlling the interaction of nanomaterials with proteins in a physiological environment. *Chem Soc Rev.* 2012;41(7):2780-2799. doi:10.1039/c1cs15233e
77. Fleischer CC, Payne CK. Nanoparticle-cell interactions: Molecular structure of the protein corona and cellular outcomes. *Acc Chem Res.* 2014;47(8):2651-2659. doi:10.1021/ar500190q
78. Fleischer CC, Payne CK. Secondary structure of corona proteins determines the cell surface receptors used by nanoparticles. *Journal of Physical Chemistry B.* 2014;118(49):14017-14026. doi:10.1021/jp502624n

79. Ritz S, Schöttler S, Kotman N, et al. Protein Corona of Nanoparticles: Distinct Proteins Regulate the Cellular Uptake. *Biomacromolecules.* 2015;16(4):1311-1321. doi:10.1021/acs.biomac.5b001083
80. Hellstrand E, Lynch I, Andersson A, et al. Complete high-density lipoproteins in nanoparticle corona. *FEBS Journal.* 2009;276(12):3372-3381. doi:10.1111/j.1742-4658.2009.07062.x
81. Pujol-Autonell I, Mansilla MJ, Rodriguez-Fernandez S, et al. Liposome-based immunotherapy against autoimmune diseases: Therapeutic effect on multiple sclerosis. *Nanomedicine*. 2017;12(11):1231-1242. doi:10.2217/nnm-2016-0410
82. Almenara-Fuentes L, Rodriguez-Fernandez S, Rosell-Mases E, et al. A new platform for autoimmune diseases. Inducing tolerance with liposomes encapsulating autoantigens. *Nanomedicine*. 2023;48. doi:10.1016/j.nano.2022.102635
83. Pujol-Autonell I, Serracant-Prat A, Cano-Sarabia M, et al. Use of autoantigen-loaded phosphatidyl-serine-liposomes to arrest autoimmunity in type 1 diabetes. *PLoS One*. 2015;10(6). doi:10.1371/journal.pone.0127057

APPENDIX

English Summary

The immune system is a complex network of cells, tissues, and molecules that work together to protect the body against harmful invaders, such as bacteria, viruses, and other pathogens. To this end, the immune system is very good at recognizing and eliminating these pathogens, while sparing healthy cells and the body's own cells and tissues. In some cases, the immune system may malfunction, causing it to attack the body's own cells and tissues. This process is also called loss of immune tolerance, which can lead to the development of autoimmune diseases, such as rheumatoid arthritis.

Rheumatoid arthritis is a chronic autoimmune disease, in which the joint tissue is affected, resulting in painful inflammation. This can lead to joint damage and limitations in movement, which means that this disease can have a significant impact on the quality of life of patients. Current treatment methods focus on reducing symptoms, thereby improving quality of life. This is accomplished, among other things, by administering disease-modifying anti-rheumatic drugs (DMARDs). DMARDs can be divided into several categories, with the ultimate goal of inhibiting the inflammatory response and thereby preventing joint damage. The downside to using this type of medication is that it can lead to general, non-specific suppression of the immune system.

To improve immunotherapy for autoimmune diseases, there is increasing interest in developing specific therapies. These therapies focus on the disease-causing antigen so that other cells, tissues, and molecules are not affected. An antigen is a foreign molecule that is often found on the surface of a pathogen. Current clinical trials investigate the therapeutic effect of dendritic cells (DCs) for treating various autoimmune diseases. Dendritic cells play an important role in triggering the specific immune system, which adapts to the pathogen. This part of the immune system is also called the adaptive immune system. These cells are extremely good at recognizing and processing pathogens and are then able to present pieces of this pathogen, called peptides, on their cell surface with the help of major histocompatibility complex (MHC) molecules. These molecules play a central role in the immune response. The MHC-peptide complex can then be recognized by receptors on the surface of T cells, called T cell receptors (TCRs). These TCRs enable T cells to generate specific immune responses. Furthermore, DCs can influence naive T cells to become a certain type of effector T cell. This process is called differentiation. Depending on the signals the T cell receives from the DC, the naive T cell becomes a pro-inflammatory T cell (T helper (Th1) or Th17) or a regulatory T cell (Treg). Tregs are very important for regulating the immune response. In the case of autoimmune diseases, the regulation of the immune response is disrupted, causing a pro-inflammatory immune response against the body's own antigens, also known as self-antigens. To counter this, researchers are now looking at optimizing the DC-T cell interaction in clinical trials to ensure that more antigen-specific Tregs are produced in the body. They do this by loading the DC with a disease-relevant antigen so that it is presented by the DC. Only T cells that can respond to this antigen, i.e. T cells with a specific TCR, will recognize this antigen and can bind to the DC. Now, it is important to skew the T cells to become Tregs. This is accomplished by treating DCs with immunomodulators, such as dexamethasone (dex), retinoic acid (RA), or vitamin D3. This stimulation turns DCs into tolerogenic DCs (tolDCs), which express lower levels of CD40 and CD86, which are the surface molecules that are essential fot T cell co-stimulation. DCs will also secrete signaling molecules, called cytokines. Normally, activated DCs increase the production of pro-inflammatory cytokines, such as interleukin (IL) 12 and interferon-gamma (IFNγ). By adding immunomodulators, DCs will secrete more anti-inflammatory cytokines such as IL-10 and TGFβ. This ensures that naive T cells will change into Tregs. In the clinical trials, researchers stimulate DCs with a disease-relevant antigen and immunomodulators, which ensures the induction of antigen-specific tolDCs, which are then able to stimulate antigen-specific Tregs in the body, i.e. *in vivo*.

There is a lot of attention for tolDCs as a new immunotherapy for autoimmune diseases. However, there are also disadvantages to the use of these cells. Generating antigen-specific tolDCs requires complicated protocols, which, in turn, require specialist centers. Furthermore, the quality and potency of tolDCs can vary from person to person, which affects the consistency and effectiveness of treatment. In **Chapter 2**, nanoparticles are discussed and described as an alternative way to induce antigen-specific immune tolerance. The emphasis is on choosing the right nanoparticles for the right application. For example, nanoparticles with a negative charge are often used for research into the treatment of autoimmune diseases. These anionic particles are often around 200nm in size, making them easy for DCs to take up. By packaging a disease-relevant antigen and an immunomodulator in a nanoparticle, both the immunomodulator and the antigen can be taken up by a DC. This may cause DCs to change into tolDCs *in vivo* and stimulate the production of antigen-specific Tregs. In **Chapter 3** it is investigated whether it is possible to induce antigen-specific Tregs using liposomes. Liposomes are nanoparticles composed of lipids that form a double layer called a bilayer. These structures can most easily be compared to a soap bubble. Within the double layer with lipids, molecules that are not easily soluble in water (hydrophobic) can be packaged. Molecules that are easily soluble in water (hydrophilic) can be packed in the center of the liposomes. By ensuring that lipids with a negative charge are used and by pressing (extruding) the liposomes under high pressure through a 200nm filter, it is ensured that the liposomes all have approximately the same size and charge. By packaging retinoic acid and the rheumatoid arthritis-relevant antigen human proteoglycan (hPG) in these liposomes, the negative charge and the size of around 180nm remained the same. Like free hPG and RA, hPG-RA liposomes were able to induce tolDCs in bone marrow-derived DCs (BMDCs) in the lab *in vitro*, but liposomes containing hPG were preferentially taken up by BMDCs than free hPG. To see whether these tolDCs were able to induce antigen-specific Tregs, DCs treated with hPG-RA liposomes were combined with naive T cells that only have a T cell receptor specific for hPG. DCs treated with hPG-RA liposomes were able to induce antigen-specific Tregs *in vitro*, and when these liposomes were injected into a wild-type mouse that had also been injected with T cells specific for hPG, it was found that the mice receiving the liposomes had an antigen-specific Treg response, as did mice treated with DCs stimulated with free hPG and RA or free hPG and RA without DCs.

Because these liposomes appeared to be a suitable way to co-transport a disease-relevant antigen and an immunomodulator to DCs *in vivo*, **Chapter 4** examined the use of dexamethasone in these liposomes for immunotherapy in a mouse model of rheumatoid arthritis. In this chapter, we linked dexamethasone to the hPG antigen with a lysine tetramer (hPG_{K4}-Dex) before packaging this complex into liposomes. The hPG_{K4}-Dex complex can convert BMDCs to tolDC in the presence of the pro-inflammatory stimulus lipopolysaccharide (LPS), both when administered freely and when packaged in liposomes. Furthermore, hPG_{K4}-Dex liposomes can induce antigen-specific Tregs *in vivo*.
To validate the effect of these hPG_{K4}-Dex liposomes in a disease model, the proteoglycan-induced arthritis (PGIA) model was used. In this model, rheumatoid arthritis is artificially induced by an injection of hPG with the adjuvant dimethyldiotadecylammonium bromide (DDA). After two injections, this causes the development of rheumatoid arthritis in the injected mice. By injecting mice with hPG_{K4}-Dex liposomes on day 17 after the first injection (4 days before the second injection), the development of rheumatoid arthritis could be prevented in 33.3% of the mice, and the development of rheumatoid arthritis was significantly less than in mice treated with hPG_{K4}-Dex-stimulated tolDCs. This was a promising discovery, which also allowed the functionality to be investigated in mice with active rheumatoid arthritis. In mice that had already developed rheumatoid arthritis, treatment with hPG_{K4}-Dex liposomes was able to significantly inhibit the development of rheumatoid arthritis compared to the control groups. Increased levels of anti-inflammatory cytokine IL-10 were found in

the paws of these mice and more Tregs were present in the spleens. These results highlight the potential of using nanoparticles to restore immune tolerance in autoimmune diseases.

In recent years, nanoparticles have gained popularity not only for the treatment of autoimmune diseases. The world of gene therapy is also interested in these multifunctional particles. In **Chapter 5**, the application of liposomes for the i*n vivo* delivery of the Cas9 protein is investigated. The Cas9 protein is a bacterial protein involved in the CRISPR-Cas9 gene editing system. The development of CRISPR has ensured that specific DNA modifications can be made, allowing mutations in genes to be repaired. This is an important goal, but the bacterial nature of the protein poses problems. The immune system will immediately recognize it as foreign and clean it up. By packaging the protein in liposomes, in combination with dexamethasone, the research in this chapter attempts to prevent an immune response against the Cas9 protein. This is successfully accomplished *in vitro*, inducing tolDCs from BMDCs stimulated with liposomes containing dexamethasone sodium phosphate (DexPhos) and Cas9. DexPhos is a water-soluble form of dexamethasone, which is easier to package in liposomes. The Cas9-DexPhos liposomes are efficiently taken up by DCs *in vitro*, and translocate to the liver and spleen *in vivo*. The liver and spleen are organs that are important in maintaining immune tolerance. More optimization is required for the *in vivo* application of Cas9-DexPhos liposomes, but a first step has been taken in this new field of research.

TolDCs and liposomes loaded with a relevant antigen and immunomodulator represent promising strategies for restoring immune tolerance in autoimmune diseases. In this thesis, anionic liposomes loaded with an immunomodulator and a disease-relevant antigen are used to induce immune tolerance *in vitro* and *in vivo* and to prevent the development of rheumatoid arthritis. Furthermore, a first effort is made towards applying these nanoparticles for optimizing CRISPR-Cas9 gene editing.

Nederlandse Samenvatting

Het immuunsysteem is een complex netwerk van cellen, weefsels en moleculen die samenwerken om het lichaam te beschermen tegen schadelijke indringers, zoals bacteriën, virussen en andere ziekte verwekkers. Daartoe is het immuunsysteem erg goed in het herkennen en elimineren van deze pathogenen, terwijl gezonde cellen en lichaamseigen cellen en weefsels gespaard blijven. In sommige gevallen kan het immuunsysteem disfunctioneren, waardoor het lichaamseigen cellen en weefsels aan gaat vallen. Dit proces wordt ook wel het verlies van immuun tolerantie genoemd, en dit leidt tot het ontstaan van auto-immuunziektes, zoals reumatoïde artritis.

Reumatoïde artritis is een chronische auto-immuunziekte, waarbij het gewrichtsweefsel wordt aangetast waardoor er pijnlijke ontstekingen ontstaan. Dit kan leiden tot gewrichtsschade, en beperkingen in bewegingsvrijheid, waardoor deze ziekte een aanzienlijke impact kan hebben op de levenskwaliteit van patiënten. Huidige behandelmethoden richten zich op het verminderen van de symptomen, waardoor de kwaliteit van leven verbeterd wordt. Dit wordt onder andere gedaan door de toediening van disease-modifying anti-rheumatic drugs (DMARDs). DMARDs kunnen worden onderverdeeld in meerdere categorieën, met het uiteindelijke doel om de ontstekingsreactie te remmen en daardoor gewrichtsschade te voorkomen. Het nadeel aan het gebruik van dit soort medicatie, is dat het kan leiden tot algehele, niet-specifieke onderdrukking van het immuunsysteem.

Om immuuntherapie voor auto-immuunziekten te verbeteren, is er steeds meer interesse in het ontwikkelen van specifieke therapieën. Hierbij richt de therapie zich op het ziekte veroorzakende antigeen, zodat andere cellen, weefsels en moleculen niet beïnvloed worden. Een antigeen is een lichaamsvreemd molecuul, dat zich vaak op het oppervlak van een pathogeen bevindt. Huidige klinische trials onderzoeken het therapeutische effect van dendritische cellen (DCs) voor het behandelen van verschillende auto-immuunziekten. Dendritische cellen spelen een belangrijke rol in het aanzetten van het specifieke immuunsysteem, dat zich aanpast aan het pathogeen. Dit deel van het immuunsysteem wordt ook wel adaptieve immuunsysteem genoemd. Deze cellen zijn uitermate goed in het herkennen en verwerken van ziekteverwekkers, en zijn vervolgens in staat stukjes van deze ziekteverwekker, genaamd peptide, te presenteren op het celoppervlak met behulp van major histocompatibility complex (MHC) moleculen. Deze moleculen spelen een centrale rol in de afweerreactie. Het MHC-peptide complex kan vervolgens herkent worden door receptoren op het oppervlak van T cellen, genaamd T cel receptoren (TCRs). Door deze TCRs zijn T cellen in staat specifieke immuunrespons te genereren. Een andere belangrijke interactie tussen DCs en T cellen, is dat DCs naïeve T cellen kunnen beïnvloeden in welk type T cel ze uiteindelijk zullen worden, ook wel differentiatie genoemd. Afhankelijk van de signalen die de T cel krijgt van de DC, wordt de naïeve T cel een pro-inflammatoire T cel (T helper (Th1) of Th17) of een regulatoire T cel (Treg). Tregs zijn erg belangrijk voor het reguleren van de immuun respons. In het geval van auto-immuunziekten, is de regulatie van de immuun respons verstoord, waardoor er een pro-inflammatoire immuunrespons tegen een lichaamseigen antigeen ontstaat. Om dit tegen te gaan, kijken onderzoekers in klinische trials nu naar het optimaliseren van de DC-T cel interactie om ervoor te zorgen dat er meer anti-geen-specifieke Tregs worden aangemaakt in het lichaam. Dit doen ze door de DC te beladen met een ziekte-relevant antigeen, zodat deze door de DC gepresenteerd wordt. Alleen T cellen die kunnen reageren op dit antigeen, dus de T cellen met een specifieke TCR, zullen dit antigeen herkennen en kunnen binden aan de DC. Vervolgens is het belangrijk dat de T cellen voornamelijk Tregs zullen worden. Dit wordt bewerkstelligd door DCs te behandelen met immunomodulatoren, zoals dexamethason (dex), retinolzuur (RA) of vitamine D3. Door deze stimulatie veranderen DCs in tolerogene DCs (tolDCs), waarbij de oppervlakte moleculen die belangrijk zijn voor de co-stimulatie

van T cellen, CD40 en CD86, minder tot expressie gebracht worden. Hierdoor zullen antigeen-specifieke pro-inflammatoire T cellen dood gaan, terwijl dit juist Tregs stimuleert. Ook zullen DCs andere signaalstoffen, genaamd cytokines, uitscheiden. Normaal gesproken zorgen geactiveerde DCs voor een verhoogde productie van pro-inflammatoire cytokines, zoals interleukine (IL) 12 en interferon gamma (IFNγ). Door het toevoegen van immunomodulatoren, zullen DCs meer anti-inflammatoire cytokines zoals IL-10 en TGFβ uitscheiden. Dit zorgt ervoor dat naïeve T cellen zullen veranderen naar Tregs. In de klinische trials stimuleren onderzoekers DCs met een ziekte-relevant antigeen en immunomodulatoren, hetgeen zorgt voor de inductie van antigeen-specifieke tolDCs, die vervolgens in staat zijn om in het lichaam, dus *in vivo*, antigeen-specifieke Tregs te stimuleren.

Er is veel aandacht voor tolDCs als nieuwe immuuntherapie voor auto-immuunziekten. Echter zitten aan het gebruik van deze cellen ook nadelen. Voor het genereren van antigeen-specifieke tolDCs zijn gecompliceerde protocollen nodig, hetgeen specialistische centra vereist. Ook kan de kwaliteit en de potentie van tolDCs van persoon tot persoon verschillend zijn, wat de consistentie en effectiviteit van de behandeling beïnvloedt. In **hoofdstuk 2** worden nanodeeltjes bediscussieerd en beschreven als alternatieve manier om antigeen-specifieke immuun tolerantie op te wekken. Hierbij wordt de nadruk gelegd op het kiezen van het juiste nanodeeltjes voor de juiste toepassing. Zo wordt er voor onderzoek naar de behandeling van auto-immuunziekten vaak gebruik gemaakt van nanodeeltjes met een negatieve lading. Deze anionische deeltjes zijn vaak rond de 200nm in grootte, waardoor ze makkelijk kunnen worden opgenomen door DCs. Door een ziekte-relevant antigeen en een immunomodulator te verpakken in een nanodeeltje, kunnen zowel de immunomodulator en het antigeen door een DC worden opgenomen. Dit kan er voor zorgen dat DCs *in vivo* veranderen in tolDCs en hierdoor kan het aanmaken van antigeen-specifieke Tregs gestimuleerd worden.

In **hoofdstuk 3** wordt er onderzocht of het mogelijk is antigeen-specifieke Tregs te induceren met behulp van liposomen. Liposomen zijn nanodeeltjes die bestaan uit lipides die een dubbele laag, genaamd bilaag, vormen. Deze structuren zijn het makkelijkste te vergelijken met een zeepbel. In de dubbele laag met lipides kunnen moleculen die niet goed in water op te lossen zijn (hydrofoob) verpakt worden. In het midden van de liposomen kunnen moleculen die goed in water oplosbaar zijn (hydrofiel) verpakt worden. Door ervoor te zorgen dat er lipides gebruikt worden met een negatieve lading en door de liposomen te onder hoge druk door een 200nm filter te persen (extruderen), wordt ervoor gezorgd dat de liposomen allemaal ongeveer de zelfde grootte en lading hebben. Door retinolzuur en het reumatoïde artritis-relevante antigeen human proteoglycan (hPG) te verpakken in deze liposomen bleef de negatieve lading en de grootte van rond de 180nm hetzelfde. Net als vrije hPG en RA waren de hPG-RA liposomen in staat tolDCs te induceren in beenmerg-verkregen DCs (BMDCs) in het lab *in vitro*, maar werden de liposomen met hPG beter opgenomen door BMDCs dan vrije hPG. Om te kijken of deze tolDCs in staat waren om antigeen-specifieke Tregs te induceren, werden DCs behandeld met hPG-RA liposomen samen gevoegd met naïeve T cellen die alleen een T cel receptor hebben die specifiek is voor hPG. DCs behandeld met hPG-RA liposomen waren in staat antigeen-specifieke Tregs te induceren *in vitro*, en wanneer deze liposomen ingespoten werden in een wildtype muis die ook een injectie had gehad met T cellen specifiek voor hPG, werd er gevonden dat de muizen die de liposomen hadden gehad een antigeen-specifieke Treg respons hadden, net als muizen behandeld met DCs gestimuleerd met vrije hPG en RA of vrije hPG en RA zonder DCs.

Doordat deze liposomen een geschikte manier leken om een ziekte-relevant antigeen en een immunomodulator samen te vervoeren naar DCs *in vivo*, werd er in **hoofdstuk 4** gekeken naar het gebruik van dexamethason in deze liposomen voor immuuntherapie in een muismodel voor

reumatoïde artritis. In dit hoofdstuk hebben we dexamethason gelinkt aan het hPG antigeen met een lysine tetrameer (hPG_{K4}-Dex) alvorens het in liposomen te verpakken. Het hPG_{K4}-Dex complex is in staat BMDCs in de aanwezigheid van de pro-inflammatoire stimulus lipopolysaccharide (LPS) te veranderen naar tolDC, zowel wanneer het vrij toegediend is als wanneer het verpakt is in liposomen. Bovendien kunnen hPG_{K4}-Dex liposomen antigeen-specifieke Tregs induceren *in vivo*. Om het effect van deze hPG_{K4}-Dex liposomen te valideren in een ziekte model, werd het proteoglycan-induced arthritis (PGIA) model gebruikt. In dit model wordt reumatoïde artritis kunstmatig geïnduceerd door een injectie van hPG met het hulpmiddel dimethyldiotadecylammonium bromide (DDA). Na twee injecties zorgt dit voor de ontwikkeling van reuma in de geïnjecteerde muizen. Door muizen te injecteren met hPG_{K4}-Dex liposomen op dag 17 na de eerste injectie (4 dagen voor de tweede injectie), kon de ontwikkeling van reuma voorkomen worden in 33.3% van de muizen, en was de ontwikkeling van reuma significant minder dan in muizen behandeld met hPG_{K4}-Dex tolDCs. Dit was een veelbelovende ontdekking, waardoor ook de functionaliteit in muizen met actieve reuma onderzocht kon worden. In muizen die al reuma hadden ontwikkeld was behandeling met hPG_{K4}-Dex liposomen in staat om de ontwikkeling van reuma significant te remmen ten opzichte van de controle groepen. In de poten van deze muizen werden verhoogde niveaus van anti-inflammatoire cytokine IL-10 gevonden en in de milten waren meer Tregs aanwezig. Deze resultaten benadrukken de potentie van het gebruik van nanodeeltjes in het herstellen van immuun tolerantie in auto-immuunziekten.

Niet alleen voor de behandeling van auto-immuunziekten hebben nanodeeltjes aanzien verkregen over de afgelopen jaren. Ook de wereld van de gentherapie is geïnteresseerd in deze multifunctionele deeltjes. In **hoofdstuk 5** wordt de toepassing van liposomen voor het *in vivo* bezorgen van het Cas9 eiwit onderzocht. Het Cas9 eiwit is een bacterieel eiwit dat betrokken is bij het CRISPR-Cas9 gen editing systeem. De ontwikkeling van CRISPR heeft ervoor gezorgd dat er specifieke DNA modificaties gedaan kunnen worden, waardoor mutaties in genen hersteld kunnen worden. Dit is een belangrijk doel, maar de bacteriële aard van het eiwit brengt problemen met zich mee. Het lichaam zal het direct herkennen als lichaamsvreemd en het gaat opruimen. Door het verpakken van het eiwit in liposomen, in combinatie met dexamethason, wordt in dit hoofdstuk geprobeerd te voorkomen dat er een immuun respons optreed tegen het Cas9 eiwit. *In vitro* wordt dit goed bewerkstelligd, met het induceren van tolDCs in BMDCs die gestimuleerd zijn met liposomen die dexamethason natrium fosfaat (DexPhos) en Cas9 bevatten. DexPhos is een wateroplosbare vorm van dexamethason, die makkelijker te verpakken is in liposomen. De Cas9-DexPhos liposomen worden efficiënt opgenomen door DCs *in vitro*, en verplaatsen zich *in vivo* naar de lever en milt. De lever en milt zijn organen die belangrijk zijn bij het in stand houden van immuun tolerantie. Voor de *in vivo* toepassing van Cas9-DexPhos liposomen is meer optimalisatie nodig, maar een eerste stap is gezet in dit nieuwe onderzoeksveld.

TolDCs en liposomen beladen met een relevant-antigeen en immunomodulator vormen veelbelovende strategieën voor het herstellen van immuun tolerantie in auto-immuunziekten.
In dit proefschrift worden anionische liposomen beladen met immunomodulator en ziekte-relevant antigeen gebruikt om immuun tolerantie te induceren *in vitro* en *in vivo*, en om de ontwikkeling van reumatoïde artritis te voorkomen, en wordt de eerste stap gezet naar het toepassen van deze nanodeeltjes voor het optimaliseren van CRISPR-Cas9 gen editing.

List of Publications

2024

Autoantigen-dexamethasone conjugate-loaded liposomes halt the development of arthritis in mice
N. Benne*, **D. ter Braake***, D. Porenta, C.Y.J. Lau, E. Mastrobattista, F. Broere
Published in Advanced Healthcare Materials – January 2024
** Shared first author*

2022

Modulating albumin-mediated transport of peptide-drug conjugates for antigen-specific Treg induction
C.Y.J. Lau, N. Benne, B. Lou, O. Zharkova, H.J. Ting, **D. ter Braake**, N. van Kronenburg, M.H. Fens, F. Broere, W.E. Hennink, J. Wang, E. Mastrobattista
Published in Journal of Controlled Release – August 2022

Nanoparticles for Inducing Antigen-Specific T Cell Tolerance in Autoimmune Diseases
N. Benne, **D. ter Braake**, A.J. Stoppelenburg, F. Broere
Published in Frontiers in Immunology – March 2022

Tuning Surface Charges of Peptide Nanofibers for Induction of Antigen-Specific Immune Tolerance: An Introductory Study
C.Y.J. Lau, N. Benne, B. Lou, **D. ter Braake**, E. Bosman, N. van Kronenburg, M.H. Fens, F. Broere, W.E. Hennink, E. Mastrobattista
Published in Journal of Pharmaceutical Science – January 2022

2021

Retinoic Acid-Containing Liposomes for the Induction of Antigen-Specific Regulatory T Cells as a Treatment for Autoimmune Diseases
D. ter Braake*, N. Benne*, C.Y.J. Lau, E. Mastrobattista, F. Broere
Published in MDPI Pharmaceutics – November 2021
** Shared first author*

2020

Influenza-like illness is associated with high pneumococcal carriage density in Malawian children
T.K. Nyazika, A. Law, T.D. Swarthout, L. Sibale, **D. ter Braake**, N. French, R.S. Heyderman, D. Everett, A. Kadioglu, K.C. Jambo, D.R. Neill
Published in Journal of Infection – October 2020

Acknowledgements

I am truly grateful for the amazing time I've had these past years. Not only was I very fortunate to have the opportunity to work on a fascinating project, but I also got to meet incredible, inspiring people along the way. This Ph.D. thesis would not have been possible without them.

First, I'd like to thank my promotors **Willem** and **Femke**. **Willem**, thank you for your valuable input in scientific discussions and interesting talks these past years. You were always there to encourage the scientific progress we made. I wish you and Margreet all the best. It was wonderful to get to know you. **Femke**, thank you for being such an involved promotor and supervisor. You were always available for advice, whether this was about experiments, paper writing, or whenever I did something clumsy again. During our meetings, you were never short of ideas and suggestions, which contributed greatly to the thesis you see before you. You allowed me the freedom to join several committees and explore the field of education more, which I really appreciated. Not only did I thoroughly enjoy the Ph.D. project, these side activities brought me a lot of joy and energy. Thank you for everything!

Naomi, I don't even know where to start thanking you. Your work ethic, knowledge, and skills are incredible and taught me so much regarding experimental planning and execution. I've been very lucky that you were willing to be my co-promotor and I couldn't have wished for a better collaboration. You truly are one of the highlights of my Ph.D. period, as I not only had the honor of working with an amazing scientist, but I also met a great friend in you! From not so stealthily sneaking out during the keynote lecture at the NVVI to building a vegetable garden bed together, my memories of spending time with you are only happy ones. I am sure you will do amazing things in the world of nanoparticle-based immunotherapies, and I am looking forward to seeing these things come to fruition. When you (inevitably) win the Nobel prize for one of your ideas, I will for sure be there to cheer you on. You and **Adrian** are two of the kindest people I know, and I wish you both all the best things in the world.

My lovely paranymphs, **Lobna** and **Emanuele**, you both made my Ph.D. the incredible experience it was. We have visited many amazing places over the years, including beautiful Africa. You quickly became two of my closest friends and I really cannot imagine life without you anymore. Thank you for everyting. **Lobna**, your bubbly personality and unending warmth make you an incredible friend. The first time we met, we were both waiting for a bus to the Christmas Markets in Düsseldorf. By the end of the day, it felt like I had known you all my life. From doing silly accents (omagaaa), laughing uncontrollably, dancing and singing along to our favorite songs, the day is just better when you are in it. You are a fiercely strong and independent woman. Your energy and enthusiasm are infectious and made my time at the lab and outside of it so much more enjoyable. **Emanuele**, your humor, kindness, and sassiness are only a few of the things that make you such a wonderful friend. Over the years we did so many fun things, but I think the highlight might have been our trip to see ABBA live in London. That was such an incredible experience! You introduced me to many great Italian artists, such as Myss Keta and Elodie, and I always really enjoy judging the San Remo contestants together. I am incredibly happy to have you in my life. Your hugs are the best and you are truly one of the most thoughtful people I know. Baci stellari sempre!

Irene, you truly are one of the most knowledgeable people I've ever met (both science-wise and during pub quizzes), so I felt lucky to be sharing an office with you. Whenever I was looking for something or tried to solve an issue in the lab, you were always there to help. I'm 100% convinced the lab would fall apart without you and I am sure there will be many more future moments where I am stuck and will think 'Irene would know'. Thank you for everything!

Arjan, you were the first person I met after my interview at the department, and you made me feel so welcome. Your dedication to science and the passion with which you speak about immunology are truly admirable and very contagious. Your insightful comments and suggestions were of great help over the past years. You were always there for me when I needed advice, and having a friend in the lab who can make you laugh without even saying a complete sentence made many days even more enjoyable. Thank you!

Deja, you are such an incredibly motivated, kind, and intelligent person. I thoroughly enjoyed working with you and singing along to various songs during late-night *in vivo* experiments. I am afraid that the horrible music at the animal facility will probably always haunt us, especially 'Bleeding Love'. I will definitely miss our daily discussions about flow cytometry, plants, gardening, and cooking, and I am looking forward to more gardening trips soon!

Elise, what a pleasure to get to know you better over these past years. When you came back to the lab in Utrecht you helped me organize the lab Christmas party. It was so much fun arranging everything together, and even though keys got lost, the party was a great success. We should have known this was the start of all the crazy situations we would end up in over the years (definitely not our fault). Every time I hear Taylor Swifts' Anti-Hero on the radio I am reminded of the Halloween party, and it fills me with a lot of joy. You are an amazing person, and I am looking forward to our future adventures.

Aad, you have been a great friend during these past years. Your positive outlook on life brought me so much happiness, and your laugh is incredibly contagious. I enjoyed the time we spent together a lot, whether it was singing 'Ring of Fire' from the top of our lungs at a late-night karaoke session, or discussing the latest football matches of Liverpool, Barcelona, and Feyenoord/Ajax. I loved how our lab VrijMiBo's were attended by your whole family, and you & Denise's enthusiasm for the disco bingo's made them a lot more fun to host. Also, thank you for all your help in the lab. **Denise**, thank you for all your advice, kindness, and limoncello these past years. Hopefully, there will be many more tennis matches and drinks nights to catch up in the future.

Adil, you're such a wonderful and warm person. I truly believe that if kindness was a person, it would be you. You taught me to speak in Urdu (Mehra nam "bohot" Daniëlle he) and always joined in with all the singing and sports at the lab. Your voice is beautiful and so soothing, it's great to listen to. Thanks for all the great times we've spent together, and I am already looking forward to trying the famous Pakistani carrots when visiting you there.

Ali, you're just a beacon of joy. Your kind heart combined with your sharp wit makes you a great friend to hang out with. It's been such a pleasure to work and laugh with you daily. Thanks to both you and Adil for introducing me to the world of Cricket, and it was great fun to visit the Pakistan vs. Netherlands match in 2022 together with Aad.

Qingkang, my always enthusiastic office mate. I really enjoyed all our tea chats and discussions about all things in life. Your willingness to join every event we organized, from football to pub quizzes, made you a great coworker to have around. You're a very hard worker, and I wish you, your wife, and your daughter Mengran all the best in Georgia, USA.

Fatemeh, you've been such a great friend to work with. From enjoying the sun together and eating some raisins to our weekly Careless Whisper hour in the blue lab, I thoroughly enjoyed working with you. You're such a warm and caring person, and you always made me feel so comfortable. Thank you for being you and I hope to see many more pictures of Bakhtiar and Shapur soon.

Fenne, when you came to our lab I just knew it would become an even nicer place to be. In many ways, you remind me of a true Disney princess. You are kind, incredibly creative, and your singing voice is gorgeous. Thank you for the joy you bring!

Esther, I was very happy when you decided to join our lab for a Ph.D. after your internship. The dedication and resilience you show on the football field translates to the lab, and it's been a pleasure to have you around these years.

Dylan, you joined our lab relatively late during my Ph.D. but I cannot imagine the lab without you anymore. You are an amazingly funny person, and your (often dark) humor brought a lot of joy to many days at the lab. I have thoroughly enjoyed practicing playing different instruments together over the past year and I am looking forward to many more Dungeons & Dragons sessions in the future!

Jairo, cariño, you are such a warm and fun person to be around. When I first met you, you were a ray of sunshine and you've continued to brighten every day that I have seen you. It is so inspiring to see how you manage to combine a job at the department with successfully completing every course of your studies. It is something you definitely should be proud of. Thank you for all your kind words when I needed them, I truly appreciate it.

Edwin, when we first met, you told me about your amazing singing voice, and how lucky we were that you would join our department. Half of that turned out to be true. Your unique sense of humor always made me laugh way too much, even if I didn't want to. Your enthusiasm made organizing lab activities and arranging goodbye videos so much more fun. I truly appreciate your commitment to 100% being you. Thank you for often arranging trips to watch Fc Utrecht lose at the Galgenwaard, and for introducing me to the Friday PubPopQuiz. You have been a joy to have around.

Estefanía, I am really happy you joined our department. Your positive energy and infectious smile brighten up the department. Your knowledge of flow cytometry has been experiment-saving on many occasions, and I am very grateful for that. The positive energy you bring to any situation is incredibly appreciated. Every time I see turtles at the Griftpark, they remind me of talking to you, and that makes walking through the park even better.

Ger, thank you for all your help with our flow cytometry experiments. It was a pleasure to work with you, and thank you for improving many lunches with Monty Python quote and great stories about gardening and playing the trumpet in the big band. I hope you thoroughly enjoy your well-deserved retirement.

Nathalie, thank you for not only being a friend in the lab but also outside of it. From going to football matches and weaponized Mariah Carey to going for drinks and walks around Utrecht, I am really happy we stayed in touch after you left the department. Thank you for your support and motivating talks, and we should really plan that run soon.

Miriam, you joined our lab only briefly, but I am very happy you did. Your determination is inspiring. I enjoyed all our coffee/tea moments over the years and hope that there are many yet to come.

Sergio, even though you visited our lab for only 8 months, it was a real pleasure to get to know you. Thank you for showing me around Tenerife. I wish you all the best for your Ph.D. and I hope we run into each other again soon!

The general atmosphere at the lab made me enjoy the Ph.D. so much. **Alice**, **Andreja**, **Bart**, **Cécile**, **Daphne**, **Ilaria**, **Kitty**, **Lena**, **Lindert**, **Marja**, **Peter**, **Robin**, **Tabitha**, **Victor**, thank you for everything.

Over the years, I had the opportunity to supervise some wonderful students. **Lisa**, **Heleen**, **Bonne**, **Billy** & **Hester**, I am sure you will all do great things in the future! **Hanako**, **Pepijn**, **Stef**, **Tom**, and all other students who visited our department over the past years, you were a pleasure to have around, and I hope we run into each other again in the future.

Another thing that made this Ph.D. special to me was the opportunity to collaborate with other scientists. **Jerry**, thank you for sharing your expertise on peptide synthesis, and your willingness to help out during our experiments. I wish you all the best during your postdoc in Japan. **Johanna**, you have been a wonderful person to work with. It is nice to share knowledge on different parts of the nanoparticle field and share Ph.D.-related challenges. Good luck in your future endeavors. **Enrico**, thank you for many lively discussions. You have been a pleasure to collaborate with.

For help and support during animal studies, I would like to thank **Trudy** & **Anja** from the Gemeenschappelijk Dieren Laboratorium. Your thorough care for the mice ensured the best possible living conditions for them. Thank you!

My days always started off great due to the incredible people at the reception/security. **Gerda**, **Jolanda**, **Doreen**, **Augusto**, and all other security guards/reception colleagues, thank you.

Jan, **Pieter-Bas**, and colleagues at the postal department, thank you for your never-lacking positivity when delivering packages to our floor. Your enthusiastic energy never failed to put a smile on my face.

I would also like to thank the cleaning staff that kept our department clean over the past years. You are very much appreciated.

During my Ph.D. I had the pleasure of contributing to several committees. I would like to thank the Veterinary Ph.D. Council of the Faculty of Veterinary Medicine and the Graduate School of Life Sciences Ph.D. Retreat Committee for valuable experiences. **Alain**, **Anne-Marie**, **Debbie**, **Katrien**, **Mathijs**, **Michelle**, **Saskia**, **Theo**, and **Wieke**, thank you for being such inspiring members of the Equality, Diversity and Inclusivity (EDI) committee of the Faculty of Veterinary Medicine. It's been a great pleasure to work with you all and I look forward to seeing the great things you will achieve in the EDI field at the Faculty.

In my spare time, I had the great honor of improving my teaching skills by being a Dutch teacher through the UU-International Neighbor Group (ING). **Pablo**, **Eduardo**, **Lena**, and all other participants, thank you for being such wonderful students.

I have thoroughly enjoyed teaching you. **Femke**, **Bettina**, **Erna**, **Paula**, and my fellow UU-ING board members, thank you for all you do for this organization. Your work is important to more people than you might realize.

Of course, not only my colleagues and friends at the immunology department made this Ph.D. such a great experience.

Priyanka, you have always been so supportive, even from Australia. I enjoyed our (for me) early morning calls throughout these years. I'll make sure to come visit you soon!

Emma & **Marjolein**, thank you for countless fun adventures in Groningen and throughout the world in the past years. Berlin, Stockholm, and Milan (amongst many), the past 4 years we've visited so many great spots together, and I am sure there are many more places that we will add to our ever-growing list. You're amazing friends and I wish you both all the best in life.

Patrick, **Willem** and **Yvar**, we've known each other for about 15 years now, and I am very grateful that we have remained friends all this time. Living far apart is never easy, but you made sure we always stayed in touch. Of course, our yearly tradition of 'carbid schieten' at Patrick's is always a highlight. Thank you for your support these past years and I really appreciate all of you.

Tjeu, you always know exactly what to say to make me laugh. Thank you for always being such a positive influence in my life.

Josephine & **Wouter**, thank you for all the welcome distractions over the year. Whether it was a trip to Giethoorn, climbing in the woods, or just having some tea in the garden, I could always count on you to do something fun. You are the best parents to sweet **Jonas**.

Nina, thank you for everything. We've been friends for over half our lives now, and I am very grateful for that. You were always available for a call, and I loved our catch-ups. You and **Chiel** have continuously been there for me. Now you have become parents to little **Elias**, and I am sure he will grow up to be just as lovely as the two of you.

Eva, you are an amazing friend. Even though we live far apart, when we hang out it feels like we saw each other just yesterday. I love how we can laugh about the silliest things. Thank you for all the support over the past years!

Nicole & **Jareau**, where do I start thanking you? You are always there for me, and I appreciate that greatly. You are absolute sweethearts. When I first moved to Utrecht, you guys made me feel really at home in this new city and I enjoyed our many dinners and trips these past years. I always feel so welcome with you. Thank you for everything!

Ellen, thank you for always being there for me. We have been on the phone so much these years, and I feel like you know everything that happened throughout my Ph.D.. I appreciate that I was able to share even the smallest things with you, and you always made me feel understood. Thank you for our many calls, support, and encouragement.

Hubert, **Wiebe**, **Charlie**, **Momo**, **Mitzu**, love you.

Mike, talking to you always puts me in a great mood. Whether it is showing off a new haircut or getting into trouble again, you always have something fun to share. Thank you for all your support and many distractions these past years!

Juliët, thank you for always being there for me. You have a talent for getting into the strangest situations, and your stories never cease to amaze me. Even if I think, 'this won't happen to anyone', it probably already has happened to you. Your infectious energy is admirable and inspiring… until we were in Scotland and you had to slide down a muddy mountain in light blue jeans. Nevertheless, you did it with a smile. I am very proud of you and feel very lucky that you are my sister. Love you!

Pap & **Mam**, I am extremely grateful for everything. I love how your humor, creativity, and resourcefulness can make even the simplest situations into something fun, which definitely helped me these past years. Your never-ending support means the world to me and it is truly lovely to know that there always is a place I can call home. You are the best parents. Love you, or as mom would write 'luf joe', and thank you for everything.

Oma Betsie, my best friend and the loveliest grandma anyone could have wished for. You were always there for me, and you have shown me nothing other than unconditional love and happiness my whole life. We went on many fun adventures together, including an incredible weekend in Newcastle. It is truly special to have a drink and laugh with your grandma in a random pub in the UK on a Saturday evening. I miss you so much, every day, and I am also incredibly grateful for all the beautiful moments we shared. I feel indescribably lucky to be able to call you my grandma. You are such an inspiration. This thesis for sure wouldn't have been possible without our daily calls. Love you lieverd!

About the Author

Daniëlle ter Braake was born on March 5th 1995 in Almelo, The Netherlands. She obtained her high school VWO degree at St. Canisius Almelo in 2013. Hereafter, she moved to Groningen to start a bachelor's degree in Biology at the Rijksuniversiteit Groningen. During her research internship, she studied the Tat-pathway translocation of SNAP-tag bound E. coli DH5α proteins OmpA, DmsA and MdoD at the Department of Molecular Bacteriology at the University Medical Centre Groningen (UMCG). During her minor, she happily obtained her qualification for teaching Biology to high school students at Winkler Prins in Veendam. She successfully concluded her Bachelor's Degree with a thesis on 'Oncolytic Adenoviruses' in 2016.

She continued with a Master's degree in Biomedical Sciences at the Rijksuniversiteit Groningen, starting with a research internship at the Department of Cell Biology at the UMCG. This research focussed on assessing a possible role for heat shock protein 70 in DNA damage repair. Wanting to shift her focus to immunology, she moved to England to conduct a second research internship at the University of Liverpool. Here, she studied the role of respiratory viral infection in immune tolerance to Streptococcus pneumoniae, which contributed to a 2020 publication in the Journal of Infection. She finished her Master's degree in 2018, with a thesis entitled 'The Role of Regulatory T Cells in Autoimmune Vasculitis'.

Searching for a Ph.D. position that would combine her interests in immunology and autoimmunity, she found exactly what she was looking for at the Department of Immunology and Infectious Diseases at Utrecht University. Her research focussed on the induction of antigen-specific immune tolerance in mice, specifically through the modulation of dendritic cells. This dendritic cell modulation was achieved through the use of immunomodulators and liposomes that encapsulate both an immuno-modulator and autoantigen. The results of this work are described in this thesis. Besides her scientific work, she enjoyed teaching students of Veterinary Medicine on a variety of immunological topics and put her efforts into making Utrecht University more accessible and inclusive through actively participating in the Equality, Diversity & Inclusivity committee and volunteering as a Dutch teacher through the UU-International Neighbour Group.

She is continuing her work in the field of Immunology with a Postdoc at the department of Immunopathology at Sanquin in Amsterdam. During her postdoc, she will participate in the European ImmuTol consortium and contribute to their aim of developing a novel tolerogenic dendritic cell-based immunotherapy for the treatment of Multiple Sclerosis.