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FACULTEIT GENEESKUNDE EN FARMACIE

Immunogenicity of a mRNA-based vaccine encoding the House Dust Mite allergen Derp1

Master thesis submitted to obtain the degree of Master in Medicine

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Abstract

Background

The prevalence of allergy has strongly increased in the last decades. In Europe, the prevalence of allergic rhinitis was found to be around 25 %. One of the mechanisms contributing to the development of allergy is a T_H1-T_H2 balance that is dominated by T_H2 cells. Because these subsets counteract each other's proliferation and activation, one of the proposed therapies is to tilt this balance in favor of the T_H1 subset.

Aim of the study

To evaluate whether injection of mRNA encoding the house dust mite allergen Derp1 and TriMix in the inguinal lymph node of mice can skew the immune response towards a T_H1-dominated one.

Methods

Eight BALB/c mice were immunized three times at a ten day interval through intranodal injection of mRNA encoding Derp1 or OVA, Firefly luciferase and TriMix. *In vivo* bioluminescence imaging was performed 24 hours after each immunization to evaluate the success of the intranodal injection. Blood samples were taken five and nine days after the last immunization. Spleens were isolated nine days after the last immunization and single cell suspensions were prepared. The presence of IFN-γ producing cells was evaluated with ELISPOT, intracellular staining and ELISA. Secretion of IL-4 was measured with ELISA. Results were analyzed statistically with a Mann-Whitney U test or a Kruskal-Wallis test followed by Dunn's Multiple Comparison Test.

Results

ELISPOT for IFN-γ demonstrated the presence of antigen-specific, IFN-γ producing T-cells in two out of four mice for those immunized with OVA and TriMix and three out of four mice for those immunized with Derp1 and TriMix. Intracellular staining for IFN-γ showed similar results for the OVA-immunized mice, and IFN-γ secreting T-cells were present in one out of four mice immunized with Derp1. ELISA showed a rise in IFN-γ in all the mice, except in one of the OVA-immunized group. ELISA for IL-4 showed that the amount of secreted cytokine was negligible in all mice.

Conclusion

We can conclude that intranodal delivery of mRNA is a feasible route of vaccine administration, induces IFN-γ producing T-cells and does not induce antigen-specific IL-4 producing cells. We can also say that mRNA vaccines encoding antigen and TriMix seem to be able to evoke a T_H1-dominated immune response. Whether this vaccine will be applicable in the clinical practice, depends on the results of further investigations.

Key words: *Allergy, Derp1, mRNA, TriMix, Intranodal*

List of abbreviations

APC: Antigen-presenting cell

ARIA: Allergic Rhinitis and its Impact on Asthma

BLI: Bioluminescence imaging

CCL2: Chemokine (C-C motif) ligand 2

DC: Dendritic cell

Derp1: Dermatophagoides pteronyssinus group 1

ECRHS: European Community Respiratory Health Survey

eGFP: Enhanced Green Fluorescent Protein

ELISA: Enzyme-Linked Immuno Sorbent Assay

ELISPOT: Enzyme-Linked ImmunoSpot assay

FITC: Fluorescein isothiocyanate

FLT3 ligand: Fms-like tyrosinase kinase 3 ligand

GM-CSF: Granulocyte/Macrophage colony-stimulating factor

HDM: House dust mite

ICS: Intracellular staining

IFN-γ: Interferon-γ

Ig: Immunoglobulin

IL-4/5/8/10/13: Interleukin-4/5/8/10/13

ILIT: Intralymphatic immunotherapy

INI: Intranodal injection

ISAAC: International Study of Asthma and Allergies in Childhood

LPS: Lipopolysaccharide

MACS: Magnetically activated cell sorting

NHANES: National Health and Nutrition Examination Survey

OVA: Ovalbumin

PGD2: Prostaglandin D2

QOL: Quality of life

RAST: Radio-allergosorbent test

SFU: Spot forming units

TAA: Tumor-associated antigen

TCR: T-cell receptor

TGF-β: Transforming growth factor β

T_H1/2: T-helper 1 or T-helper 2 cell

TLR: Toll-like receptor

TNF-α: Tumor necrosis factor-α

Treg: Regulatory T-cell

SCIT: Subcutaneous immunotherapy

SIT: Specific immunotherapy

SLIT: Sublingual immunotherapy

SPT: Skin prick test

TNF-α: Tumor necrosis factor-α

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Conflict of interest

The use of dendritic cells electroporated with tumor-associated antigen mRNA and TriMix is the topic of a patent (W2009/034172) on which Dr. A. Bonehill and Prof. Dr. K. Thielemans are filed as inventors. None of the authors receive any support or remuneration related to this platform. There are no further potential conflicts of interest.

Background

1. What is allergy?

Allergy is an abnormal adaptive immune response directed against non-infectious environmental substances, so-called allergens.[1] Allergy can be seen clinically as an allergic rhinitis, allergic asthma, food allergies, contact dermatitis, etc.[1] The type I, type II and type III hypersensitivity reactions are antibody-mediated, while the type IV hypersensitivity reaction is independent of immunoglobulins (Ig).[2]

As we are going to work in the context of house dust mite (HDM) allergy, we will take a closer look at the type I, IgE-dependent allergic reaction. Type I or immediate hypersensitivity is a reaction mediated by allergen-specific IgEs, which are bound to mast cells, waiting for the allergen to come.[2] When this occurs, they activate the mast cells to release their mediators (*e.g.* histamine). This starts an inflammatory reaction with activation of eosinophils, neutrophils and basophils through different kinds of cytokines.[2] These reactions occur quickly after the exposure to the allergen. An example of a type I hypersensitivity reaction is allergic rhinitis.[2]

1.1 Phases of an allergic reaction

Before one can have an allergic reaction, sensitization has to occur. The allergen reaches the local dendritic cells (DCs), which take it up and migrate to the regional lymph node. Here, the DCs present an allergen-derived peptide to the naïve $CD4^+$ T-cells, via the MHC class II-system. These T-cells can develop to T-helper type 2 (T_H2) cells upon recognition of the peptide/MHC II complexes by their T-cell receptor (TCR).[1] This is enhanced by Jagged-Notch interactions between the DCs and the naïve T-cells, as well as by IL-4, which is produced by activated T-cells and further by T_H2 cells.[1,2] In this way, the T_H2 cells enhance their own proliferation and differentiation. The T_H2 cells will interact with local B-cells, through CD40 ligand-CD40 and CD28-CD80/CD86 interactions.[1] IL-4 and IL-13 produced by the T_H2 cells will co-stimulate this interaction, which will make the B-cells undergo an Ig class-switch recombination.[1] In this way, the B-cells will produce allergen-specific IgE. These Igs spread through the body and will bind to a high-affinity receptor (FcεRI) for IgE on the tissue-resident mast cells.[1] Consequently the mast cells are sensitized when the host is later re-exposed to this allergen.

The accumulation of mast cells in the airway mucosa is an important event in allergic rhinitis due to pollen, as inhalation of allergens impacts the mucosa of the nose.[3] There is also infiltration of eosinophils, $CD4^+$ T-cells and Langerhans-like ($CD1^+$) cells during the pollen season.[3] In this case, the IgE-production takes place in the nasal mucosa itself.[3] During and after the pollen season, the allergen drives the switch to the IgE class. This is why there is a persistent IgE synthesis in the nasal mucosa during and just after the pollen season.[3] Patients with HDM allergy will show less nasal eosinophilia, and will not always have a rise in number of mast cells in the nasal mucosa.[3]

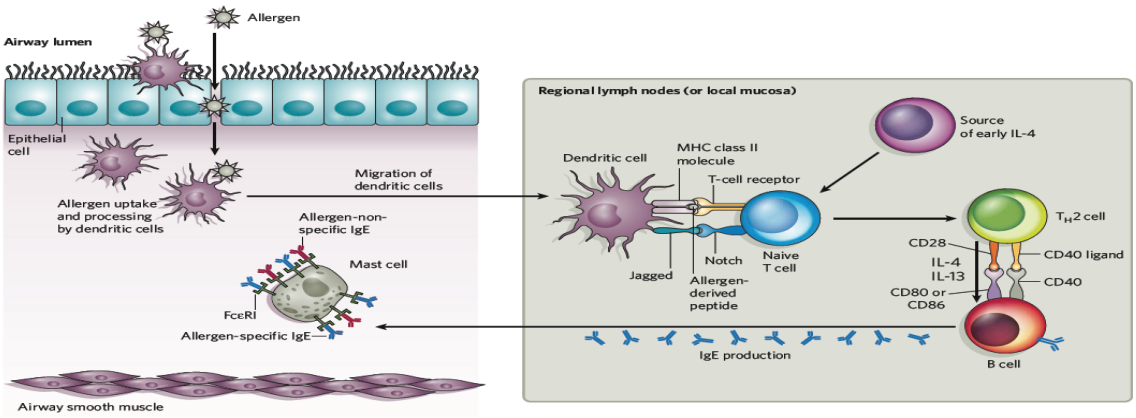

Figure 1: Sensitization is the first step in the development of an allergic reaction.[1] Local DCs take up the allergen and transport it to the regional lymph nodes, where they present the allergen to naïve T-cells, through MHC II-TCR interaction. This stimulates the T-cells to develop into T_H2 cells, which interact with local B-cells, causing an immunoglobulin class-switch: the B-cells now produce allergen-specific IgE. The IgE spreads through the body and binds to mast cells in the tissues, waiting for a second exposure to the specific allergen.

After one is sensitized, a new exposure to the allergen will cause an allergic reaction. Such an allergic reaction consists of different phases: the early phase, the late phase and the chronic allergic inflammation.

The early phase occurs within minutes after allergen exposure. The allergen binds the FcεRI-bound IgE, which causes cross-linking of these Igs and aggregation of FcεRI.[1] This activates mast cells to release various enzymes, cytokines and growth factors. These substances can cause different signs and symptoms, according to the site of the reaction. It can be seen locally as erythema, edema, an increased mucus production or coughing.[1] In some cases, an anaphylactic reaction occurs. Mast cells also release proteases like tryptase and chymase, which lead to tissue damage. They also recruit leukocytes (T-cells, neutrophils, eosinophils, basophils) by secreting chemotactic mediators and by up-regulating adhesion molecules on vascular endothelial cells. This can be considered as the transition to the late-phase reaction.[1]

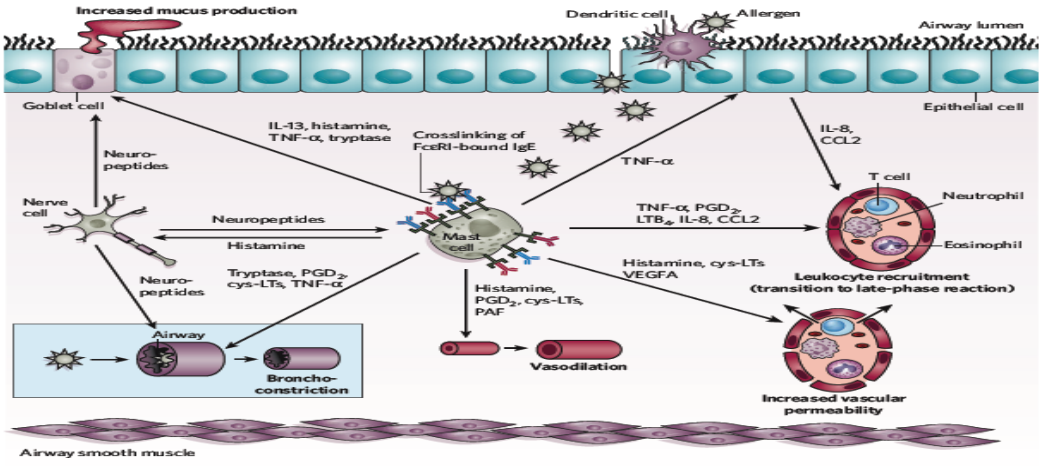

Figure 2: Early phase reaction of allergic inflammation.[1] The allergen binds IgE attached to the mast cells, which causes a degranulation and release of their mediators. Histamine causes symptoms through an increased vascular permeability, vasodilatation, stimulation of nociceptors and an increased mucus production. TNF-α, PGD_2, IL-8 and CCL2 cause a recruitment of leukocytes (T_H2 cells, neutrophils, basophils and eosinophils), a step that initiates the transition to the late-phase reaction.

The late phase usually develops 2-6 hours after allergen exposure, peaks after 6-9 hours and resolves in 1-2 days.[1] It can be seen as redness, pain, edema and warmth of the skin, or as airway narrowing if the symptoms are respiratory. During the late phase, the mast cells continue to release mediators: IL-8 and TNF-α still recruit and stimulate neutrophils, which in turn release tissue destroying elastase. The mast cells also release cytokines contributing to a favorable environment for the inflammatory cells. They release prostaglandins, leukotrienes and platelet-activating factor. All these mediators are released in a more gentle way than during the early phase of allergy.

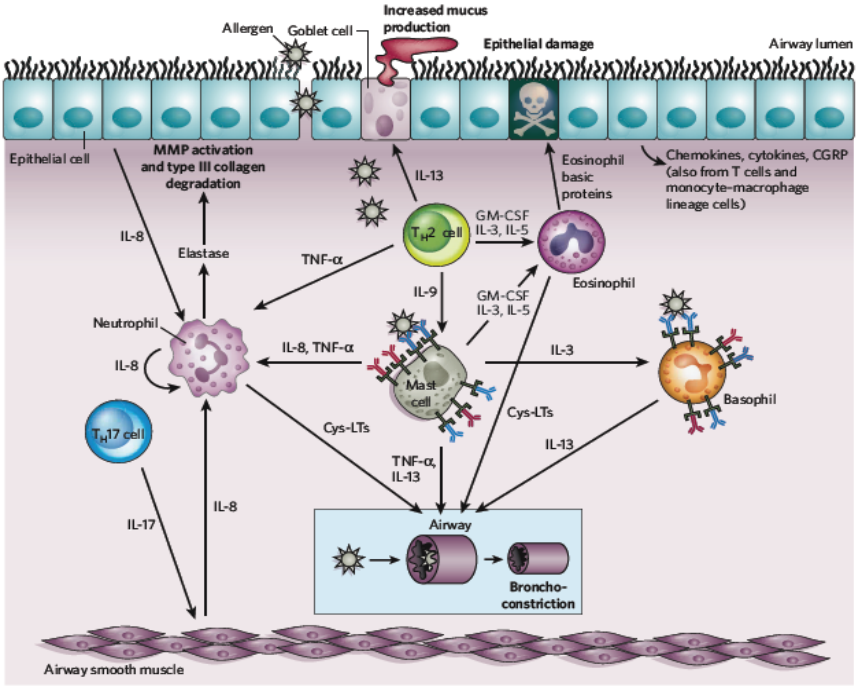

Figure 3: Late phase reaction of allergic inflammation.[1] The clinical effects in this phase are caused by both innate and adaptive immune cells that have been recruited in the early phase reaction, as well as by tissue-resident T-cells and mast cells. Neutrophils release elastase and eosinophils secrete basic proteins, which will both damage the airway epithelium. T_H2 cells maintain the inflammatory environment and induce mucus production at the respiratory epithelium. Basophils and T_H17 cells contribute to airway broncho-constriction. The mast cells still release IL-8 and TNF-α, as well as cytokines contributing to a favorable environment for the inflammatory cells.

If the patient is continuously or repetitively exposed to the allergen, he will develop a chronic allergic inflammation. The tissues undergo structural changes, and the affected site is infiltrated with a large amount of immune cells.[1] In patients with allergic rhinitis, epithelial damage of the nasal mucosa is only minimal. This damage is more pronounced in the bronchi of patients suffering from allergic asthma.

1.2. Immunologic players in allergy

In general, inflammatory reactions can be dominated by T_H1 cells or by T_H2 cells. Other T-cells are present when inflammation occurs, so this T_H1-T_H2 model may be somewhat simplistic, but it's a good model to get a global idea of the most important players in allergy. The environment of cytokines early in the differentiation of T-cells determines in which direction T-cells will evolve. Each T-cell subset cross-regulates the other one. So when an immune response goes into one direction, this response will further be polarized.[2]

IL-4 is the predominant cytokine in allergy, secreted by mast cells, basophils and T_H2 $CD4^+$ T-cells.[2] By producing IL-4, the T_H2 cells stimulate their own maintenance. They also produce IL-5 and IL-13, which activates the mast cells and eosinophils, and stimulates the B-cells to produce IgE.[2] The IL-4-induced signal transduction cascade in the T helper cells also leads to silencing of their IFN-γ expression, which would shift the T_H1-T_H2 balance rather to a T_H1-dominated one.[4]

When an infection with a virus or bacteria occurs, IL-12 is secreted by the activated macrophages and the DCs, which causes the helper T-cells to become T_H1 cells.[2] These cells produce IFN-γ, which further promotes the development of T_H1 cells and inhibits the proliferation of T_H2 cells.[2] IFN-γ is also an important marker to measure the T_H1 response, as it is not secreted by T_H2 cells.[2] Because of this cytokine, macrophages are activated, and there is more IgG production, especially IgG2a.[2]

CD4+ T-cell	Signature cytokines	Immune reactions
T_H1 cells	IFN-γ	Macrophage activation IgG2a production
T_H2 cells	IL-4	IgE production, mast cell activation Isotype switching of B-cells to IgE and IgG1
	IL-13	Mucosal secretions
	IL-5	Eosinophil activation

Table 1: Helper T-cell subsets, their secreted cytokines and their effect.

1.3 Hygiene hypothesis

The prevalence of allergy has strongly increased in the last decades. According to the hygiene hypothesis, this originates from the decreasing incidence of infections due to a more hygienic lifestyle.[5] Underlying mechanisms are complex and involve various T-cell subsets and Toll-like receptor (TLR) stimulation. The first presented theory was a T_H1-T_H2 deviation. Microbial infections normally favour a strong T_H1-biased immunity. Given the reciprocal down-regulation of T_H1 and T_H2 cells, the immune response is redirected towards a T_H2 phenotype in countries with a lack of microbial burden.[5] Other theories of underlying mechanisms have been proposed, and the observations leading to the hygiene hypothesis are probably the result of a sum of different immunologic events. Of course, genetic factors also play a role in the development of allergy. But in spite of this, the T_H1-T_H2 deviation is a rationale that is still often used in allergology research.

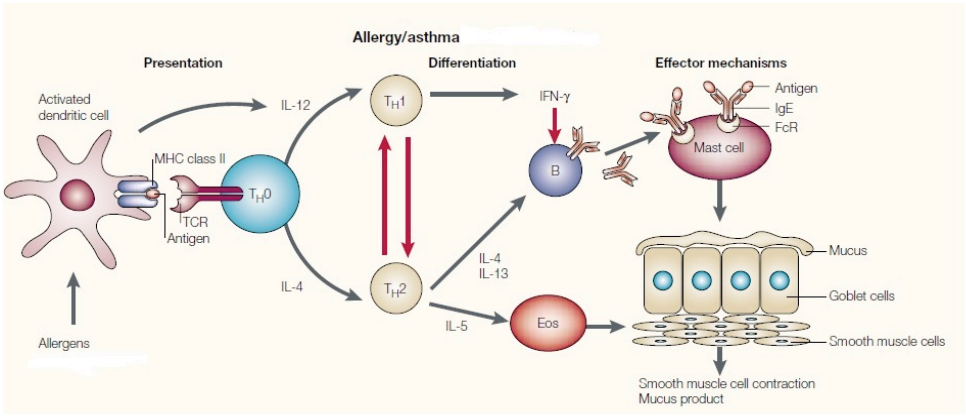

Figure 4: Different T helper cell subsets are induced in different cytokine environments and counteract each other's development. When an antigen is presented in the presence of IL-12, the T_H1 subset is predominant. The T_H2 subset develops in a context of IL-4. Both subsets inhibit each-other's development. The red arrows indicate inhibitory signals.[6]

2. Allergic rhinitis: Definition, prevalence, impact on quality of life and current therapies

2.1. Definition

Allergic rhinitis is a disease in which the patient can present with rhinorrhea, post-nasal drip, bilateral nasal obstruction, nasal itching and sneezing.[3] Mild hyposmia and ocular symptoms can also occur. The allergy can present with less typical symptoms like snoring, sleep problems or chronic cough due to post-nasal drip. Causing allergens can be pollen, HDM, epithelial cells from domestic animals, etc.

Nasal endoscopy, skin prick tests (SPT) to specific allergens or measuring allergen-specific IgE in the blood should be done to distinguish patients with allergic rhinitis from those with non-allergic rhinitis. In the end, the diagnosis is based upon the concordance between a typical history of allergic symptoms and the results of supplementary diagnostic tests.

Intermittent Symptoms are present: < 4 days/week or < 4 consecutive weeks	**Persistent** Symptoms are present: > 4 days/ week and > 4 consecutive weeks
Mild None of the following are present: Sleep disturbance Impairment of daily activities, leisure and/or sport Impairment of school or work The symptoms are present but not troublesome	**Moderate/Severe** One or more of the following are present: Sleep disturbance Impairment of daily activities, leisure and/or sport Impairment of school or work Troublesome symptoms

Table 2: Classification of allergic rhinitis according to ARIA.[3]

Allergic rhinitis is divided into "intermittent" or "persistent" disease. According to the ARIA (Allergic Rhinitis and its impact on Asthma), the severity of the disease can be classified as "mild" or "moderate to severe" (Table 2).[3]

2.2. Prevalence

Allergic rhinitis and allergic asthma are often studied together, as one can be associated with the other. The prevalence of allergic asthma and rhinitis has been investigated by many national and multinational studies like ISAAC I and III, NHANES and ECRHS. They investigated whether the prevalence of serum IgE, positive SPT, as well as symptoms of allergic asthma and rhinitis have increased.

An increased serum allergen-specific IgE or a positive SPT is prevalent in 40 % to 50 % of the population of Europe, USA and Australia - New Zealand.[2, 3] The most frequently detected allergen in Europe is grass pollen (52 % of patients with allergic rhinitis), followed by HDM (49 %).[8] In Europe, the prevalence of allergic rhinitis was found to be around 25 %[7,8], the highest prevalence being in Belgium at 29 %.

It is clear that allergic rhinitis is a very common disease in westernized, developed countries, and that it also becomes more common in developing countries. However, more studies on the epidemiology of allergic rhinitis are required, to have a clearer view on the spread of this modern disease as well as to provide useful information for the interpretation of immunologic abnormalities associated with allergic diseases.

2.3. Impact on quality of life

The general impact of allergy on the quality of life (QOL) is not negligible. It can cause significant fatigue and mood changes, sleep disturbances, impairment of cognitive function, and even depression or anxiety.[3] The fatigue can be caused by sleep disturbances, but also by sedating oral H1-antihistamines. Because of more work absenteeism as well as reduction in work productivity, the economic cost of this disease also has a substantial impact.[3] These impairments are particularly present in patients with moderate to severe symptoms.

We can conclude that allergic rhinitis is a major chronic respiratory disease due to its prevalence, impact on quality of life and work/school performance, economic impact and links with asthma.

2.4. Current therapies

The management of allergic rhinitis comprises of patient education towards avoidance of the allergen, symptomatic pharmacotherapy and allergen-specific immunotherapy (SIT).[3] Hygienic measures to avoid contact with the allergen have variable success. With pharmacotherapy, only the symptoms but not the cause is treated. As soon as the patient stops taking his medication, the symptoms often re-occur. This is why allergen-SIT is an interesting alternative for most patients, as allergen-SIT aims to educate the immune system to react differently to allergens.

3. Current methods for allergen-specific immunotherapy

Allergen-SIT is currently the only allergy-therapy that provides long-term relief of symptoms.

3.1. SIT in clinical practice

Allergen-extracts are injected subcutaneously (SCIT, subcutaneous immunotherapy) or administered sublingually (SLIT, sublingual immunotherapy) in increasing doses. In SCIT the vaccines are given weekly for 8-16 weeks during an up-dosing phase, followed by monthly maintenance injections for a period of three to five years.[9] This method can give satisfying results if the patient has one or two IgE-mediated allergies.[9]

Immediate local or systemic reactions should be recorded after each injection, and the presence of any delayed local or systemic reaction should be checked at each visit. There is a risk of developing a systemic allergic reaction, anaphylactic shock or even death after the subcutaneous injection. This is why the patient has to stay in the clinic for at least thirty minutes following it. When this problem occurs, adrenaline should be given immediately.

Risk factors for developing such reactions are the presence of bronchial asthma, a history of previous systemic reactions, co-seasonal allergen exposure or an extremely high sensitivity.[9,10]

Indications	Contra-indications
Limited spectrum of allergies	Asthma
Positive SPT or RAST to the allergen	Therapy with β-blockers
Unsatisfying results from drug treatment	Pregnancy
Impossibility to avoid the allergen	Small children
	Incompliant patient

Table 3: Indications and contra-indications for allergen-SIT.[9]

3.2. Immunologic mechanism of allergen-specific immunotherapy

This method has already been used for more than 100 years, but the underlying mechanism has only recently been described. It consists of a complex interaction between the different types of T-cells, as well as B-cells, mast cells, basophils and eosinophils.[10,11]

In SCIT or SLIT the local Langerhans' cells take up the administered allergen and transport it to the draining lymph node. Allergen-specific regulatory T-cells (Treg) are then generated. These Tregs suppress the effects of T_H2 cells, mast cells, basophils and eosinophils. They inhibit inflammatory DCs and stimulate the development of tolerogenic ones. They also diminish the late-phase response, by repressing the mucus production and tissue inflammation in a direct and indirect way. The Tregs inhibit the effect of different subsets of T_H cells to varying degrees. This results in a T_H1-T_H2 balance that is dominated by the T_H1 subset. This is reflected in a rise of IFN-γ and a fall of IL-4.[12]

The composition of antibodies secreted by the B-cells changes during SIT. There is a relatively early and rapid increase in IgG4, which continues to increase for as long as the SIT is administered. IgG4 works as a blocking antibody at the level of the FcεRI-bound IgEs, thus preventing degranulation of mast cells.[11] There is a late decrease in IgE. Due to the rise in IFN-γ, there is more production of IgG2a en thus the ratio IgG2a/IgG1 increases.[2,11]

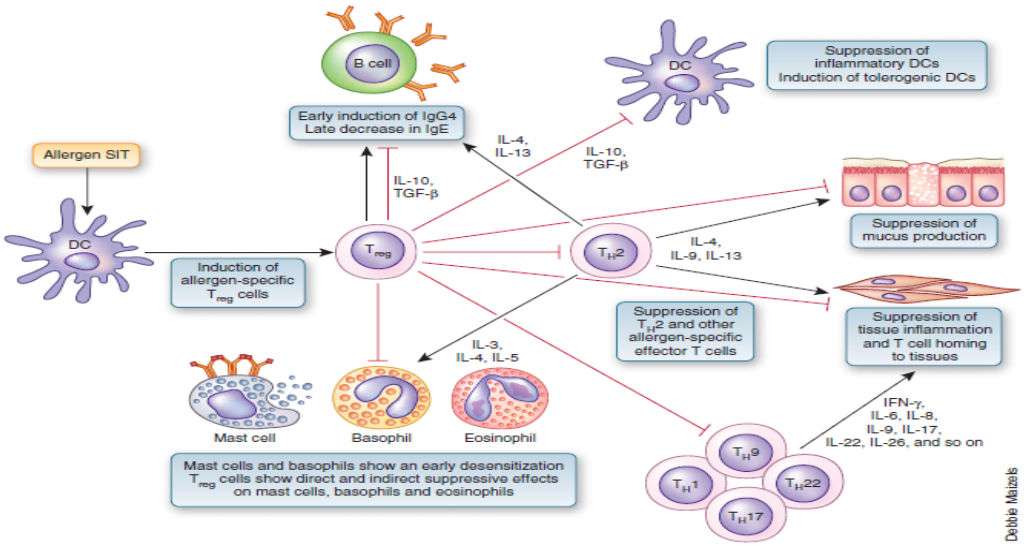

Figure 5: Mechanisms of long-term immune tolerance obtained by treatment with allergen-SIT.[11] Allergen-specific Tregs are induced and inhibit the pro-inflammatory responses by suppressing inflammatory DCs, mast cells, basophils and eosinophils. They inhibit the different subsets of T_H cells in varying degrees, causing a decrease of T_H2-cells and an increase of T_H1 cells. There is also an early increase in IgG4 and a late decrease in IgE.

3.3. Other immunomodulating strategies in allergy

Other strategies have been evaluated to modulate the immune response in allergic disease, all with the same goal: decreasing the T_H2-response that leads to allergic inflammation. This has been attempted by blocking critical T_H2-cytokines with antagonists against IL-4, IL-13, IL-9 and IL-4 receptor.[11,13] Instead of blocking the cytokines, one could block their synthesis. This has been tried with Suplatast, PPAR-γ agonists and OX40 Ligand antagonist, with varying degrees of success.[13] Another strategy was to block critical T_H2 effector molecules, with a monoclonal antibody against IgE (Omalizumab), a CD23 antagonist or a PGD_2 receptor antagonist.[13] One could also inhibit the mast cells or the eosinophils. The last attempted strategy was to stimulate the T_H1-response.[13] This is achieved by administering TLR-agonists.[13] We are going to use a strategy where we enhance the T_H1-response by giving a vaccine that stimulates DCs to present allergens in an MHC-I context. The rationale behind this strategy is that the T_H1 and T_H2 subset counteract each other's proliferation and activation. By tilting this balance in favor of the T_H1 subset, we want to obtain a decrease in the T_H2 subset. We can see this shift towards the T_H1 subset in patients who completed SLIT.[14]

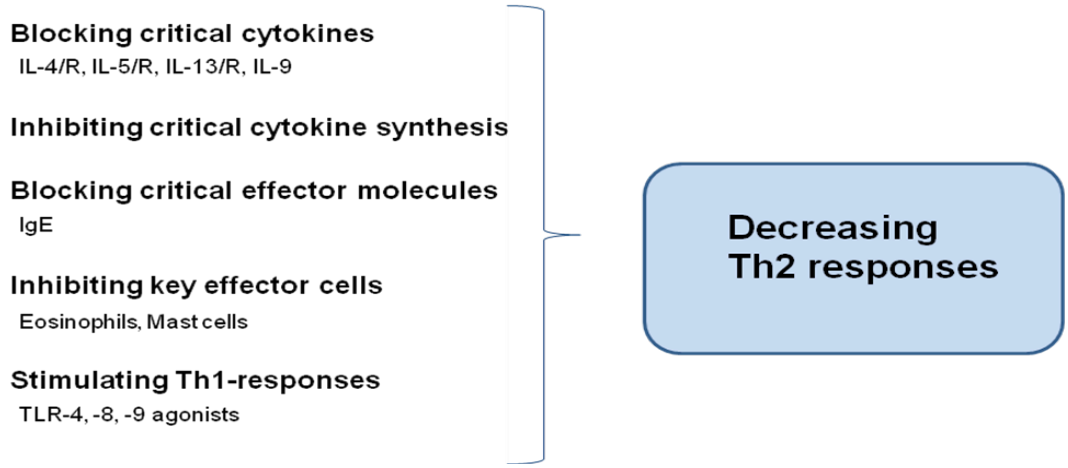

Figure 6: Strategies of immune modulation in allergic diseases (adapted from 13).

4. mRNA vaccines

Allergen-SIT is a time-consuming therapy, sometimes with disappointing long-term results. In addition to that, it is a therapeutic intervention that is only given to patients after their childhood. It would be good to have a prophylactic vaccine, which is less cumbersome to the patient. Another option is to find a vaccine that can be added to the classic allergen-SIT. A mRNA-based vaccine could be a good option to accomplish these ideas.

In the last few decades, interest is growing in the use of mRNA vaccines, especially in the field of anticancer therapy. It is a logical evolution, if we take into account the many advantages that mRNA has to offer.

In theory, any cell type can be modified with mRNA vaccines. The mRNA does not need to cross the nuclear barrier, thus making it possible to use in slowly dividing cells like DCs, who are critical players in the priming of an effective immune response.[15,16] Because the mRNA encodes the entire antigen, all of its epitopes can be presented by the DC.[15] By transfecting mRNA in these cells – *in vitro or in vivo* – one can generate all proteins of interest, and consequently obtain presentation of different types of antigenic peptides. The mRNA could contain sequences encoding proteins that influence the pathway of antigen-presentation, *e.g.* to ensure the antigen is presented in an MHC class I as well as class II context. If wanted, one could also stimulate the production of specific co-stimulatory ligands or cytokines. This means that we can influence the three signals required for T-cell differentiation: first the interaction peptide/MHC – TCR, second the interaction between co-stimulatory signals, and third the release of cytokines responsible for T-cell polarization.[16]

It is clear that these advantages can also be subscribed to DNA vaccines or vaccines using viral vectors. But mRNA vaccines are safer, as mRNA does not integrate in the genome, which could cause mutations.[15] Due to RNAses in the tissues, mRNA has a shorter half-life than the above-mentioned.[15] This results in a more controlled antigen exposure.[16] Because there are no additional sequences – *e.g.* a plasmid – immune responses are elicited only against the encoded antigen, so there is no risk of induction of pathogenic auto-antibodies.[16] Because mRNA vaccines have these features, they are not classified by the FDA as 'Gene therapy'.[16] The mRNA can be produced without using potentially problematic materials, such as animal-derived proteins.[15] The safety of the mRNA vaccine against HDM allergy has to be one of our primary concerns, because we don't want to induce life-threatening diseases by giving a therapy that is above all meant to increase the QOL.

Last but not least, mRNAs are easily produced at low cost and can be easily modified.[15] They are namely transcribed from plasmid DNA-templates by bacteriophages RNA-polymerases.

To deliver mRNA, it can be transfected into DCs *in vitro*, after which these DCs are given to the patient as a vaccine. This method of delivery was the first one established.[15] Another way to deliver the mRNA is by direct injection of the 'naked mRNA' into the body. In this way, antigen-presenting cells (APC) are modified *in situ*. Several preclinical trials on a variety of mouse models, as well as clinical trials have been performed on this subject.[15,16,17,18]

The effect of immunization *in vivo* also depends on the adjuvants within the mRNA vaccine. One of the adjuvants tested is TriMix, a mix of three mRNA molecules encoding two activation stimuli CD40 Ligand (CD40L) and a constituvely active form of TLR4 (caTLR4) as well as the co-stimulatory molecule CD70. TriMix partially mimics the events that occur in a normal cellular immune response: after presentation of antigen by the DCs to the T-cells, the latter express CD40L. This ligand binds CD40 expressed by DCs, which leads to higher expression of CD80 and CD86 on DCs and stimulates their secretion of IL-12. CD70 is a co-stimulatory molecule. caTLR4 is an active form of TLR4, which normally recognizes microbial products like lipopolysaccharide (LPS) and activates the DCs. All these elements get the DCs in the right functional status and enhance the T-cell proliferation and differentiation.

Van Lint *et al*. compared the efficacy of antigen-specific T-cell stimulation by DCs modified either *ex vivo* or *in vivo* with mRNA encoding an antigen and TriMix.[17] They showed that immunization with antigen and TriMix mRNA *in vivo* was as efficient as immunization with DCs electroporated with antigen and TriMix mRNA. They further showed that immunization of mice with ovalbumin (OVA) mRNA and TriMix resulted in an enhanced stimulation of OVA-specific T-cells when compared to mice immunized with OVA mRNA alone or combined with LPS. DCs from lymph nodes co-injected with TriMix also showed a higher expression of CD40, CD80 and CD86 than DCs from lymph nodes injected with mRNA and/or LPS.

There are different possible routes to deliver mRNA, *e.g.* subcutaneously, intradermally or intranodally. When mRNA is delivered intradermally, a major fraction is rapidly degraded. The remaining mRNA is taken up by somatic bystander cells or dermal DCs and Langerhans' cells. The DCs take up the mRNA and transport it to the draining lymph node, where the peripheral DCs can exchange their antigenic load with lymph node-resident DCs. This cross-presentation and cross-dressing is a critical event in the expansion of functional T-cells in response to the antigen. It has been demonstrated that intranodal delivery of mRNA results in the direct modification of lymph node-resident DCs, and the induction of functional antigen-specific T-cell responses.

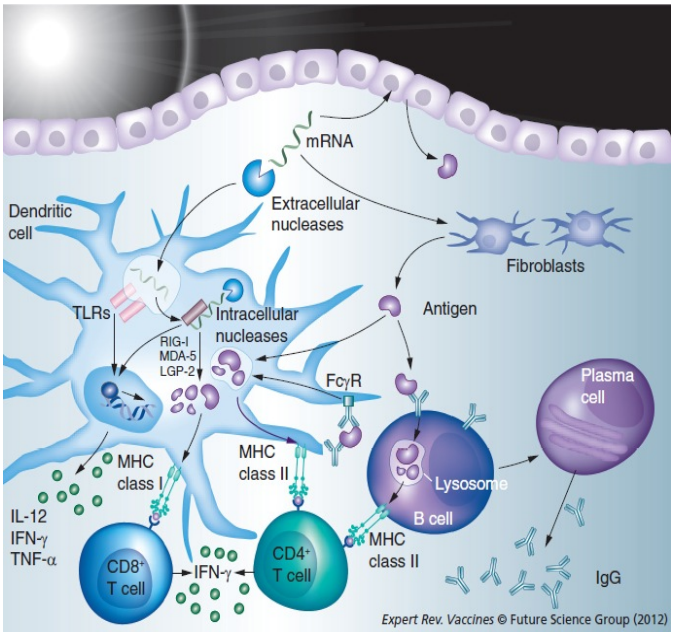

Figure 7: Events induced by intradermal injection of naked mRNA.[8] A fraction of the injected mRNA is degraded by extracellular nucleases. The remaining mRNA is taken up by APCs and somatic cells like fibroblasts. This results in an increased secretion of IL-12, IFN-α and TNF-α, which generate an inflammatory environment. The protein translated from the mRNA is presented by the APCs in MHC I molecules. Antigen from transfected somatic cells can be acquired by APCs and presented in MHC II molecules. Thus, there is presentation to $CD8^+$ and $CD4^+$ T-cells, respectively. These T-cell subsets can both contribute to a T_H1 cytokine environment. B-cells can also be activated by free antigen, leading to their differentiation into IgG-secreting and memory B cells. The B cells can also serve as APCs and further potentiate the immune response.

It is clear that DCs play a major role in the induction of an immune response against the antigen encoded by the mRNA. That is why the subject can get a pre-treatment with granulocyte macrophage-colony stimulating factor (GM-CSF) or with Fms-like tyrosinase kinase 3 (FLT3) ligand, before the mRNA is injected.[17] These factors enhance the number of DCs, thus enhancing the mRNA-induced immune response. Van Lint *et al.* compared the induction of $CD8^+$ T cells in mice pretreated with GM-CSF after intradermal or intranodal delivery of OVA and TriMix mRNA, demonstrating the superiority of intranodal vaccine delivery.[17]

There has been no research yet for the intranodal administration of antigen-encoding mRNA as a treatment of allergy. However, there has been research for the intranodal administration of allergen, which gave better results than the subcutaneous administration of it.

5. Intranodal allergen administration

To be able to elicit an immune response, an antigen must come in contact with enough B- and T-lymphocytes to find those cells that recognize it. The highest concentrations of T- and B-cells can be found in the secondary lymphoid organs, like the spleen and the lymph nodes.

The classic route of administration in allergen-SIT is by subcutaneous injections or sublingual drops. But if this allergen has to reach the lymph node to be able to elicit an immune response, why not inject the allergen directly into it?

This is what Senti *et al.* investigated.[19] They compared the efficacy and safety of intranodally injected allergen in humans, to that of a subcutaneously injected allergen vaccine. The long-lasting effects on allergy were comparable, but the group who received intranodal injections already had an improvement in their nasal provocation test after 4 months, while the group who received subcutaneous injections only had a better test after 1 year. This shorter treatment time was beneficial for the compliance in the group with intranodal injections. The pain from an intranodal injection was less than that of a venous puncture. Also, this administration route showed less allergic side effects.

Hylander *et al.* compared the intranodal administration of ALK Alutard (containing 1000 SQ-U birch pollen or grass pollen) to the intranodal administration of placebo in humans.[20] In this way, they evaluated the effects and side-effects of intralymphatic allergen-administration. They found that this method required only a few injections (3 intralymphatic injections of 0.1 ml ALK Alutard over a period of 12 weeks) to induce a reduction in IgE levels 3 months after completion of the ILIT, instead of 3 years in the case of SCIT. The total number of serum leukocytes and the leukocyte formula did not change during the course of the treatment.

In patients treated with ALK Alutard, the nasal lavage fluid contained a significantly lower number of living leukocytes, compared to the placebo group. IL-8, the most abundant inflammatory cytokine in the nose of allergic individuals, was also decreased in the ALK Alutard-group. This possibly reflects a decline in the local nasal inflammation. The nasal symptoms after allergen challenge were diminished in the actively treated group, with a significant difference compared to the placebo group. The actively treated patients also reported less seasonal allergic symptoms than before the start of the ILIT. This improvement was not seen in the placebo group. Only minor local reactions were observed after allergen administration, and none of the subjects suffered from a severe or systemic allergic event, in contrast to up to 30 % of the conventional SCIT-treated patients. The intranodal injection did not cause any major discomfort, which is in line with the notion that the sensory innervations of the lymph nodes are sparse. Furthermore, because ILIT requires less treatment visits, fewer injections and lower allergen doses, it is far less expensive than the classic SCIT.

By reviewing studies about SCIT versus ILIT with different types of vaccines (RNA, DNA, oligopeptides, proteins or live virus vaccines), Senti *et al.* found that the enhanced effect of ILIT inversely correlates with the pharmacokinetics of the vaccine (Table 4).[12] They found a difference in response between intranodally injected mRNA encoding lymphocytic choriomeningitis virus and the same vaccine injected subcutaneously, which is expected as subcutaneous injection of RNA results in part in its degradation by RNAses.

Type of vaccine	Dose required (SCIT/ILIT)	Stability *in vivo* and drainage to lymph node
Naked RNA	$> 10^6$	-
Oligopeptide	10^6	+
Naked DNA	10^4	++
Protein	10^3	+++
Live virus	1	++++

Table 4: The enhancing effect of intralymphatic immunotherapy inversely correlates with pharmacokinetics of the vaccine.[12]

Senti *et al.* found that the responses of the adaptive immune system are higher with intranodal injection than after subcutaneous administration. The responses of IgG2a antibodies were 10 to 20 times higher with only 0.1 % of the allergen dose.[9] There was also more secretion of IFN-γ, IL-2 and IL-10.[12] But this rise in IL-10 secretion could not be found in all studies. Hylander *et al.* could not show this increase by administering ALK Alutard (containing birch pollen or grass pollen) intranodally.[20] This difference could be explained by the use of different types of allergen extracts for immunization.

Aim of the thesis

Today, allergen-SIT is the only strategy available to induce allergen-specific unresponsiveness. However, SIT is not optimal as it is not always effective and carries certain risks. Thus, new strategies are needed to treat allergies. A possible strategy to prevent the development of allergy or to improve SIT is to deliver the allergen together with immune modulating stimuli to APCs in such a way that these APCs drive a T_H1-skewed allergen-specific T-cell response.

Therefore, the overall goal of the project is to investigate the effect of a novel mRNA-based allergy vaccine. In particular, a vaccine consisting of mRNA encoding Derp1 (Dermatophagoides pteronyssinus group 1) as well as mRNA encoding CD40L, caTLR4 and CD70, together referred to as TriMix, will be studied.

The hypothesis is that upon administration the mRNA-based vaccine is taken up by DCs after which the mRNA-encoded proteins dictate how the DC presents the allergen and how the DC signals to T-cells. It is predicted that a T_H1-response is evoked. Skewing the allergy response away from the typical allergic T_H2-type is believed to represent an improved strategy for the treatment of allergy.

Therefore, we will address the following question:

Does intranodal delivery of a mRNA vaccine encoding the allergen Derp1 and TriMix skew the immune response towards a T_H1-dominated one?

Materials and methods

1. Mice

Female, 6 to 12 week-old naïve BALB/c mice were purchased from Charles River (L'arbresle Cedex, France). Animals were treated according to the European guidelines for animal experimentation. Experiments were reviewed by the Ethical committee for use of laboratory animals of the "Vrije Universiteit Brussel" (VUB, Jette, Belgium; CEP 10-214-2).

2. Production and intranodal delivery of mRNA

Production. The vector, pST1 was provided by U. Sahin (Johannes- Gutenberg University, Mainz, Germany). The vectors pGEM-li80tOVA, pST1-caTLR4, pST1-mouse CD40L, pST1-mouse CD70 and pST1-FLuc have been described previously.[21] The sequence encoding Derp1 was purchased from GeneArt (Life Technologies, Ghent, Belgium) and cloned into the pST1-vector using the restriction sites *Nco*I-*Xho*I. Before *in vitro* transcription, pGEM and pST1 vectors were linearized with *Spe*I and *Sap*I, respectively. All enzymes were purchased from Fermentas (Vilnius, Lithuania). *In vitro* transcription was carried out as described by Van Meirvenne et al.[22] The mRNA was dissolved in phosphate buffered saline (PBS, Sigma-Aldrich, Diegem, Belgium), Ca^{2+}-containing Hank's balanced salt solution (HBSS, Lonza, Verviers, Belgium), or 0.8 Ringer lactate (0.8 RL; Baxter, Braine-l'Alleud, Belgium).

Intranodal delivery. Mice were anesthetized with ketamine (70 mg/kg; Ceva, Brussels, Belgium) and xylazine (10 mg/kg; Bayer, Diegem, Belgium). The inguinal lymph node was surgically exposed and injected with the mRNA, after which the wound was closed with Vicryl rapide 4.0 (Ethicon, Johnson & Johnson, Beerse, Belgium). Mice received 1 µg Firefly Luciferase (FLuc) mRNA, 10 µg antigen mRNA and 15 µg TriMix mRNA (5 µg/component).

mRNA electroporation. Spleens were isolated and reduced to single cell suspensions. These were washed twice with PBS and twice with OptiMEM (Invitrogen, Life Technologies). Subsequently, the splenocytes were resuspended at 20 x 10^6 cells in 200 µl OptiMEM containing 1 µg eGFP mRNA and 10 µg OVA or Derp1 mRNA. The cell suspension was transferred to a 4-mm cuvette and electroporated with a square-wave pulse (300 V and 500 µs) in a Genepulser (XCell, Bio-Rad, Nazareth Eke, Belgium). Immediately after electroporation, the cells were transferred to complete medium (CM), which consists of RPMI 1640 medium (Sigma-Aldrich) supplemented with 5% Foetal Clone I serum (FCI, Harlan, Horst, the Netherlands), 50 µmol/l β-mercaptoethanol and 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich).

3. *In vivo* bioluminescence imaging

Mice were imaged 24 hours post mRNA injection following the procedure described in Keyaerts *et al*.[23] After a brief sedation induction with 2% isoflurane anesthesia (VelTech Solutions, UK), mice were injected intraperitoneally with the substrate D-luciferin (Xenogen, Almeda, CA) at a weight-dependent substrate dose of 150 mg/kg. Seven minutes after substrate administration, mice were imaged using the Photon Imager (Biospace, France). Mice were placed inside the bioluminescence imaging camera. Using a nose cone, volatile anesthetics were administered during the entire acquisition. The photon emission was measured dynamically during 10 minutes. At the end of each acquisition, a photographic image was obtained. Bioluminescent pseudocolor images displayed in the figures are shown superimposed on gray-scale photographic images of the mice, with the most intense detected FLuc signal shown as red and the weakest signal shown as blue.

4. Collection of sera

Fifteen minutes prior to blood collection, mice were placed under a red-light warming lamp. Subsequently, mice were immobilized in a constrainer through which the tail was protruding. The tail was cut, allowing collection of approximately 250 µl of blood in non-heparinized tubes. This blood was left undisturbed at room temperature for approximately 30 minutes, after which it was centrifuged at 1500 g for 10 minutes. The serum was immediately transferred to new tubes and frozen at -20 °C.

5. Detection of IFN-γ producing cells in ELISPOT

Enzyme-linked immunospot (ELISPOT) plates (Millipore) were coated overnight at 4°C with 100 µl purified anti-IFN-γ antibodies (BD Pharmingen). Wells were washed with 100 µl PBS, and then blocked with 100 µl Blocking buffer (10 % FCI in RPMI). A total of 2 x 10^5 MACS sorted $CD8^+$ or $CD4^+$ splenocytes (Miltenyi, Bergish-Gladbach, Germany) or bulk splenocytes was plated per well in 100 µl CM (in duplicate). These were stimulated with 2 x 10^4 syngeneic splenocytes that were electroporated with 1 µg eGFP and 10 µg OVA or Derp1 mRNA. Splenocytes activated using anti-CD3/anti-CD28 beads (Invitrogen) or Concanavalin A (Sigma-Aldrich) served as a positive control. Splenocytes that were not stimulated, or splenocytes from OVA-injected mice stimulated with Derp1-electroporated splenocytes and *vice versa* served as a negative control (Figure 8).

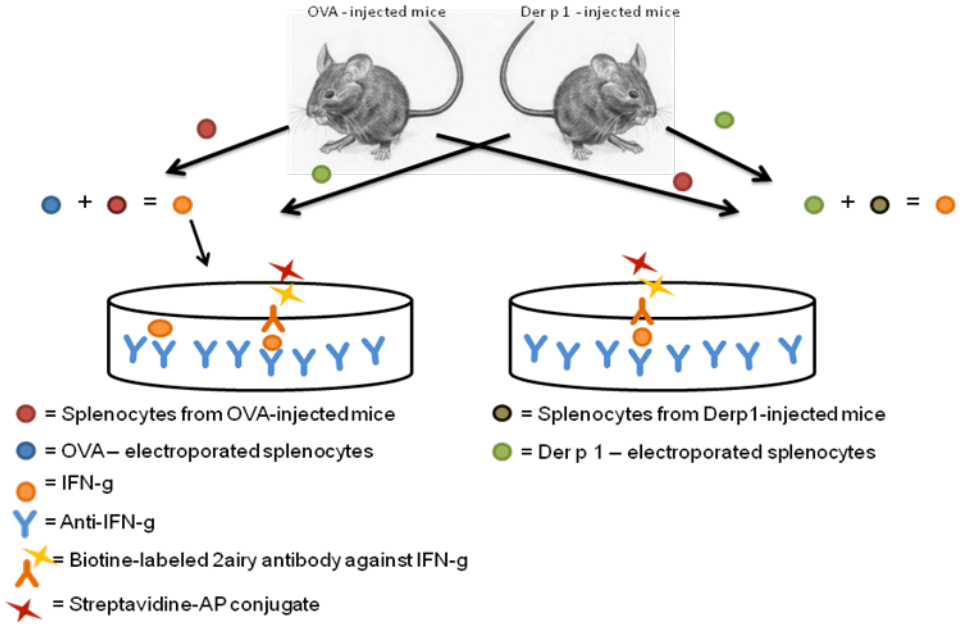

Figure 8: Schematic representation of the ELISPOT used to detect IFN-γ secretion by murine splenocytes. Splenocytes from OVA-injected mice were incubated with stimulating OVA-electroporated splenocytes. In the negative control wells, they were co-cultured with Derp1-electroporated splenocytes or medium only. The positive control consisted of splenocytes from immunized mice stimulated with anti-CD3/anti-CD28 beads or Concanavalin A. The same set-up was used for the splenocytes of Derp1-immunized mice. Visualisation of IFN-γ was obtained with biotin-labeled secondary antibodies.

Co-cultures were kept at 37°C, 5% CO_2 in a humidified atmosphere for 24 or 72 hours, after which the ELISPOT plates were developed according to the manufacturer's instructions (Diaclone, Besançon, France). Spots were counted using an ELISPOT counter (Autoimmun Diagnostika GmbH, Straβberg, Germany) and software (Autoimmun Diagnostika ELISPOT Reader 5.0).

6. Flow cytometry

Electroporation efficiency. Expression of eGFP was measured 4 hours after mRNA electroporation of splenocytes. Non-modified splenocytes were used as controls. Samples were acquired using the FACSCanto flow cytometer (BD Biosciences, Ermebodegem, Belgium), while fluorescence analysis was performed using FACSDiva software (BD Biosciences).

Intracellular cytokine staining. Co-cultures identical to those described in section 5: "Detection of IFN-γ producing cells in ELISPOT" were set up in U-bottom 96 well culture plates. These co-cultures were performed in the presence of 2 µg/ml Brefeldin A for 16 hours. Intracellular staining of IFN-γ was performed using the BD Cytofix/Cytoperm^{TM} kit and the following antibodies: Alexa Fluor 647-conjugated anti-CD3 (ImTec Diagnostics, Antwerp, Belgium), PerCP-Cy5.5-conjugated anti-CD4 (ImTec Diagnostics), FITC-conjugated anti-CD8 (eBioscience, Vienna, Austria) and PE-Cy7A-conjugated anti-IFN-γ antibodies (BD Biosciences). Samples were acquired using the Fortessa LSR flow cytometer (BD Biosciences), while fluorescence analysis was performed using FACSDiva software (BD Biosciences)

7. Detection of IFN-γ and IL-4 in ELISA

Co-cultures identical to those described in section 5: "Detection of IFN-γ producing cells in ELISPOT" were set up in U-bottom 96 well culture plates. Supernatants were collected 24 hours later and tested in a sandwich ELISA for the presence of IL-4 (eBioscience) and IFN-γ (eBioscience). The IL-4 and IFN-γ ELISA was performed according to the manufacturer's instructions.

8. Statistical analysis

Experimental data of experiment 1 and 3 were analyzed using the Mann-Whitney U test and GraphPad Prism software. Experimental data of experiment 2 were analyzed using the Kruskal-Wallis test and the post hoc Dunn's Multiple Comparison Test. P values < 0.05 were considered as significant. Throughout the manuscript, data are presented in scatter plots in which each dot represents the result of one mouse. The horizontal line depicts the median. The number of mice used in the experiments are 3 to 4 per vaccination group.

Results

1. Intranodal delivery of mRNA is a feasible route of vaccine administration

It has been suggested that immunotherapeutics capable of inducing T_H1 or Treg cells could provide a valuable means of preventing allergy or shifting the T_H2-biased immune response to a non-pathogenic reaction. Indeed, vaccines consisting of allergen encoding mRNA or DNA have been successfully employed for prophylactic immunization against type I allergy in mouse models, demonstrating induction of T_H1 or Treg cells.[8] Moreover, a first DNA vaccine has recently demonstrated its safety in a phase I clinical trial.[14] Since it was recently shown that delivery of antigen mRNA together with TriMix mRNA into the lymph node results in an enhanced induction of T_H1 cells[17], we set out to evaluate this strategy in the context of allergy (Figure 9).

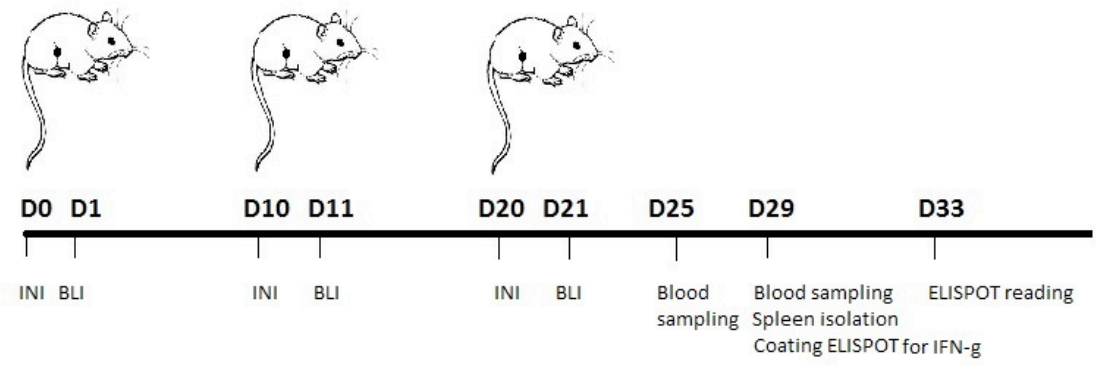

Figure 9: Schematic representation of the experimental design of experiment 1. Mice received mRNA encoding Fluc, TriMix and antigen on day 0, 10 and 20. *In vivo* BLI was performed 24 hours after each mRNA injection to evaluate proper delivery of the mRNA to the lymph node. On day 25 and 29, we collected sera and froze them for future experiments. After the last blood collection (day 29), mice were sacrificed and spleens isolated. The presence of IFN-γ producing cells in the splenocyte population was analyzed upon *in vitro* re-stimulation of the cells using ELISPOT. Abbreviations: INI = Intranodal injection. BLI = Bioluminescence imaging.

As shown in Figure 9, the inguinal lymph nodes of mice were injected with TriMix and antigen mRNA on day 0, 10 and 20. In addition, we co-delivered Fluc mRNA, as this enabled us to evaluate the success of the intranodal injection using *in vivo* bioluminescence imaging (BLI). The results of the *in vivo* BLI demonstrate expression of the enzyme Fluc, 24 hours after injection of Fluc mRNA (Figure 10), in 45 out of 60 injections (75% success rate) (Table 5). In addition, these results demonstrate that all mice – except two in each experiment – received at least two successful mRNA injections (Table 5).

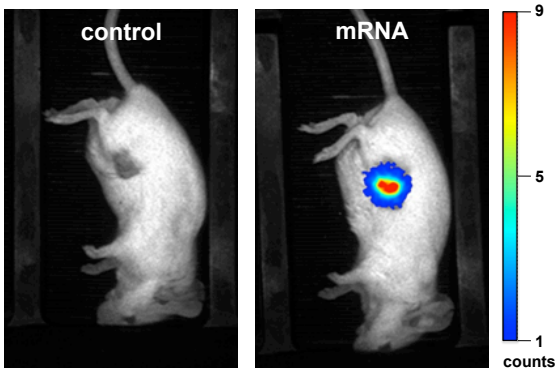

Figure 10: Intranodal delivery of mRNA is a feasible route of vaccine administration. Mice received an intranodal injection with Fluc, TriMix and antigen mRNA. Expression of Fluc was evaluated 24 hours after mRNA injection using *in vivo* BLI. The photo's show a mouse that did not receive Fluc mRNA (control) and a mouse that received Fluc, TriMix and antigen mRNA. These are representative for the 60 injections that were performed.

	Experiment 1						Experiment 2				Experiment 3					
	Derp1			OVA			OVA		OVA+ TriMix		Derp1			OVA		
Injection n°	1	2	3	1	2	3	1	2	1	2	1	2	3	1	2	3
Mouse n°																
1	+	+	-	-	+	+	+	+	+	+	+	+	+	+	-	+
2	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+
3	-	-	+	+	+	+	-	+	+	-	-	+	-	+	+	-
4	+	+	+	-	-	-	ND	ND	ND	ND	-	+	+	+	-	-

Table 5: Intranodal delivery of mRNA is a feasible route of vaccine administration. The table summarizes the results of the *in vivo* BLI. Abbreviations: ND, not done.

2. Intranodal delivery of antigen and TriMix mRNA induces IFN-γ producing T-cells

The rationale behind the intranodal delivery of antigen and TriMix mRNA is to induce a T_H1-skewed immune response. In order to investigate this, we evaluated the presence of IFN-γ producing cells in the splenocyte population of immunized mice. The antigens used for immunization were the allergen Derp1 and the model antigen OVA. The latter served as a control, since it was previously shown in the host laboratory that intranodal delivery of OVA either or not combined with TriMix mRNA resulted in the induction of IFN-γ producing $CD4^+$ T-cells as well as IFN-γ producing $CD8^+$ T-cells.[17]

In the first experiment, immunized mice were sacrificed ten days after the last mRNA injection. Spleens were isolated and single cell suspensions prepared. Subsequently, $CD8^+$ T-cells were sorted by magnetically activated cell sorting (MACS) to high purity (Figure 11A). These cells (2×10^5) were re-stimulated *in vitro* for three days to evaluate the specific production of IFN-γ in ELISPOT. As stimulator cells, we used splenocytes of syngeneic animals that were electroporated with eGFP and OVA mRNA, or eGFP and Derp1 mRNA.

The inclusion of eGFP allowed us to evaluate the electroporation efficiency by flow cytometry before addition of the stimulator cells (2 x 10^4) to the responder cells (= $CD8^+$ sorted cells from immunized mice). Flow cytometric analysis demonstrated that about 30 % of the splenocytes electroporated with eGFP and OVA mRNA, or eGFP and Derp1 mRNA were modified (Figure 11B). Of note, sorted $CD8^+$ T-cells were stimulated with anti-CD3/anti-CD28 antibody coated beads as a positive control. As negative controls the $CD8^+$ T-cells were either not stimulated, or the $CD8^+$ T-cells of OVA immunized mice were re-stimulated with Derp1 presenting cells, whereas the $CD8^+$ T-cells of Derp1 immunized mice were re-stimulated with OVA presenting cells (Figure 8). In this experiment, antigen-specific IFN-γ producing $CD8^+$ T-cells could only be detected in one out of four mice both in the OVA immunized group as well as in the Derp1 immunized group (360 and 535 spot forming units [SFU] per 10^6 cells, respectively) (data not shown).

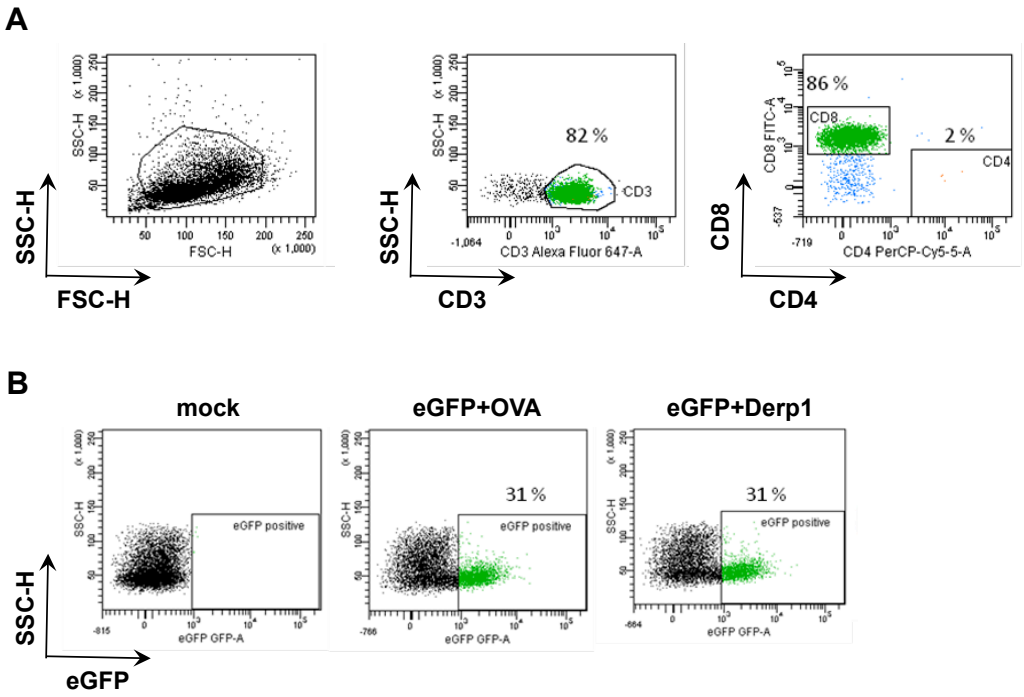

Figure 11: Flow cytometric analysis of the responder (A) and stimulator cells (B). (A) $CD8^+$ T-cells were sorted from the splenocytes of immunized mice. The cells were stained for CD3, CD4 and CD8. After acquisition the data were analyzed. The graphs depict the scatter characteristics (FSC-H/SSC-H, left), $CD3^+$ cells (middle) and $CD4^+$ versus $CD8^+$ cells within the $CD3^+$ population (right). More than 80 % of the spleen cells were $CD3^+$. From these cells more than 80 % were $CD8^+$ T-cells. The graphs are representative for the results obtained for the 8 immunized mice. **(B)** Splenocytes of syngeneic mice were electroporated with eGFP and antigen mRNA. Four hours later we evaluated the expression of eGFP in flow cytometry. The graphs depict the expression of eGFP by cells electroporated without addition of mRNA (mock) and cells electroporated with eGFP and OVA mRNA (eGFP+OVA) or eGFP and Derp1 mRNA (eGFP+Derp1). 31 % of the electroporated cells were positive for eGFP, in the OVA-group as well as in the Derp1-group.

Since Van Lint *et al.* previously demonstrated the expansion of OVA-specific T-cells upon intranodal delivery of OVA and TriMix mRNA[17], we expected to detect antigen-specific IFN-γ producing $CD8^+$ T-cells in all immunized mice. However, Van Lint *et al.* used different assays to demonstrate the presence of OVA-specific T-cells, amongst which a $CD4^+$ and $CD8^+$ T-cell proliferation assay and *in vivo* cytotoxicity assay. In addition, the ELISPOT is not yet fully optimized in the host laboratory. Therefore, we questioned whether technical issues might be at the basis of the negative results. Therefore, we decided in a second experiment to only immunize mice with OVA and TriMix mRNA. For the sake of time, we only performed two immunizations. Ten days after the last immunization, the ELISPOT assay was performed using optimized conditions (Figure 12).

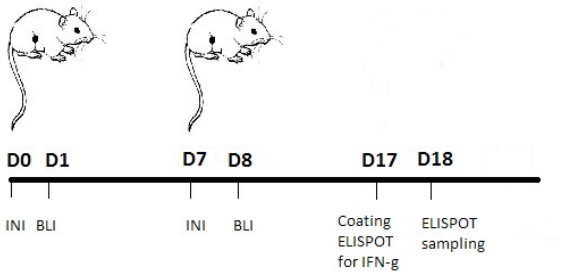

Figure 12: Schematic representation of the experimental design of experiment 2. Mice received mRNA encoding OVA, TriMix and Fluc on day 0 and day 7. *In vivo* BLI was performed 24 hours after each mRNA injection to evaluate proper delivery of the mRNA to the lymph node. On day 18, we evaluated the presence of IFN-γ producing cells in an ELISPOT. Mice were therefore sacrificed and spleens isolated, after which the splenocytes were restimulated and incubated in ELISPOT plates. The ELISPOT was analyzed 24 hours after the start of this co-culture. Abbreviations: INI = Intranodal injection. BLI = Bioluminescence imaging.

Several changes were made based on the experience of other lab members. Firstly, we used total splenocytes as responder cells rather than sorted $CD8^+$ T-cells. Secondly, we used protein and peptide pulsed syngeneic splenocytes as stimulator cells. Moreover, we titrated the amount of antigen presented to the responder cells by pulsing stimulator cells with 25 or 1 µg/ml OVA protein or 1, 0.25 or 0.1 µg/ml of the immunodominant peptide of OVA, SIINFEKL. Splenocytes that were not stimulated or that were stimulated with concanavalin A served as a negative and positive control, respectively. Thirdly, the ELISPOT was analyzed one day after the start of the co-culture rather than three days later. We observed antigen-specific IFN-γ producing T-cells in all conditions irrespective of the stimulator cells used (Figure 13), leading us to the conclusion that the lack of response in the first experiment is most likely due to the conditions used in the ELISPOT assay.

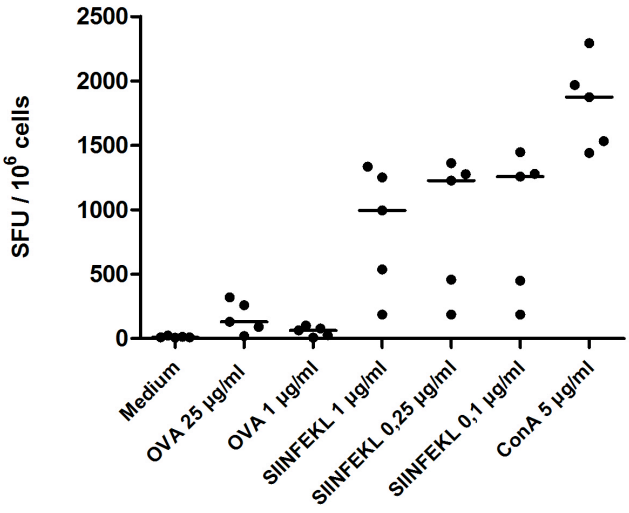

Figure 13: Repeated intranodal delivery of OVA and TriMix mRNA results in the stimulation of IFN-γ producing CD8$^+$ T-cells. Mice were immunized twice at an interval of 7 days through intranodal injection of OVA and TriMix mRNA. Spleens were isolated 10 days after the second immunization, single cell suspensions prepared and re-stimulated with different concentrations of OVA protein or the OVA peptide SIINFEKL in the ELISPOT assay. Injection of OVA mRNA with TriMix resulted in IFN-γ producing CD8$^+$ T-cells, independent of the concentration of the stimulating protein or peptide. Concanavalin A as stimulans was used as a positive control, whilst effector cells incubated with medium only were used as a negative control. The graphs depict the number of SFU and their median per 10^6 cells for each test group. The difference between the negative control and the test samples was not significant (p-value > 0.05).

Based on these results, we repeated the immunizations as described for experiment 1, isolated the spleen ten days later and analyzed the presence of antigen-specific IFN-γ producing T-cells in ELISPOT using the conditions determined in experiment 2 with one modification (Figure 14). As stimulator cells we used syngeneic splenocytes electroporated with eGFP and OVA mRNA, or eGFP and Derp1 mRNA as we did not have Derp1 protein or peptides. In addition, we set up extra co-cultures, a first to analyze the cells in flow cytometry by intracellular staining for IFN-γ, a second to analyze the supernatants for the presence of IFN-γ (as well as IL-4) in ELISA (Figure 14).

The ELISPOT results demonstrated the presence of antigen-specific, IFN-γ producing T-cells in two out of four mice immunized with OVA and TriMix and three out of four mice immunized with Derp1 and TriMix (Figure 15A). The difference between the number of SFU in OVA-injected mice stimulated with OVA and those stimulated with Derp1 or the negative control, was however not statistically significant (Mann-Whitney U test; p values: 0.4 and 0.3 respectively). The same is true for the Derp1-immunized mice. The number of SFU was similar for the OVA-immunized mice and the Derp1-immunized ones.

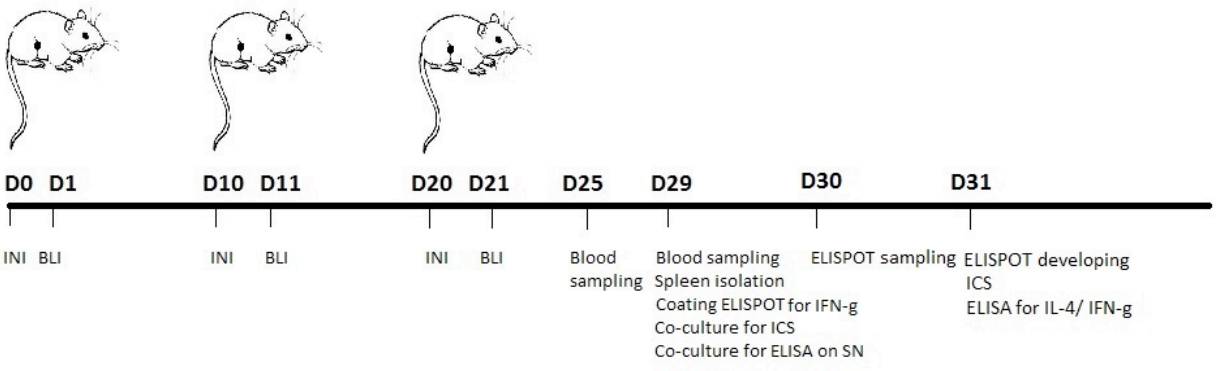

Figure 14: Schematic representation of the experimental design of experiment 3. Mice received mRNA encoding Fluc, TriMix and antigen on day 0, 10 and 20. *In vivo* BLI was performed 24 hours after each mRNA injection to evaluate proper delivery of the mRNA to the lymph node. On day 25 and 29, we collected sera and froze these for future experiments. After the last blood collection (day 29), mice were sacrificed and spleens isolated. The presence of IFN-γ producing cells in the splenocyte population was analyzed upon *in vitro* re-stimulation of the cells using ELISPOT, intracellular cytokine staining and ELISA. Abbreviations: INI = Intranodal injection. BLI = Bioluminescence imaging.

The data for mice immunized with OVA and TriMix were confirmed in flow cytometry. Intracellular staining for IFN-γ demonstrated that IFN-γ was mainly produced by $CD8^+$ T-cells and to a lesser extent by $CD4^+$ T-cells (Figure 15B). In contrast with the ELISPOT, intracellular cytokine staining only revealed IFN-γ producing T-cells in one out of four mice immunized with Derp1 and TriMix (Figure 15B). Although not all mice showed a response using ELISPOT and intracellular cytokine staining as a read out, we observed in ELISA that the supernatants of all co-cultures except one contained high levels of IFN-γ (Figure 15C). These contradicting data need to be clarified in future experiments.

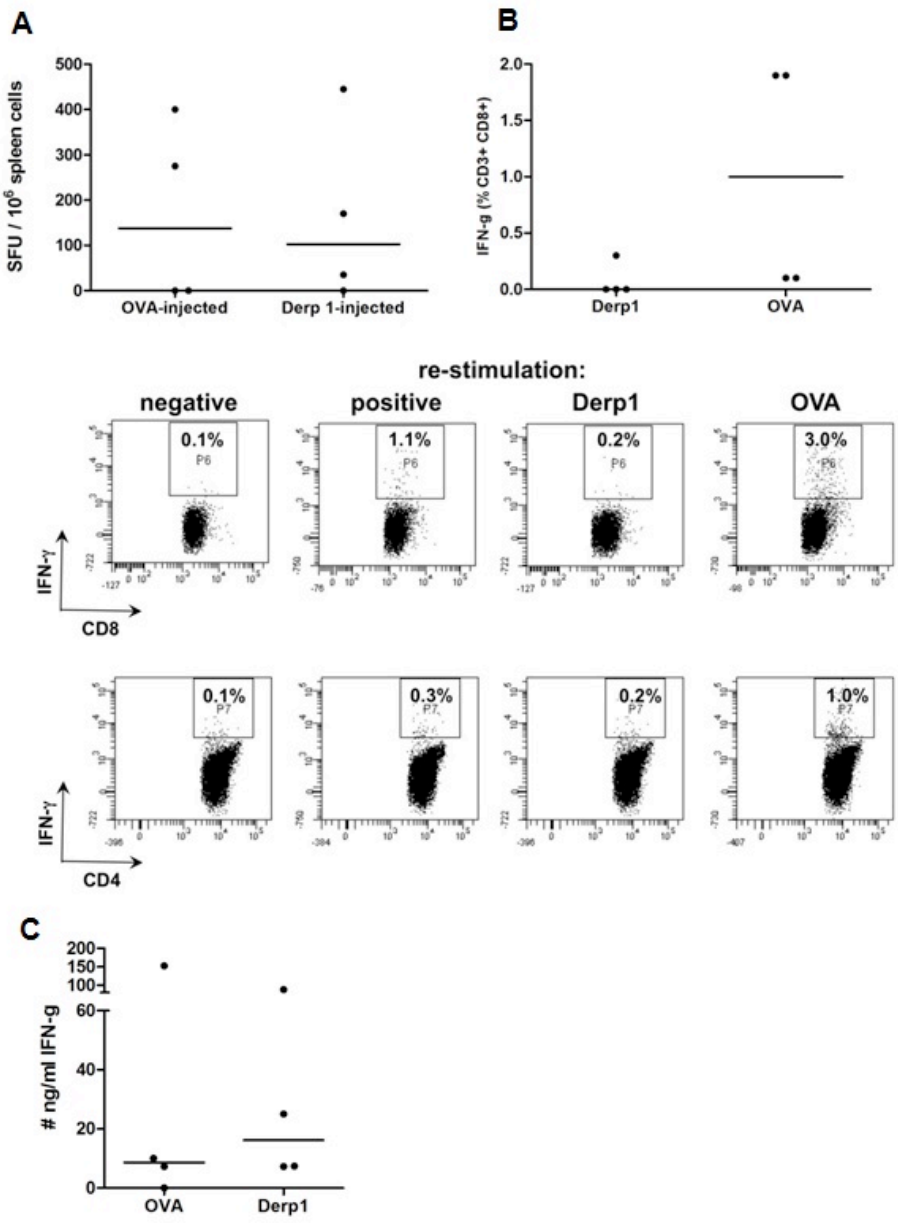

Figure 15: Intranodal delivery of antigen and TriMix mRNA induces IFN-γ producing T-cells. (A) Splenocytes from mice immunized with OVA or Derp1 mRNA were co-cultured in an ELISPOT plate with splenocytes of naïve mice that were electroporated with OVA or Derp1 mRNA. Twenty-four hours later, the number of spots was determined. The graphs show the number of SFU and their median per 10^6 spleen cells. The difference in SFU between OVA-injected and Derp1-injected mice is not significant (p value: 0.8) **(B)** A co-culture of splenocytes from immunized mice and electroporated splenocytes was established for 24 hours, wherein Brefeldin A at a 1/1000 dilution was added for a period of 16 hours. Cells were stained for CD3, CD4 and CD8, after which anti-IFN-γ antibodies were used to detect the intracellular IFN-γ. Cells were analyzed in flow cytometry. The normalized ratio's of IFN-γ for the OVA and the Derp1 injected mice are indicated in the graphs. The difference between the IFN-γ ratio's of OVA-immunized mice and Derp1-immunized mice is not significant (p value: 0.1) **(C)** An ELISA was conducted to further evaluate the amount of secreted IFN-γ. The graph depicts the concentration of secreted IFN-γ (ng/ml) for OVA as well as for Derp1 injected mice. The difference between the IFN-γ secretion of OVA-immunized mice and Derp1-immunized mice was not significant (p value: 0.7)

3. Intranodal delivery of antigen and TriMix mRNA does not induce antigen-specific IL-4 producting cells

IL-4 is the predominant cytokine in an allergic context and in general in inflammatory reactions regulated by the T_H2 subset of T-cells. These T_H2 cells stimulate their own maintenance by further producing IL-4. This cytokine also leads to silencing of the IFN-γ expression. It is thus a cytokine that promotes the development of a T_H2 subset and inhibits that of a T_H1 subset. As immunization of the mice with antigen and TriMix mRNA was developed to induce a T_H1 response, we expect to observe less of T_H2 cells and only low amounts of IL-4.

To evaluate this, we conducted an ELISA on the supernatants of co-cultures of spleen cells from immunized mice and stimulating spleen cells. Supernatants were collected after 24 hours and tested in a sandwich ELISA. We observed that the production of IL-4 was not detectable in three out of four mice in both mice immunized with OVA and TriMix, or Derp1 and TriMix mRNA. Production of low amounts of IL-4 was detected in one out of four mice in both vaccination groups. These amounts were around the lower detection limit of the ELISA (less than 14 pg/ml) (Figure 16). These data indicate that the vaccination strategy did not induce antigen-specific T_H2 cells.

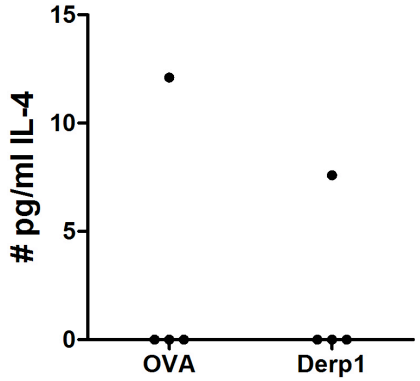

Fig 16: Intranodal delivery of antigen and TriMix mRNA does not induce IL-4 producing T-cells. A sandwich ELISA to measure IL-4 was conducted on spleen cells from immunized mice, after being co-cultured with OVA or Derp1 electroporated splenocytes. The graph depicts the concentrations of secreted IL-4 by the spleen cells of OVA and Derp1 injected mice. The amount of secreted IL-4 in the OVA-immunized mice was not significantly different from that in the Derp1-immunized mice (p-value: 1).

Discussion

Allergen-SIT is currently the only therapy with the promise for a cure for allergy. However it is far from perfect, as it carries certain risks and requires a long-term patient compliance. Furthermore, the obtained results aren't always satisfactory. This is why patients at risk should be able to get a prophylactic vaccine against allergy. Another possible approach is to ameliorate the results of allergen-SIT by co-delivering immune modulating stimuli with the allergen. These stimuli could alter the APCs in such a way that these APCs drive a T_H1-skewed allergen-specific T-cell response. Before such a vaccine can be designed, research is necessary concerning the conveyance of the allergen and the route of administration.

The route of administration of mRNA vaccines can influence their efficacy. When injected directly into the lymph node, mRNA can make contact with a relative high amount of APCs, which in turn can immediately interact with lymphocytes to evoke an immune response. Because mRNA is rapidly degraded by RNAses, the advantage of injecting it directly into the lymph node is even bigger than it is with viruses, proteins, peptides or DNA. *In vivo* bioluminescence imaging 24 hours after intranodal injection of mRNA encoding Fluc showed us that injecting mRNA in murine lymph nodes is a feasible route of administration, with a success rate of 75 % in our hands and 100 % in the hands of experienced researchers.[17, 18] In human subjects, the inguinal lymph node is easily accessible. Ultrasound is already used in the clinical practice to assist fine needle aspiration, and thus could also be used to guide intranodal vaccine injections. Moreover, the pain from an intranodal injection is less than that of a venous puncture.[19] In addition, strategies are under way to deliver mRNA to lymph nodes without the need for intranodal injection. One of these strategies uses gas-filled microbubbles that are currently used in the clinic as a contrast agent. When injected intradermally these migrate to lymph nodes, a process that can be followed by ultrasound-guided imaging. Interestingly, mRNA packaged into lipoplexes can be coupled to these microbubbles and ultrasound can be applied to image these bubbles and furthermore initiate a process that is called sonoporation. During sonoporation the microbubbles implode resulting in shear forces that temporarily damage the cell membrane of surrounding cells. In addition the mRNA is released and as such can immediately enter the cells cytoplasm.[24]

mRNA can theoretically generate all proteins of interest and modify any cell type. We used mRNA encoding Derp1 or OVA. Of note, the genetic code of the antigen was fused to a class II targeting signal (that of DC-LAMP). This ensures that the antigen is not only presented in the MHC I but also in the MHC II pathway, thus activating $CD8^+$ and $CD4^+$ T-cells, respectively. When given with an appropriate adjuvant, mRNA vaccines can induce a T_H1-dominated cellular response, similar to the natural protective response that is acquired in the presence of microbial burden early in life. TriMix, a mixture of three mRNA molecules of which two encode DC activation stimuli, CD40L and caTLR4, and the third encodes a co-stimulatory molecule, CD70 has proven its efficacy as adjuvant in the context of cancer in earlier studies both in a mouse and human setting.[17,25,26] Injecting mRNA along with TriMix offers the advantage that the latter activates DCs without having a negative impact on the bio-availability of the mRNA encoded antigen, in contrast to other adjuvants like LPS.[17] LPS immediately activates the DCs. Consequently, the DCs are no longer able to engulf mRNA as this is performed through macropinocytosis, a process that is rapidly down-regulated upon DC activation. In contrast, the activation stimuli included in TriMix are only expressed after translation. At that time, the antigen mRNA is already engulfed and translated. As such the DCs are optimal equipped for antigen-presentation.

The aim of this study was to investigate whether a T_H1-dominated immune response could be elicited by intranodal delivery of a mRNA vaccine encoding the HDM allergen Derp1 and TriMix. To evaluate this skewing in the T-cell balance, we measured the amount of IFN-γ producing cells with ELISPOT and ICS, as well as the amounts of IFN-γ in ELISA. We also measured the amount of secreted IL-4.

In experiment 1 we performed an ELISPOT where we detected a significant amount of IFN-γ secretion by $CD8^+$ T-cells only in one out of four mice both in the OVA immunized group (360 SFU/10^6 cells) as well as in the Derp1 immunized group (535 SFU/10^6 cells). As the positive control with anti-CD3/anti-CD28 antibody coated beads was clearly positive, this could not be attributed to a failed development of the ELISPOT plate. But a suboptimal protocol or a human mistake during the manipulations of the splenocytes – between the moment of isolating the spleen and the moment of putting the cells in co-culture with their stimulator cells – cannot be excluded.

In search for a more successful protocol, we performed a second experiment where mice were immunized twice with OVA and TriMix mRNA. An ELISPOT assay was performed ten days after the last immunization. In this experiment, we used total splenocytes as effector cells instead of sorted $CD8^+$ T-cells. The stimulating splenocytes were pulsed with protein or peptide instead of with mRNA modified cells. The analysis of the ELISPOT was carried out 24 hours after the start of the co-culture instead of 72 hours. With these modifications, we found IFN-γ producing cells in all conditions. Consequently, we made the following adjustments when repeating the first experiment: we used bulk splenocytes, and developed the ELISPOT 24 hours after setting up the co-cultures between stimulator and effector cells. When using this protocol, we found that two out of four mice immunized with OVA and TriMix and three out of four mice immunized with Derp1 and TriMix demonstrated antigen-specific IFN-γ producing cells. These results confirm that the lack of IFN-γ secreting cells probably resulted from a suboptimal protocol. On separate co-cultures, we performed an ICS for IFN-γ, followed by flow cytometry. This showed us that IFN-γ was mainly produced by $CD8^+$ T-cells. Similar to the results of the ELISPOT, IFN-γ producing $CD8^+$ T-cells were found in two out of four mice immunized with OVA and TriMix. However, only one out of four mice immunized with Derp1 and TriMix showed IFN-γ production on ICS. As ICS has a lower sensitivity than ELISPOT, it is possible that some of the mice were falsely rated as negative.[27] Also, secretion kinetics of individual cells scarcely influence the ELISPOT assay, as the cytokine is continuously captured during the entire incubation of the cells on the plate. In ICS cells are blocked at a supposed ideal time point to measure the amount of intracellular cytokine.[27] It is possible that secretion of IFN-γ for ICS purposes was performed too early or too late. ELISA was also conducted to measure the amount of secreted IFN-γ in supernatants of separate co-cultures. This showed high levels of IFN-γ in all co-cultures except one in the OVA-immunized group. This is a remarkable result, as ELISPOT is at least 200 times more sensitive than ELISA.[27]However, ELISPOT determines how many cells secrete IFN-γ, whilst ELISA provides information on the amount of secreted IFN-γ. It is possible that a modest number of $CD8^+$ T-cells secreted an extensive amount of IFN-γ. Nonetheless, the amounts measured in ELISA were unexpectedly high. The fact that IFN-γ was produced by $CD8^+$ T-cells, suggests that a T_H1-response was induced. The proliferation and activation of $CD8^+$ T-cells is stimulated by the T_H1-subset of $CD4^+$ T-cells. Thus, a high amount of IFN-γ secreting $CD8^+$ T-cells is an indirect sign of a shift of the T_H cells towards the T_H1 phenotype. This is further supported by the absence of IL-4, an indication that the vaccination strategy did not induce a pronounced T_H2-response.

The high amount of IFN-γ detected in the ELISA is a point of concern, as we don't want to induce a too strong T_H1 response. In fact, we probably don't even need to have a very strong response in the context of allergy. Weiss *et al.* remarked that – to develop a prophylactic vaccine – a subtle change in the T_H1-T_H2 balance in the advantage of T_H1 could be enough to prevent sensitization to an allergen.[14] Repeated exposure of the subject to environmental allergens would maintain this T_H1-dominated response. Thus, it is possible that the addition of TriMix to the mRNA vaccine generates a too strong T_H1 response in a context of allergy. It is fair to state that the biggest challenge in the development of an effective allergy vaccine is to generate a T_H1-response that is strong enough to protect from sensitization or to help desensitization, but not that strong that it could provoke adverse events. In this regard, the most feared side effect of a too pronounced T_H1-deviation is to induce autoimmunity.

The effect of IFN-γ on the development of auto-immune diseases is not yet fully understood, because it is a multipotent cytokine with complex biological functions.[28,29] In comparison with the classic immunotherapy, we could consider this mRNA vaccination strategy as safe, because there is less risk of developing severe adverse reactions like anaphylaxis and death.[13] It is although very important not to engender new diseases when treating allergy.

We can conclude that mRNA vaccines encoding antigen and TriMix seem to be able to evoke a T_H1-dominated immune response. However – due to contradictory results in this study – it is unclear yet if this vaccine would be applicable in the prevention or treatment of allergy. More tests should be done to examine the cellular response of these mice. We froze sera of all mice, which makes it possible to perform an ELISA to measure IgG2a, IgG1 and IgE. A protocol for this assay is found in annex. In future experiments, IL-10 and/or TGF-β could also be measured to look at the response of Tregs to the vaccination.

Conclusion

The results of this study are inconclusive and further research needs to be done on this topic. If further studies confirm the utility of this vaccine in a context of allergy, mice immunized with this vaccine should be challenged with the allergen to confirm the effects and side effects of this therapy. Does the vaccination really protect the subject from developing allergy? Is this vaccination therapy safe? Many questions remain on this subject, and future studies will hopefully provide answers. But as G. B. Shaw said: "Science is always wrong. It never solves a problem without creating ten more."

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Annex 1: Detection of immunoglobulins by ELISA

The amount of OVA- or Derp1-specific IgG2a, IgG1 and IgE in the murine sera can be measured using an enzyme-linked immunosorbent assay (ELISA). To that end, ELISA plates are coated over night at 4°C with 100 µl of a 10 µg/ml solution of OVA or Derp1 protein (both purchased from Indoor Biotechnologies, Cardiff, UK). The next day the plates are washed five times using PBS containing 0.05% Tween (Sigma-Aldrich, Diegem, Belgium) after which the non-specific binding sites are blocked for 1 hour using PBS containing 1% bovine serum albumin (BSA, Sigma-Aldrich). Subsequently, the plates are washed five times, after which 50 µl of the serum is added (in duplicate). After two hour of incubation at room temperature, the plates are washed five times and a secondary antibody is added. The secondary antibodies used are: horse-radish peroxidase (HRPO)-conjugated goat anti-murine IgG1 (Serotec, Oxford, UK), HRPO-conjugated rat anti-murine IgG2a (PharMingen, San Diego, CA) or HRPO-conjugated rat anti-mouse IgE (Serotec, Oxford, UK). After two hours, the plates are washed five times and TMB (Tetramethylbenzidine) is added to visualize the bound antibodies. Throughout the assay, antibodies with an IgG1 or IgG2a isotype are used as a positive control. In the negative control wells, we perform the same steps as in the other wells but without addition of serum.

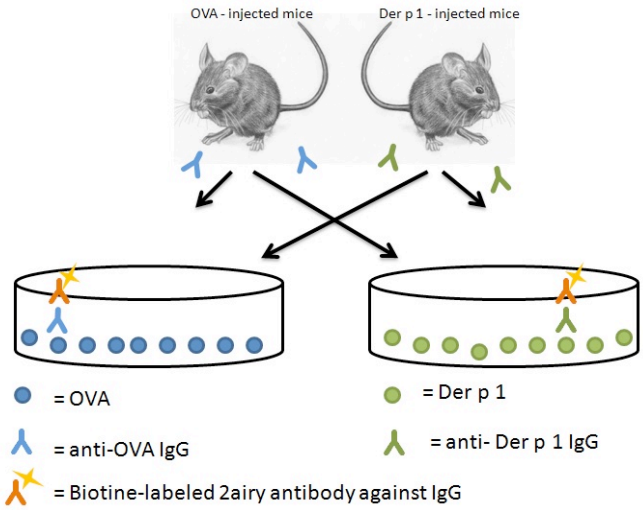

Figure A. Schematic representation of the ELISA used to detect OVA- and Derp1-specific IgG2a and IgG1 antibodies in murine sera.