Immune modulation by vaccination in chronic arthritis

A balancing act

Evelien Zonneveld-Huijssoon

About the cover
'a balancing act' door Marjoke de Heer

Cover: Vincent Zonneveld

Layout and printing: Optima Grafische Communicatie

ISBN: 978-94-6169-222-1

Address of correspondence
Evelien Zonneveld-Huijssoon, Wilhelmina's Children's Hospital, University Medical Center Utrecht, Department of Pediatric Immunology, PO Box 85090, 3508 AB Utrecht, the Netherlands. E-mail: e.zonneveld-huijssoon@umcutrecht.nl

Immune modulation by vaccination in chronic arthritis
A balancing act

Immuunmodulatie door vaccinatie bij chronische arthritis
Een kwestie van balans
(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. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties in het openbaar te verdedigen
op dinsdag 24 april 2012 des middags te 4.15 uur

door

Evelien Zonneveld-Huijssoon

geboren op 4 maart 1976
te Deventer

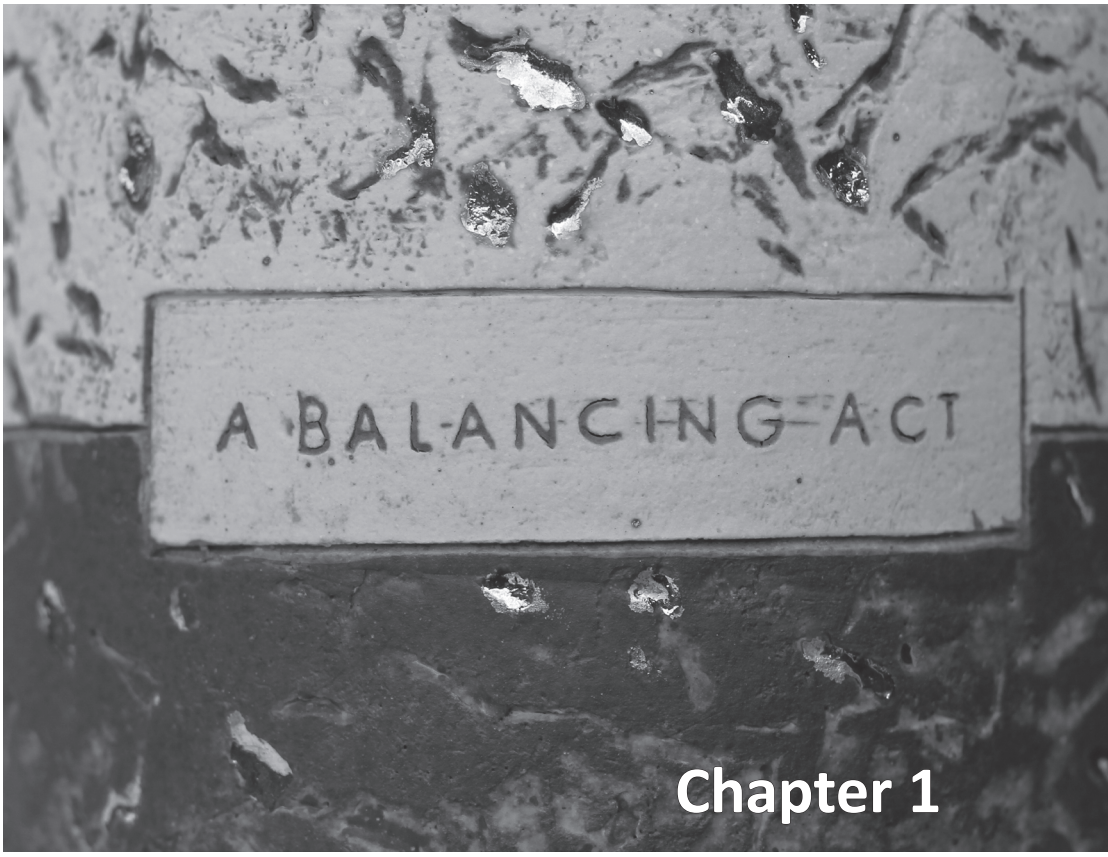
Promotoren: Prof.dr. B.J. Prakken
Prof.dr. W. Kuis

Co-promotoren: Dr. N.M. Wulffraat
Dr. F. van Wijk

The printing of this thesis was supported by ABBOTT B.V., J.E. Jurriaanse stichting, Reumafonds, GlaxoSmithKline, Sanofi Pasteur MSD N.V., Thermo Fisher Scientific en Stichting Kind en Afweer

Aan Emma en Julia

TABLE OF CONTENTS



Chapter 1

Introduction

INTRODUCTION

Part I Vaccination against infection in arthritis

Juvenile idiopathic arthritis

Juvenile idiopathic arthritis (JIA) is the most common childhood chronic rheumatic disease worldwide affecting 16-150 children per 100 000 per year.[1,2] JIA is assumed to develop in genetically susceptible individuals exposed to an unknown environmental trigger leading to an uncontrolled adaptive immune response towards a self antigen. A self-perpetuating loop of activation of both innate and adaptive immune responses ensues this initial trigger, leading to persistent synovial inflammation causing joint damage, disability and impaired quality of life.[1] By definition, JIA starts in children below 16 years of age, so joint damage resulting from arthritis at such an early age can have a great lifetime impact. JIA is a heterogeneous disease, with subtypes classified according to international criteria (table 1).[3] The subtypes described in this classification vary greatly regarding severity and long term outcome. However, factors predicting long term outcome are largely lacking. The most favourable outcome is seen in the self-limiting subtype, known as persistent oligoarticular JIA (OA-JIA), in which a maximum of four joints is affected. The self limiting nature in this subtype has been associated with adequate immune regulation by which the immune system has apparently found a way to restore immunological tolerance against auto-antigens.[4] A comparison with immunological processes in polyarticular JIA (PA-JIA), affecting more than four joints within the first half year of the disease, could reveal new insights in the possible defects in immune regulation in PA-JIA. Although the inflammation in OA-JIA is self-limiting and the disease can reside after puberty, the inflammation may cause permanent joint damage causing lifelong disability. Prevention of such damage should clearly be a reason to search for optimal early treatment strategies, also for this relatively mild subtype.

Current treatment of juvenile idiopathic arthritis is based on a combination of medication, physical and occupational therapy supporting an active lifestyle, and psychosocial support. Commonly used drugs are nonsteroidal anti-inflammatory drugs (NSAIDs), disease modifying anti-rheumatic drugs (DMARDS) like methotrexate and corticosteroids (systemic or local intra-articular injection). Since 2000, targeted immunosuppressive and highly effective biologicals (like the in JIA most commonly used anti TNFα receptor blocking drugs, but also Interleukin 1 (IL-1) or IL-6 receptor blocking agents) have been added for more severe cases of JIA. Over the past years the use of these cytokine blocking agents has been greatly increased. It must however be noted that long term outcome studies are scarce, especially studies focussing on possible long term side effects.

Table 1 Description, frequency and age at onset of the International League of Associations for Rheumatology (ILAR) categories of juvenile idiopathic arthritis.

JIA subtype	Description	Frequency*	Onset age
Systemic arthritis	quotidian fever with arthritis and one or more of evanescent, non-fixed erythematosus rash hepatomegaly or splenomegaly, lymphoadenopathy, or serositis	4–17%	Throughout childhood
Oligoarthritis	arthritis affecting 1-4 joints during the first months of disease, with two subtypes: persistent oligoarthritis which does not extend further and extended oligoarthritis in which arthritis extends to more than four joints after the first 6 months of disease	27–56%	Early childhood; peak at 2–4 years
Rheumatoid-factor-positive polyarthritis	arthritis affecting ≥ 5 joints in the first 6 months of disease with positivity for IgM RF on at least two occasions more than 3 months apart	2–7%	Late childhood or adolescence
Rheumatoid-factor-negative polyarthritis	arthritis affecting ≥ 5 joints in the first 6 months of disease in the absence of IgM RF	11–28%	Biphasic distribution; early peak at 2–4 years and later peak at 6–12 years
Enthesitis-related arthritis	arthritis with enthesitis, mostly HLAB27 positive	3–11%	Late childhood or adolescence
Psoriatic arthritis	simultaneous presence of arthritis and a typical psoriatic rash, or arthritis and at least two of a family history of psoriasis in a first-degree relative, dactylitis or nail pitting	2–11%	Biphasic distribution; early peak at 2–4 years and later peak at 9–11 years
Undifferentiated arthritis	arthritis with an unknown cause that persists for ≥ 6 weeks but that either does not fulfil criteria for any category or meets the criteria for more than one	11–21%	

*Reported frequencies refer to percentage of all juvenile idiopathic arthritis.
Adapted from [2]

Patients with rheumatic diseases are at increased risk of infections, possibly in part due to the disease itself, but mainly due to the immunosuppressive effect of its treatment.[5-7] The current early use of immunosuppressive drugs and the increasing utilization of cytokine blocking biologicals have further enlarged the susceptibility to infections and contribute to a changed pattern of infections, now also including tuberculosis and opportunistic infections.[8-11] Vaccination programs from the national health authorities have reduced the risk of infection substantially in the general population of healthy children. Development of similar guidelines for children with rheumatic diseases has been hampered by the lack of

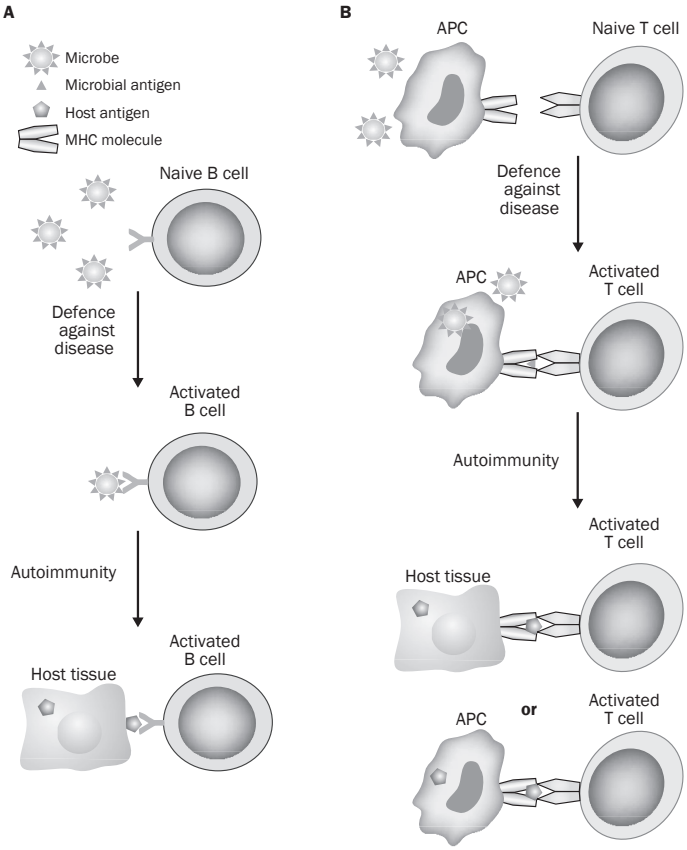


Figure 1 Mechanisms of molecular mimicry. (A) Microbe-specific B lymphocytes are activated by direct recognition of microbial antigen. Activated B cells then cross-react with antigens expressed by host tissues, leading to autoimmunity. (B) Microbe-specific T lymphocytes are activated by recognition of microbial antigens presented by MHC molecules on APCs. These activated microbe–specific T cells cross-react with self-antigens expressed by host tissue or presented by APCs, leading to autoimmunity. APC=antigen presenting cell. Reprinted from [27] Copyright (2003), with permission from Elsevier.

clinical studies. Recently a first proposal has been done by a taskforce from the European League Against Rheumatism (EULAR).[12]

The two major issues to address when considering vaccination of children with an autoimmune disease like JIA are the safety and efficacy of the vaccine.

Safety of vaccination in autoimmune disease

The increasing numbers of immunisations at ages at which some autoimmune diseases are known to occur has led to a debate whether these immunisations might be the cause of onset of the disease rather than a coincidence.[13,14] Several natural occurring infectious agents like rubella, parvovirus, and incidentally also acute meningococcal disease have

been associated with reactive arthritis.[15-17] Thus, it seems logical to consider especially immunisations against such organisms as possible causes of arthritis. Indeed several case reports exist of such an association after rubella vaccination.[18,19]

Although many links between autoimmune diseases and vaccinations have been suggested, only a few, such as Guillain Barré Syndrome after the 1976 swine influenza vaccination[20-22] and idiopathic thrombocytopenic purpura after MMR vaccination fulfil all criteria of causality (consistency strength, specificity and temporal relation).[23-25] As the occurrence of autoimmune diseases is quite rare, registration studies of new vaccines are usually underpowered to detect such possible adverse events. The currently available case reports cover a total of 16 cases of arthritis that have been reported after vaccination against hepatitis B, tetanus, anthrax and diphtheria-tetanus-polio combination vaccine (reviewed by [26]).

Considering the contribution of vaccination to the induction or aggravation of autoimmune disease, two potential mechanisms have been described: antigen specific molecular mimicry and antigen non-specific bystander activation.[27]
Molecular mimicry between a microbial antigen and a host antigen can cause microbe-specific B or T cells to cross-react with host tissue expressing the mimicking antigen, resulting in autoimmune responses (figure 1).[27,28] The increased incidence of arthritis after intravesical BCG immunotherapy for bladder carcinoma could for example be due to molecular mimicry, as in rats a *M. tuberculosis* specific T cell clone has been shown to cross-react with human joint proteins.[29]
Autoimmune responses can also be evoked by bystander activation (figure 2). The release of sequestered self antigen from damaged host cells after vaccination with the simultaneous activation of antigen presenting cells (APC) enhances the presentation of self peptides on activated APC, leading to autoimmune responses by autoreactive T cells that were not directly involved in the initial response.[27] The possibility to induce autoimmune diseases in transgenic mice containing high numbers of autoreactive T cells with non-specific triggers like inflammatory mediators or a physical insult illustrates this mechanism.[30,31]

However, cross-reactive immune responses alone do not necessarily lead to autoimmune disease. New onset auto-antibodies like a transient rise in rheumatoid factor have been detected after vaccinations that were not accompanied by clinical disease.[32,33] Moreover, it is thought that each T cell is able to recognize multiple distinct peptide epitopes,[34] increasing the likelihood of T cell cross-reactivity. If this T cell cross-reactivity would remain unregulated, one could wonder why not vaccinations lead more often to autoimmune disease by molecular mimicry.

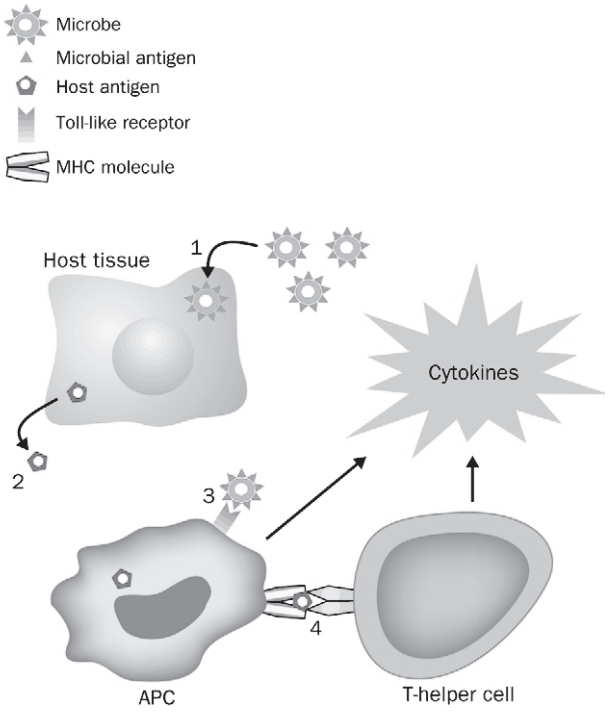


Figure 2 Mechanisms of autoimmunity resulting from bystander activation. Microbial infection of host tissue leads to tissue damage and release of self-antigen by host cells. Microbial antigens engage Toll-like receptors on APCs, resulting in up-regulation of MHC and co-stimulatory molecule expression and secretion of cytokines. Up-regulation of self-antigen expression by APCs activates autoreactive T cells, leading to a burst of cytokine secretion, local inflammation, and recruitment of additional autoreactive lymphocytes. Reprinted from [27] Copyright (2003), with permission from Elsevier.

Additional conditions are needed for autoimmune disease to arise, like genetic susceptibility and failure of peripheral tolerance mechanisms. In healthy individuals, down regulation of the immune response after infection or vaccination is regulated by several mechanisms. One of the mechanisms of peripheral tolerance that is of importance for the regulation of inflammation of human autoimmune diseases is provided by FoxP3 expressing regulatory T cells (Treg).[35,36] Self antigens are cognate antigens of Tregs[37] that upon recognition in the tissues inhibit effector T cell responses through cell-cell contact and cytokines.

Efficacy of vaccination in autoimmune disease

Another issue of debate in immunising patients with autoimmune diseases is a possible lack of immunogenicity of the vaccine due to the use of immunosuppressive drugs.[38,39] The severity of such an acquired immune deficiency is dose- and drug dependent. It can vary from lower antibody titres not reaching protective levels to an overt infection particularly

caused by live-attenuated vaccines.[40] Based on the increased risk of inducing infection by live-attenuated vaccines, it is currently recommended in adults to avoid these vaccines in immunocompromised patients whenever possible.[41] Together with the possible lower rate of protection after vaccination and the lack of specific guidelines for children with rheumatologic diseases, this explains why physicians are less likely to advice vaccination in children on higher levels of immunosuppressive therapy.[42] In Germany, about one third of the children with rheumatologic diseases have been incompletely vaccinated.[43] Of the non-live vaccines, the meningococcal C conjugate vaccine would even be withheld somewhat more often than other vaccines in JIA.[42]

Meningococcal disease

In 1999, an increasing number of cases of group C Meningococcal disease in the United Kingdom (UK) and the Netherlands were reported.[44,45] In the same year, a nation-wide vaccination campaign in the UK was initiated. Hereafter, the Netherlands initiated their own campaign with the tetanus toxoid (TT) conjugated form of the vaccine, Neisvacc-C.[46] All children at the age of 14 months were vaccinated and a catch up vaccination program was implemented for older children up to 18 years of age. This catch up vaccination program for all Dutch children including the children with JIA gave us the opportunity to set up the studies performed in chapter 2 and 3.

Part II Vaccination against autoimmunity in arthritis

Current treatment of chronic arthritis

As discussed in the previous part, current treatment of chronic arthritis mainly depends on life-long non-specific immune suppression. Although a step forward has been made in clinical efficiency by the introduction of biologicals that can block pro-inflammatory cytokines, autoimmunity revives as soon as therapy is discontinued. Also, over years people can become resistant to this therapy. Moreover, the considerable immune depression evoked by cytokine blockade has unfortunately been associated with reports of severe side-effects like tuberculosis, serious opportunistic infections or malignancies like lymphoma.[10,47-51] A more directed approach could overcome back draws of non-specific immune suppressive therapy. By specific targeting of auto-aggressive T cells in autoimmunity, side effects may be reduced and possibly, a longer lasting effect may be achieved.

Antigen specific immunotherapy has been shown effective in multiple animal models of autoimmunity, without severe side effects (reviewed by [52]). Translation of these findings into humans had promising results but efficacy has been less than expected (chapter 4, table 1).

Improvement of antigen specific therapy

To enhance the efficacy of antigen specific therapy in clinical autoimmune diseases, three issues concerning the choice of antigen, route of administration and peptide immunogenicity need to be considered.

1. Which antigen to use?

The choice of antigen in animal models and allergy is facilitated by the fact that the disease inducing antigen is known. The identification of such an antigen in human autoimmunity is more challenging as the disease inducing antigen in many autoimmune diseases remains unknown and it is even unsure whether this one disease inducing agent really exists. New targets for antigen specific therapy are therefore needed.

Bystander antigens

Shortly after an unknown trigger evokes inflammation in autoimmune diseases, the resulting cell damage leads (like in infection or vaccination) to the release of many different (self) antigens. This pro-inflammatory context may give rise to autoaggressive T cells (epitope spreading). These self antigens expressed at the site of inflammation (bystander antigens) could be alternative candidates for peptide immunotherapy as long as they are immunogenic and selectively upregulated during inflammation. Unlike in vaccination against infectious diseases when pro-inflammatory responses are pursued, for the vaccination against autoimmunity the induction of a regulatory immune response is necessary. Deviation of

the bystander antigen-specific immune response towards a regulatory phenotype would generate tolerogenic T cells specific for the antigen. These antigen-specific T cells might home to the site of inflammation as their cognate antigen is selectively expressed there, and then could suppress the local inflammation via bystander suppression by cytokine release like IL-10 and/or TGFβ and the induction of multiple kinds of regulatory T cells.[53]

Heat shock protein as a bystander antigen

Heat shock proteins (HSPs) are evolutionary conserved self-antigens that are strongly immunogenic and selectively upregulated during stress.[54] As they fulfil the criteria for bystander antigens, they seem suitable candidates for antigen specific immunotherapy (reviewed in chapter 4). The immune response evoked by antigens is thought to be dependent on the context in which it takes place.[55] HSPs therefore have a dual role. During arthritis, self antigens like HSPs will be expressed by stressed cells in the inflamed joint evoking autoreactive/autoaggressive T cell responses. In this proinflammatory context this would lead to proinflammatory responses, while in a tolerogenic environment antigen specific T cell responses to HSPs will be more skewed towards a regulatory response.

From protein to peptide

Administration of the whole protein for therapy of autoimmunity would avoid having to select specific epitopes to suit individuals with different HLA molecules. However, whole protein can activate mast cells by cross-linking IgE in allergic individuals[56] or induce pathogenic cytotoxic lymphocytes in patients with autoimmune disease.[57,58,58] Moreover, most proteins are produced in microbes, which entails the risk of contamination with bacterial products like LPS. Without thorough purity testing, the results of protein induced immune responses can therefore be hard to interpret. The use of synthetic peptides increases specificity reduces side effects and is not biased by potential contamination of bacterial products.

HLA-binding epitopes

One of the consequences of the use of peptides for antigen specific therapy in patients is that the selected peptide should be able to bind a diverse range of HLA molecules in order to be recognized by the immune system. Using computer algorithms, potential pan HLA-DR binding T cell epitopes derived from human and mycobacterial HSP60 have been identified that are recognised by the majority of patients with rheumatoid arthritis (RA) or juvenile idiopathic arthritis (JIA).[59] As effective peptide immunotherapy with one of these peptides would be directly translatable to clinical practice, results of HLA-binding epitopes in experimental models will be of particular interest.

2. Route of administration

The second issue to consider for further optimization of antigen specific therapy is the route of antigen administration. In many clinical trials of peptide immunotherapy against autoimmune diseases the antigen was administered subcutaneously (table 1, chapter 4) while the mucosal route may be more effective.[52,53]

Mucosal tolerance

Peripheral tolerance to antigens can be induced by administration of exogenous antigen to a tolerogenic site like the gut or nasal mucosa (mucosal tolerance).[53] Both orally and nasally induced mucosal tolerance utilize natural routes of immunologic exposure and importantly, lack toxicity. The mechanism by which tolerance is achieved is dependent on the dose of antigen administered: low dose leading to active suppression and high dose to deletion or anergy of antigen specific T cells.[60] Also the route of antigen administration plays a role as nasal administration of antigen seems to be more effective than oral.[61] This difference may be explained by the fact that by nasal administration the administered antigen is directly placed into contact with the inductive site, the nasal-associated lymphoid tissue (NALT). The antigen therefore is not exposed to the aggressive environment of gastric and gastrointestinal degrading enzymes as is the case in oral exposure. In addition, the NALT and gut associated lymphoid tissue (GALT) also have internal differences such as having different types of antigen presenting cells (APCs) that may play a role in the efficacy of tolerance induction.[62] Antigen specific mucosal tolerance induction has been shown to be effective in multiple animal models of autoimmunity.[63-65]

A proof of concept of mucosal antigen specific therapy in humans has been shown in allergic patients. By repeated mucosal administration of the allergen, subsequent exposures to this allergen are better tolerated and long-term tolerance can be induced.[66] The main mechanism seems to be a shift of allergen-specific immune responses from pro-inflammatory to anti-inflammatory with IL-10 being the major regulatory cytokine. Whereas allergen specific T cells remain present, the balance shifts toward IL-10 producing (regulatory) T cells.[66] However, in allergy T cell responses are skewed towards Th2 associated responses whereas the immunological balance is skewed towards Th1/Th17 responses in autoimmunity. Furthermore in allergy, disease free episodes are identifiable offering a window of opportunity for antigen administration in a non-inflammatory environment. However, in autoimmune disease, inflammation is an ongoing process, possibly hampering subtle immune modulation by peptide immunotherapy when administered in this pro-inflammatory state. Maybe therefore, results of clinical trials studying peptide therapy in advanced autoimmune disease have been suboptimal.

3. Enhancement of peptide immunogenicity

The major back draw to overcome when peptides are administered via the mucosal route may be the supposed limited immunogenicity, indicating a need for enhancement of peptide recognition.[67,68]

In vaccination against infectious diseases, adjuvants like alum have been successfully used to increase immunogenicity of poorly immunogenic antigens. Also the use of mucosal adjuvants has been established to increase vaccine efficacy.[69,70] The use of a mucosal adjuvant stimulating the innate immune system may therefore increase the adaptive immunologic response generated by the mucosally administered peptide.

On the other hand, the ongoing inflammation in autoimmune disease may be too strong for refined antigen-specific immune modulation. Combination of non-specific immune suppression dampening inflammation with peptide specific immunotherapy might therefore set the stage for peptide specific immune modulation. The success of combination therapy with anti CD3 in experimental models of new-onset diabetes underscores this notion.[71,72] In children with JIA, TNFα blockade is currently the most commonly used monoclonal antibody treatment with potent immune suppressive capacity. Besides clinical improvement of disease, TNFα blockade has been shown to have a beneficial effect on regulatory T cell number and function in adult arthritis patients.[73-75] This could make anti TNFα treatment a suitable target for combination therapy in this treatment group.

For a more extensive description of the concept of HSP peptides as bystander antigens in antigen specific therapy and strategies to further improve clinical and immunological efficacy of antigen specific immunotherapy in autoimmune diseases we refer to the review on this subject in chapter 4.

The adjuvant-induced arthritis model, a history

In search for an immunity enhancing agent against tuberculosis, Jules Freund developed in 1947 a mixture that consisted of mineral oils, heat-killed mycobacteria and an emulsifying agent. This mixture was designated as complete Freund's adjuvant (CFA), and proved to be an efficient enhancer of immune responses towards antigens that were emulsified in CFA.[76] It was also demonstrated that immunization with CFA in combination with auto-antigen could break peripheral tolerance, and thereby induce autoimmune diseases. The first experimental adjuvant-induced arthritis (AIA) model was established in 1956 by Pearson. He showed that administration of CFA induced joint inflammation in Lewis rats and that the induced symptoms showed similarities with the pathogenesis of rheumatoid arthritis in humans.[77] The adjuvant-induced arthritis in rats is a subacute polyarthritis which is self-limiting and usually does not last longer than one month. The first clinical symptoms

appear approximately 10-14 days after inoculation. Symptoms of arthritis affecting the extremities are almost always the first to be detected. The arthritis reaches a peak of severity around day 20 to 25 and then gradually declines.[76] At the site of inflammation, infiltrating T cells express Th1-associated cytokines such as IFNγ and TNFα.

Adjuvant induced arthritis can be adoptively transferred by CD4+ T cells of arthritic rats. More specifically, transfer of a T cell clone, called A2b, recognizing the 180-188 amino acid sequence in mycobacterial HSP60 was found to induce AA.[78] T cells of this A2b clone did not cross-react with self-HSP60, but with cartilage proteoglycan.[79] Nasal administration of the 15-mer mycobacterial HSP60 176-190 peptide (containing the core 180-188 sequence), prior to induction of the disease is able to delay the onset and decrease the severity of arthritis.[80] HSP peptides with a high homology to self HSP that were able to induce self cross-reactive T cell responses were capable of suppressing adjuvant induced arthritis, leading to the theory that self cross-recognition is required for the induction of peripheral tolerance mechanisms.[81]

For the studies in the second part of this thesis, we chose to use this adjuvant-induced arthritis model (AIA) in the Lewis rat. Although collagen induced arthritis is known to induce joint lesions with the closest resemblance to human rheumatoid arthritis, we chose the AIA-model as the distribution and extent of the inflammation in joints is more reproducible than in CIA.[82] Moreover, AIA has been more extensively used for pharmacological testing in the past so more data exist for cross-species comparison of anti-arthritic efficacy. Finally, the fact that the role of HSPs in antigen specific immunomodulation has been defined in this model enabled us to further build upon this large body of evidence in the same model.

Aim and outline of the thesis

The studies described in this thesis have been performed to gain more insight in the clinical and immunological outcome of modulating the immune system by vaccination in chronic arthritis and to explore strategies to improve antigen specific therapy in arthritis.

Part I

Activation of the immune system by vaccination in an autoimmune disease like arthritis is a challenging and could even be potentially dangerous. Challenging, as immune suppressive medication frequently used in this patient group can hamper the efficacy of vaccination and potentially dangerous as molecular mimicry in combination with bystander activation and loss of tolerance in autoimmunity can lead to disease flare.

In the first part of this thesis we hope to acquire more insight in the potential danger and efficacy of immune activation by vaccination in Juvenile Idiopathic Arthritis. The

simultaneous vaccination against Meningococcal C of children with JIA during the Dutch vaccination campaign is used as a tool to study this. In **chapter 2** clinical arthritis activity before and after MenC vaccination is evaluated as well as immunological efficacy in relation to different treatment modalities.
In **chapter 3** is investigated how T cell responses to the MenC vaccine and HSP60, a disease related auto-antigen, differ between patients with remitting (oligoarticular) and progressive (polyarticular) JIA.

Part II

In part two of this thesis we investigated the potential of antigen administration to treat experimental arthritis and explored strategies to improve efficacy of this antigen specific immunotherapy. We used the rat adjuvant arthritis model for these studies.
Leaving the search for the single disease inducing antigen in human autoimmune disease, we hypothesized that bystander antigens derived from heat shock protein could be alternative targets for antigen specific therapy (**chapter 4**). In **chapter 5** the clinical and immunological effects of mucosal administration of such HSP60 bystander antigens are evaluated in the rat adjuvant arthritis model. To be able to translate the eventual results to patients, panDR binding epitopes of bacterial and self HSP60 have been used.
The supposed limited immunogenicity of a peptide delivered via a tolerogenic route like the nasal mucosa may hinder clinical efficacy of antigen specific therapy. In **chapter 6** a mucosal adjuvant with regulatory capacities (CpG) is added to the bacterial HSP60 peptide to enhance peptide immunogenicity by activating innate immunity.

Although preventive strategies of this antigen specific therapy in experimental arthritis are effective, therapeutic efficacy after disease onset is currently limited to slowing down the progression of disease. In patients with diabetes type I, the effect of mucosal tolerance induction is better when administered early in the disease course. However, in patients treatment is often not initiated before the disease has progressed into a state in which generalized non-specific inflammation may hamper antigen specific modulation of the immune response. Combination treatment with short duration generalized immune modulators may aid in dampening the non-specific inflammation and also enable the induction of suppressive Treg. In **chapter 7**, this concept is tested by combining HSP peptide specific immunotherapy with TNFα blockade shutting down the pro-inflammatory environment. Finally, the results and significance of our findings are discussed in **chapter 8**.

REFERENCE LIST

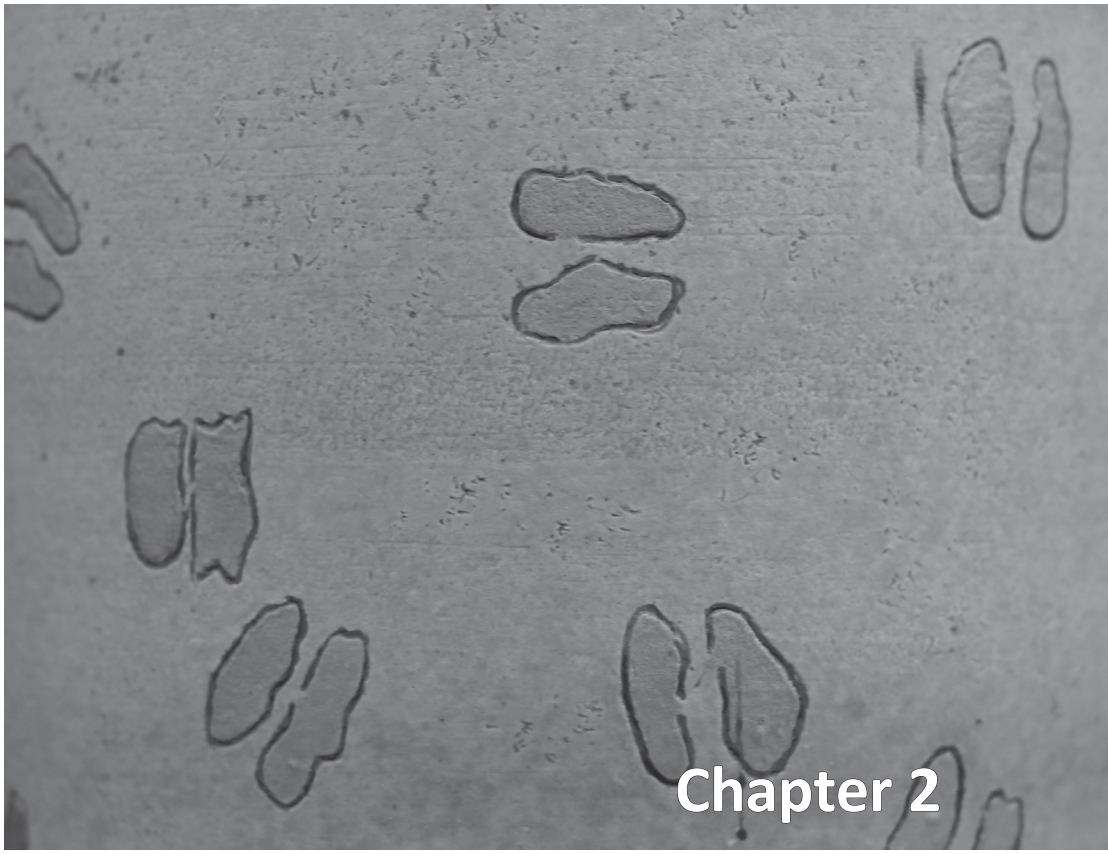
1. Prakken B, Albani S, Martini A. Juvenile idiopathic arthritis. *Lancet.* 2011;377:2138-2149.
2. Ravelli A, Martini A. Juvenile idiopathic arthritis. *Lancet.* 2007;369:767-778.
3. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. *J Rheumatol.* 2004;31:390-392.
4. de Kleer I, Kamphuis SM, Rijkers GT, Scholtens L, Gordon G, de Jager W, et al. The spontaneous remission of juvenile idiopathic arthritis is characterized by CD30+ T cells directed to human heat-shock protein 60 capable of producing the regulatory cytokine interleukin-10. *Arthritis Rheum.* 2003;48:2001-2010.
5. Atzeni F, Bendtzen K, Bobbio-Pallavicini F, Conti F, Cutolo M, Montecucco C, et al. Infections and treatment of patients with rheumatic diseases. *Clin Exp Rheumatol.* 2008;26:S67-S73.
6. Fessler BJ. Infectious diseases in systemic lupus erythematosus: risk factors, management and prophylaxis. *Best Pract Res Clin Rheumatol.* 2002;16:281-291.
7. Breda L, Del Torto M, De Sanctis S, Chiarelli F. Biologics in children's autoimmune disorders: efficacy and safety. *Eur J Pediatr.* 2011;170:157-167.
8. Morishita K, Petty R, Cairns R, Bolaria R, Cabral D, Turvey S. Serious musculoskeletal infections in children receiving anti-tumor necrosis factor-alpha therapy: a case series. *Clin Rheumatol.* 2010;29:677-681.
9. Fitch PG, Cron RQ. Septic abscess in a child with juvenile idiopathic arthritis receiving anti-tumor necrosis factor-alpha. *J Rheumatol.* 2006;33:825-827.
10. Dekker L, Armbrust W, Rademaker CM, Prakken B, Kuis W, Wulffraat NM. Safety of anti-TNFalpha therapy in children with juvenile idiopathic arthritis. *Clin Exp Rheumatol.* 2004;22:252-258.
11. Gomez-Reino JJ, Carmona L, Angel DM. Risk of tuberculosis in patients treated with tumor necrosis factor antagonists due to incomplete prevention of reactivation of latent infection. *Arthritis Rheum.* 2007;57:756-761.
12. Heijstek MW, Ott de Bruin LM, Bijl M, Borrow R, van der Klis F, Koné-Paut I, et al. EULAR recommendations for vaccination in paediatric patients with rheumatic diseases. *Ann Rheum Dis.* 2011;70:1704-1712.
13. Variation and trends in incidence of childhood diabetes in Europe. EURODIAB ACE Study Group. *Lancet.* 2000;355:873-876.
14. Wynn DR, Rodriguez M, O'Fallon WM, Kurland LT. A reappraisal of the epidemiology of multiple sclerosis in Olmsted County, Minnesota. *Neurology.* 1990;40:780-786.
15. Likitnukul S, McCracken GH, Jr., Nelson JD. Arthritis in children with bacterial meningitis. *Am J Dis Child.* 1986;140:424-427.
16. Schaad UB. Arthritis in disease due to Neisseria meningitidis. *Rev Infect Dis.* 1980;2:880-888.
17. Franssila R, Hedman K. Infection and musculoskeletal conditions: Viral causes of arthritis. *Best Pract Res Clin Rheumatol.* 2006;20:1139-1157.
18. Ray P, Black S, Shinefield H, Dillon A, Schwalbe J, Holmes S, et al. Risk of chronic arthropathy among women after rubella vaccination. Vaccine Safety Datalink Team. *JAMA.* 1997;278:551-556.
19. Korematsu S, Miyahara H, Kawano T, Yamada H, Akiyoshi K, Sato K, et al. A relapse of systemic type juvenile idiopathic arthritis after a rubella vaccination in a patient during a long-term remission period. *Vaccine.* 2009;27:5041-5042.

20. Schonberger LB, Bregman DJ, Sullivan-Bolyai JZ, Keenlyside RA, Ziegler DW, Retailliau HF, et al. Guillain-Barre syndrome following vaccination in the National Influenza Immunization Program, United States, 1976--1977. *Am J Epidemiol.* 1979;110:105-123.
21. Safranek TJ, Lawrence DN, Kurland LT, Culver DH, Wiederholt WC, Hayner NS, et al. Reassessment of the association between Guillain-Barre syndrome and receipt of swine influenza vaccine in 1976-1977: results of a two-state study. Expert Neurology Group. *Am J Epidemiol.* 1991;133:940-951.
22. Marks JS, Halpin TJ. Guillain-Barre syndrome in recipients of A/New Jersey influenza vaccine. *JAMA.* 1980;243:2490-2494.
23. Miller E, Waight P, Farrington CP, Andrews N, Stowe J, Taylor B. Idiopathic thrombocytopenic purpura and MMR vaccine. *Arch Dis Child.* 2001;84:227-229.
24. Black C, Kaye JA, Jick H. MMR vaccine and idiopathic thrombocytopaenic purpura. *Br J Clin Pharmacol.* 2003;55:107-111.
25. Beeler J, Varricchio F, Wise R. Thrombocytopenia after immunization with measles vaccines: review of the vaccine adverse events reporting system (1990 to 1994). *Pediatr Infect Dis J.* 1996;15:88-90.
26. Salemi S, D'Amelio R. Could autoimmunity be induced by vaccination? *Int Rev Immunol.* 2010;29:247-269.
27. Wraith DC, Goldman M, Lambert PH. Vaccination and autoimmune disease: what is the evidence? *Lancet.* 2003;362:1659-1666.
28. Regner M, Lambert PH. Autoimmunity through infection or immunization? *Nat Immunol.* 2001;2:185-188.
29. van Eden W, Holoshitz J, Nevo Z, Frenkel A, Klajman A, Cohen IR. Arthritis induced by a T-lymphocyte clone that responds to Mycobacterium tuberculosis and to cartilage proteoglycans. *Proc Natl Acad Sci U S A.* 1985;82:5117-5120.
30. Kissler S, Anderton SM, Wraith DC. Antigen-presenting cell activation: a link between infection and autoimmunity? *J Autoimmun.* 2001;16:303-308.
31. Goverman J, Woods A, Larson L, Weiner LP, Hood L, Zaller DM. Transgenic mice that express a myelin basic protein-specific T cell receptor develop spontaneous autoimmunity. *Cell.* 1993;72:551-560.
32. Toplak N, Avcin T. Vaccination of healthy subjects and autoantibodies: from mice through dogs to humans. *Lupus.* 2009;18:1186-1191.
33. D'Amelio R, Tagliabue A, Nencioni L, Di Addario A, Villa L, Manganaro M, et al. Comparative analysis of immunological responses to oral (Ty21a) and parenteral (TAB) typhoid vaccines. *Infect Immun.* 1988;56:2731-2735.
34. Fowell D, Mason D. Evidence that the T cell repertoire of normal rats contains cells with the potential to cause diabetes. Characterization of the CD4+ T cell subset that inhibits this autoimmune potential. *J Exp Med.* 1993;177:627-636.
35. Hori S, Takahashi T, Sakaguchi S. Control of autoimmunity by naturally arising regulatory CD4+ T cells. *Adv Immunol.* 2003;81:331-371.
36. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet.* 2001;27:20-21.
37. Hsieh CS, Liang Y, Tyznik AJ, Self SG, Liggitt D, Rudensky AY. Recognition of the peripheral self by naturally arising CD25+ CD4+ T cell receptors. *Immunity.* 2004;21:267-277.
38. Kasapcopur O, Cullu F, Kamburoglu-Goksel A, Cam H, Akdenizli E, Calykan S, et al. Hepatitis B vaccination in children with juvenile idiopathic arthritis. *Ann Rheum Dis.* 2004;63:1128-1130.

39. Abu-Shakra M, Press J, Varsano N, Levy V, Mendelson E, Sukenik S, et al. Specific antibody response after influenza immunization in systemic lupus erythematosus. *J Rheumatol.* 2002;29:2555-2557.
40. Bijl M, Kallenberg CG, van Assen S. Vaccination of the immune-compromised patients with focus on patients with autoimmune-inflammatory diseases. *Neth J Med.* 2011;69:5-13.
41. Lobermann M, Borso D, Hilgendorf I, Fritzsche C, Zettl UK, Reisinger EC. Immunization in the adult immunocompromised host. *Autoimmun Rev.* 2011.
42. Davies K, Woo P. Immunization in rheumatic diseases of childhood: an audit of the clinical practice of British Paediatric Rheumatology Group members and a review of the evidence. *Rheumatology (Oxford).* 2002;41:937-941.
43. Minden K, Niewerth M, Borte M, Singendonk W, Haas JP. [Immunization in children and adolescents with rheumatic diseases]. *Z Rheumatol.* 2007;66:111-8, 120.
44. Kaczmarski EB. Meningococcal disease in England and Wales: 1995. *Commun Dis Rep CDR Rev.* 1997;7:R55-R59.
45. Conyn-van Spaendonck MA, Reintjes R, Spanjaard L, van Kregten E, Kraaijeveld AG, Jacobs PH. Meningococcal carriage in relation to an outbreak of invasive disease due to Neisseria meningitidis serogroup C in the Netherlands. *J Infect.* 1999;39:42-48.
46. van Furth AM, Vandenbroucke-Grauls CM. [Universal vaccination against group C meningococci and pneumococci; advice from the Health Council of the Netherlands]. *Ned Tijdschr Geneeskd.* 2002;146:932-933.
47. Maini RN, Taylor PC. Anti-cytokine therapy for rheumatoid arthritis. *Annu Rev Med.* 2000;51:207-229.
48. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med.* 2001;345:1098-1104.
49. Bloom BJ. Development of diabetes mellitus during etanercept therapy in a child with systemic-onset juvenile rheumatoid arthritis. *Arthritis Rheum.* 2000;43:2606-2608.
50. Lepore L, Marchetti F, Facchini S, Leone V, Ventura A. Drug-induced systemic lupus erythematosus associated with etanercept therapy in a child with juvenile idiopathic arthritis. *Clin Exp Rheumatol.* 2003;21:276-277.
51. FDA Drug Safety Communication: Safety Review update on reports of Hepatosplenic T-Cell Lymphoma in adolescents and young adults receiving tumor necrosis factor (TNF) blockers, azathioprine and/or mercaptopurine. 2011.
52. Sabatos-Peyton CA, Verhagen J, Wraith DC. Antigen-specific immunotherapy of autoimmune and allergic diseases. *Curr Opin Immunol.* 2010;22:609-615.
53. Faria AM, Weiner HL. Oral tolerance. *Immunol Rev.* 2005;206:232-259.
54. van Eden W, van der Zee R, Prakken B. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol.* 2005;5:318-330.
55. Matzinger P, Kamala T. Tissue-based class control: the other side of tolerance. *Nat Rev Immunol.* 2011;11:221-230.
56. Genain CP, Abel K, Belmar N, Villinger F, Rosenberg DP, Linington C, et al. Late complications of immune deviation therapy in a nonhuman primate. *Science.* 1996;274:2054-2057.
57. Blanas E, Carbone FR, Allison J, Miller JF, Heath WR. Induction of autoimmune diabetes by oral administration of autoantigen. *Science.* 1996;274:1707-1709.
58. Hanninen A, Braakhuis A, Heath WR, Harrison LC. Mucosal antigen primes diabetogenic cytotoxic T-lymphocytes regardless of dose or delivery route. *Diabetes.* 2001;50:771-775.

59. Kamphuis S, Kuis W, de Jager W, Teklenburg G, Massa M, Gordon G, et al. Tolerogenic immune responses to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis. *Lancet.* 2005;366:50-56.
60. Miller SD, Turley DM, Podojil JR. Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. *Nat Rev Immunol.* 2007;7:665-677.
61. Metzler B, Wraith DC. Inhibition of experimental autoimmune encephalomyelitis by inhalation but not oral administration of the encephalitogenic peptide: influence of MHC binding affinity. *Int Immunol.* 1993;5:1159-1165.
62. Bai XF, Link H. Nasal tolerance induction as a potential means of immunotherapy for autoimmune diseases: implications for clinical medicine. *Clin Exp Allergy.* 2000;30:1688-1696.
63. Reynolds J, Abbott DS, Karegli J, Evans DJ, Pusey CD. Mucosal tolerance induced by an immunodominant peptide from rat alpha3(IV)NC1 in established experimental autoimmune glomerulonephritis. *Am J Pathol.* 2009;174:2202-2210.
64. Souroujon MC, Maiti PK, Feferman T, Im SH, Raveh L, Fuchs S. Suppression of myasthenia gravis by antigen-specific mucosal tolerance and modulation of cytokines and costimulatory factors. *Ann N Y Acad Sci.* 2003;998:533-536.
65. Staines NA, Harper N, Ward FJ, Malmstrom V, Holmdahl R, Bansal S. Mucosal tolerance and suppression of collagen-induced arthritis (CIA) induced by nasal inhalation of synthetic peptide 184-198 of bovine type II collagen (CII) expressing a dominant T cell epitope. *Clin Exp Immunol.* 1996;103:368-375.
66. Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy. *J Allergy Clin Immunol.* 2011;127:18-27.
67. Decrausaz L, Domingos-Pereira S, Duc M, Bobst M, Romero P, Schiller JT, et al. Parenteral is more efficient than mucosal immunization to induce regression of human papillomavirus-associated genital tumors. *Int J Cancer.* 2011;129:762-772.
68. Bouvet JP, Decroix N, Pamonsinlapatham P. Stimulation of local antibody production: parenteral or mucosal vaccination? *Trends Immunol.* 2002;23:209-213.
69. Holmgren J, Adamsson J, Anjuere F, Clemens J, Czerkinsky C, Eriksson K, et al. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. *Immunol Lett.* 2005;97:181-188.
70. Ivanov AP, Dragunsky EM, Chumakov KM. 1,25-dihydroxyvitamin d3 enhances systemic and mucosal immune responses to inactivated poliovirus vaccine in mice. *J Infect Dis.* 2006;193:598-600.
71. Bresson D, Togher L, Rodrigo E, Chen Y, Bluestone JA, Herold KC, et al. Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs. *J Clin Invest.* 2006;116:1371-1381.
72. Bresson D, Fradkin M, Manenkova Y, Rottembourg D, von Herrath M. Genetic-induced variations in the GAD65 T-cell repertoire governs efficacy of anti-CD3/GAD65 combination therapy in new-onset type 1 diabetes. *Mol Ther.* 2010;18:307-316.
73. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood.* 2006;108:253-261.
74. Toubi E, Kessel A, Mahmudov Z, Hallas K, Rozenbaum M, Rosner I. Increased spontaneous apoptosis of CD4+CD25+ T cells in patients with active rheumatoid arthritis is reduced by infliximab. *Ann N Y Acad Sci.* 2005;1051:506-14.:506-514.
75. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med.* 2004;200:277-285.

76. Waksman BH, Pearson CM, Sharp JT. Studies of arthritis and other lesions induced in rats by injection of mycobacterial adjuvant. II. Evidence that the disease is a disseminated immunologic response to exogenous antigen. *J Immunol.* 1960;85:403-417.
77. Pearson CM. Development of arthritis, periarthritis and periostitis in rats given adjuvants. *Proc Soc Exp Biol Med.* 1956;91:95-101.
78. van Eden W, Thole JE, van der Zee R, Noordzij A, van Embden JD, Hensen EJ, et al. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature.* 1988;331:171-173.
79. van Eden W, Waksman BH. Immune regulation in adjuvant-induced arthritis: possible implications for innovative therapeutic strategies in arthritis. *Arthritis Rheum.* 2003;48:1788-1796.
80. Prakken BJ, van der Zee R, Anderton SM, van Kooten PJ, Kuis W, van Eden W. Peptide-induced nasal tolerance for a mycobacterial heat shock protein 60 T cell epitope in rats suppresses both adjuvant arthritis and nonmicrobially induced experimental arthritis. *Proc Natl Acad Sci U S A.* 1997;94:3284-3289.
81. Anderton SM, van der Zee R, Prakken B, Noordzij A, van Eden W. Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *J Exp Med.* 1995;181:943-952.
82. Bolon B, Stolina M, King C, Middleton S, Gasser J, Zack D, et al. Rodent preclinical models for developing novel antiarthritic molecules: comparative biology and preferred methods for evaluating efficacy. *J Biomed Biotechnol.* 2011;2011:569068.



Safety and efficacy of Meningococcal C vaccination in Juvenile Idiopathic Arthritis

Evelien Zonneveld-Huijssoon, Arash Ronaghy, Marion AJ van Rossum, Ger T Rijkers, Fiona RM van der Klis, Elisabeth AM Sanders, Patricia E Vermeer-de Bondt, Arno W Hoes, Jan Jaap van der Net, Carla Engels, Wietse Kuis, Berent J Prakken, Maarten JD van Tol, Nico M Wulffraat

Arthritis and Rheumatism 2007;56:639-646

ABSTRACT

Objective

To determine whether vaccinations aggravate the course of autoimmune diseases such as juvenile idiopathic arthritis (JIA) and whether the immune response to vaccinations may be hampered by immunosuppressive therapy for the underlying disease.

Methods

In third multicentre cohort study, 234 patients with JIA (ages 1-19 years) were vaccinated with meningococcal serogroup C (MenC) conjugate to protect against seropgroup C disease (caused by *Neisseria meningitides*). Patients were followed up for disease activity for 1 year, from 6 months before until 6 months after vaccination. IgG antibody titres against MenC polysaccharide and the tetanus carrier protein were determined by enzyme-linked immunosorbent assay and toxin binding inhibition assay, respectively. A serum bactericidal assay was performed to determine the function of the anti-MenC antibodies.

Results

No change in values for any of the 6 components of the core set criteria for juvenile arthritis disease activity was seen after MenC vaccination. Moreover, no increase in the frequency of disease relapse was detected. Mean anti-MenC IgG concentrations in JIA patients rose significantly within 6-12 weeks after vaccination. Of 157 patients tested, 153 were able to mount anti-MenC IgG serum levels >2µg/ml, including patients receiving highly immunosuppressive medication. The 4 patients with a lower anti-MenC antibody response displayed sufficient bactericidal activity despite receiving highly immunosuppressive medication.

Conclusion

The MenC conjugate vaccine does not aggravate JIA disease activity or increase relapse frequency and results in adequate antibody levels, even in patients receiving highly immunosuppressive medication. Therefore, patients with JIA can be vaccinated safely and effectively with the MenC conjugate.

INTRODUCTION

The induction or worsening of autoimmune disease by vaccination has been a matter of debate for many years.[1-4] Although most controlled studies fail to demonstrate any link between vaccination and autoimmune disease, concerns about possible adverse effects hamper compliance.[5-12] The decreasing herd immunity poses increased risks for patients with chronic autoimmune diseases.[13,14]

Another concern is the potentially diminished efficacy in patients being treated with immunosuppressive drugs.[10] In the UK, physicians were less likely to vaccinate those children with juvenile idiopathic arthritis (JIA) who received higher levels of immunosuppressive drugs.[15] Guidelines of the British Society for Rheumatology state that the immune response to meningococcal serogroup C (MenC) conjugate vaccine in immunosuppressed patients with rheumatic disease may be suboptimal and therefore they may require boosters.[16]

In 2002, the Dutch health authorities initiated a nationwide campaign in which all children between 1 and 19 years of age were vaccinated against meningococcal serogroup C disease, caused by *Neisseria meningitidis*.[17,18] The guidelines for exclusion were nonspecific with regard to autoimmune diseases or the use of immunosuppressive drugs. The aim of this study was to document disease activity and immune responses in JIA patients before and after MenC vaccination.

PATIENTS AND METHODS

Study design

A multicentre cohort study was performed in which patients served as their own controls. For each patient, the study period covered 1 year starting 6 months before MenC vaccination. Since most autoimmune reactions reported by others occurred within 1 month of vaccination, we defined this period as the period of exposure.[1,3,19-22] The remaining 11 months of the study period were defined as the unexposed period.

Study population

All patients between 1 and 19 years of age who had been diagnosed as having JIA according to the criteria of the International League of Associations for Rheumatology (ILAR) were eligible.[23] Before the start of the national vaccination campaign with the MenC conjugate, patients from pediatric rheumatology outpatient clinics at the University Medical Centers of Utrecht, Leiden and Amsterdam, the Jan van Breemen Institute and the Juliana Children's Hospital (The Hague) were invited by mail to participate in this study. Written

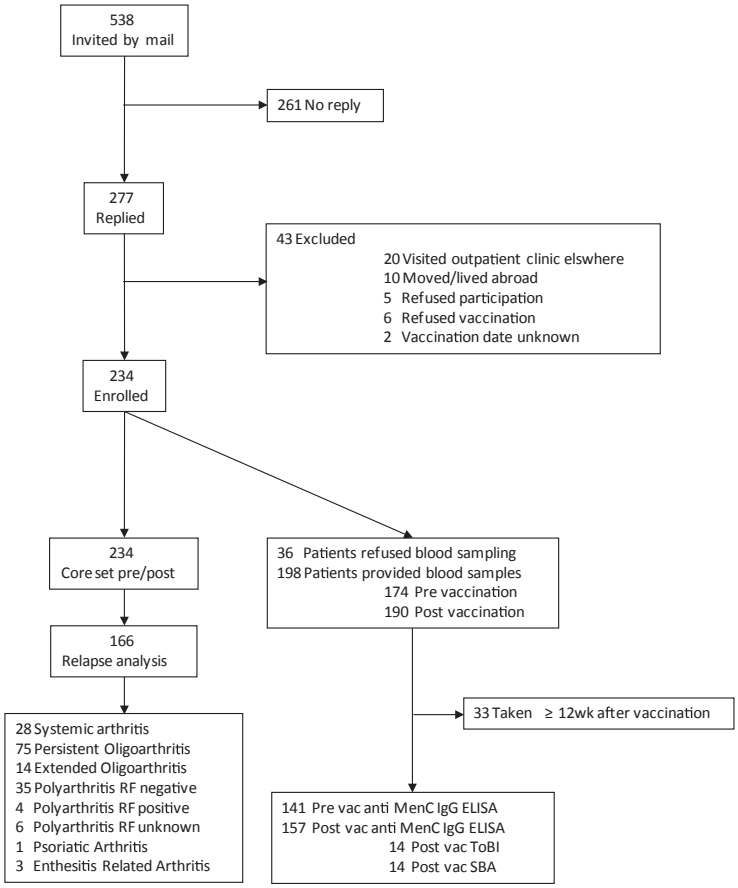


Figure 1 Number of patients with juvenile idiopathic arthritis assessed and enrolled in the study. RF = rheumatoid factor; Pre vac = prevaccination; MenC = meningococcal serogroup C; ELISA = enzyme-linked immunosorbent assay; Post vac = postvaccination; ToBI = toxin-binding inhibition test; SBA = serum bactericidal assay.

informed consent was obtained from patients or their parents. Approval by the medical ethical boards of the participating centers was acquired.

Of 538 invited patients, 277 replied (51.5%). Replying and non-replying patients were comparable in age, sex and JIA disease type. Twenty of the replying patients attended outpatient clinics elsewhere, 10 patients moved elsewhere, 11 refused to participate, and the vaccination dates of 2 patients could not be retrieved. Thus, 234 patients from 5 centers in The Netherlands were enrolled (figure 1). Sixty-five percent of the study subjects were female. At the vaccination date, the mean ± SEM age of the patients was 11.1 ± 4.2 years (range 1.5-18.9 years) and mean ± SEM disease duration was 5.9 ± 3.5 years (range

0.2-16.0 years). The mean age at onset of JIA was 5.3 ± 3.7 years (range 0.5-15.9 years). The group of patients tested for relapse frequency (n=166) was not statistically different from the total cohort (n=234) with respect to demographic, disease, and treatment characteristics.

Definition of medication groups

Postvaccination blood samples were available from 157 patients. The patients were classified based on medication use at the time of MenC vaccination. Group 1 (n=47) received no medications, group 2 (n=41) received nonsteroidal anti-inflammatory drug (NSAID) monotherapy, group 3 received low dosages of methotrexate (MTX) (<10mg/m^2/week) (n=36) or sulfasalazine (n=7) with or without NSAIDs, and group 4 received high dosages of MTX (≥10mg/m^2/week) (n=15), infliximab (n=2), etanercept (n=6), cyclosporin A (n=1) or a combination of MTX and sulfasalazine (n=2) with or without NSAIDs (table 1).Patients in the various medication groups did not differ in age, sex, duration of JIA, or age at onset of JIA. As expected, medication group 4 contained most patients with severe forms of JIA. As expected, medication group 4 contained the most patients with severe forms of JIA (i.e. extended oligoarticular JIA and polyarticular JIA), whereas group 1 contained the most patients with persistent oligoarticular JIA. Consequently, disease activity before vaccination was highest in medication group 4.

MenC conjugate vaccination

The NeisVac-C vaccine (Baxter Healthcare, Vienna, Austria) contains the *N. meningitidis* Z2491 serogroup C polysaccharide (20 µg/ml) conjugated to tetanus toxoid (TT) (20-40 µg/ml). Patients received 1 intramuscular dose of 0.5 ml NeisVac-C during the Dutch national vaccination campaign. All patients were vaccinated, irrespective of disease activity. Vaccination dates were obtained by questionnaire.

Patients were vaccinated between June 1 and December 26, 2002. In 3 patients, MenC vaccination was postponed 6-12 months because of participation in a drug trial in which vaccination was not allowed (n=2) or because of severe uveitis during the national vaccination campaign (n=1). Their clinical and serologic results were included in the analysis.

Outcome measures

Disease relapse was the primary outcome measure and was defined using the internationally validated core set criteria for juvenile arthritis disease activity.[24] Within this core set, a paediatric rheumatologist used a physician global assessment (PGA) to provide an overall impression of disease activity. The PGA was measured on a 10-cm visual analogue scale and converted to scores on a 0-3 scale. The Childhood Health Assessment Questionnaire (C-HAQ) was used to determination overall well-being (C-HAQ well-being) and functional

Table 1 Base line characteristics of the JIA patients*

		Patients tested <12 weeks after MenC vaccination†			
	Total enrolled	**Group 1**	**Group 2**	**Group 3**	**Group 4**
	(N=234)	(N=47)	(N=41)	(N=43)	(N=26)
JIA subtype					
Systemic arthritis	34 (14.5)	11 (23.4)	3 (7.3)	5 (11.6)	3 (10.3)
Persistent oligoarthritis	103 (44.0)	26 (55.3)	18 (43.9)	14 (32.6)	2 (7.7)
Extended oligoarthritis	25 (10.7)	2 (4.3)	5 (12.2)	6 (14.0)	7 (26.9)
Polyarthritis	59 (25.2)	5 (10.6)	10 (24.4)	16 (37.2)	14 (53.8)
Rheumatoid Factor positive/total typed	5/53	0/3	0/9	0/15	3/13
Psoriatic arthritis	4 (1.7)	0	1 (2.4)	2 (4.7)	0
Enthesitis-related arthritis	7 (3.0)	2 (4.3)	3 (7.3)	0	0
Undifferentiated arthritis	2 (0.9)	1 (2.1)	1 (2.4)	0	0
PGA before vaccination‡					
Severely active	8 (3.4)	0	1 (2.4)	4 (9.3)	2 (7.7)
Moderately active	16 (6.8)	1 (2.1)	4 (9.8)	3 (7.0)	5 (19.2)
Mildly active	46 (19.7)	0	13 (31.7)	11 (25.6)	8 (30.8)
Inactive	164 (70.0)	46 (97.9)	23 (56.1)	25 (58.1)	11 (42.3)
Patients taking oral steroids	5 (2.1)	0	0	2 (4.7)	2 (7.7)
Mean ± SD dosage, mg/kg/day	0.11 ± 0.08			0.14 (0.13)	0.08 (0.03)
Range, mg/kg/day	0.05-0.40				

*Except when indicated otherwise, values are the number (%). JIA = juvenile idiopathic arthritis; MenC = meningococcal serogroup C.
†Group 1 = no medication; group 2 = nonsteroidal anti-inflammatory drug (NSAID) monotherapy; group 3 = low-dosage methotrexate (MTX)(< 10mg/m^2/week) (n=36) or sulfasalazine (SSZ) (n=7) with or without NSAIDs; group 4 = high dosage MTX (≥10mg/ m^2/week) (n=15), infliximab (n=2), etanercept (n=6), cyclosporin A (n=1) or a combination of MTX and SSZ (n=2) with or without NSAIDs.
‡Physician's Global Assessment (PGA) of disease activity (0-3 scale), where 0 = inactive, 0.1-1,4 = mildly active, 1.5-2.4 = moderately active and 2.5-3.0 = severely active.

ability (C-HAQ disability), both expressed on a 0-3 scale.[25,26] Active joints were defined as all joints with swelling or with any 2 other signs of inflammation (heat, limited range of motion, tenderness or painful range of motion).[24,27] Limited range of motion was defined for each joint as a loss of at least 5 degrees in any articular movement with respect to the normal amplitude. Erythrocyte sedimentation rate (ESR) completed the core set of 6 criteria. A disease relapse was defined as a worsening of ≥40 % in at least 2 of 6 core set criteria without an improvement of ≥30% in more than 1 of the remaining criteria.[28]

Disease activity parameters as assessed by paediatric rheumatologists during at least 1 visit before and 1 visit after vaccination were compared. For the detection of disease relapses in a large subset of patients (n=166, all from the University Medical Center Utrecht), this assessment of core set criteria was extended to all available outpatient clinic visits and hospitalizations during the entire study period. Not every core set criterion was routinely evaluated, but PGAs and joint counts were performed in all cases. Patients who did not consult their physician between scheduled visits were assumed not to have experienced a disease relapse during that time. This was always confirmed at the next visit.

Serologic analysis

Blood samples were drawn from 198 patients before and after MenC vaccination. Thirty-three of 198 patients were excluded from serologic analysis because their post-vaccination blood sampling was delayed to > 12 weeks after vaccination. We analysed 141 prevaccination and 157 postvaccination samples, of which 133 were paired.

Anti-MenC total IgG antibodies were quantified in serum by enzyme-linked immunosorbent assay using the Centers for Disease Control 1992 reference serum, assigned a value of 24.1 μg/ml anti-MenC IgG.[29,30] The lower limit of antibody detection was 0.24 μg/ml. Serum with undetectable anti-MenC IgG levels was assigned a value of 0.23 μg/ml for mathematical purposes. Low responders were defined based on postvaccination anti-MenC IgG levels of ≤2 μg/ml.[31,32]

The level of anti-TT antibodies was measured using a tetanus toxin-binding inhibition assay at the Laboratory of Vaccine Preventable Diseases, Bilthoven, The Netherlands, as previously described.[33] The lower limit of detection was 0.01 IU/ml.

Serum bactericidal assays (SBAs) against the serogroup C strain (C11, phenotype C:16:P1.7a,1) were performed with baby rabbit serum (Pel-Freez, Rogers, AR) as an exogenous complement source. SBA titres were expressed as the reciprocal of the final serum dilution giving ≥ 50% killing at 60 minutes.[34] Postvaccination bactericidal titres < 8 were considered to predict susceptibility to MenC infection.[32,35-39]

Both the toxin binding inhibition assay and SBA were performed on blood samples from the 4 low responders (anti-MenC IgG ≤ 2μg/ml) and a random sample of 10 out of 153 high responders (anti-MenC IgG > 2μg/ml).

Statistical analysis

To compare the uniformity of the subset of 166 patients included in the relapse analysis with the total cohort (n=234), chi-square tests with expected frequencies of the total

cohort for distribution of categorical variables and one sample T-test for means were used. Changes in paired prevaccination and postvaccination values of core set criteria components were assessed by Wilcoxon signed ranks test.

Risk of relapse was quantified by dividing the number of detected relapses in the exposed or unexposed period by the number of patient-months within that period. Observed patient-months were calculated by multiplying the number of patients (n=166) by the duration of the observed period in months (exposed period n=1 month, unexposed period n=11 months). Relative risk (RR) of relapse was calculated by dividing the risk of relapse during the exposed period by the risk of relapse in the unexposed period. The 95% confidence interval (95% CI) was calculated using the following equation: $e^{\ln RR \pm 1.96\sqrt{1/A1 + 1/A0}}$, in which A_1 represents the number of relapses in the exposed period and A_0 the number of relapses in the unexposed period. Chi-square tests were used to analyse seasonal variability of relapses. The MIXOR program (version 2.0) was used for logistic regression analysis of longitudinal data.[40]

Distribution of geometric mean concentrations and geometric mean titers were extremely skewed. Therefore, we used nonparametric tests such as Mann-Whitney U test or the Kruskall-Wallis test for comparisons between 2 or multiple groups, Wilcoxon's signed ranks test for paired variables and chi-square test for ordinal variables. For comparison of patient characteristics and anti-MenC IgG geometric mean concentrations between medication groups, one-way analysis of variance was performed on (natural log -transformed) data. The Bonferroni adjustment was applied for multiple comparisons. Pearson's correlation coefficient was calculated for natural log transformed titer data.

Statistical analysis was performed using SPSS software, version 12.0.2 (SPSS, Chicago, IL). P values less than 0.05 were considered significant.

RESULTS

JIA disease activity

No worsening of disease activity was seen, based on mean core set criteria values during 6 months after MenC vaccination compared with the 6 months before vaccination as measured in 234 JIA patients (figure 2). A significant amelioration in PGA and limited range of motion was observed after MenC vaccination, but this was too small to be of clinical significance.

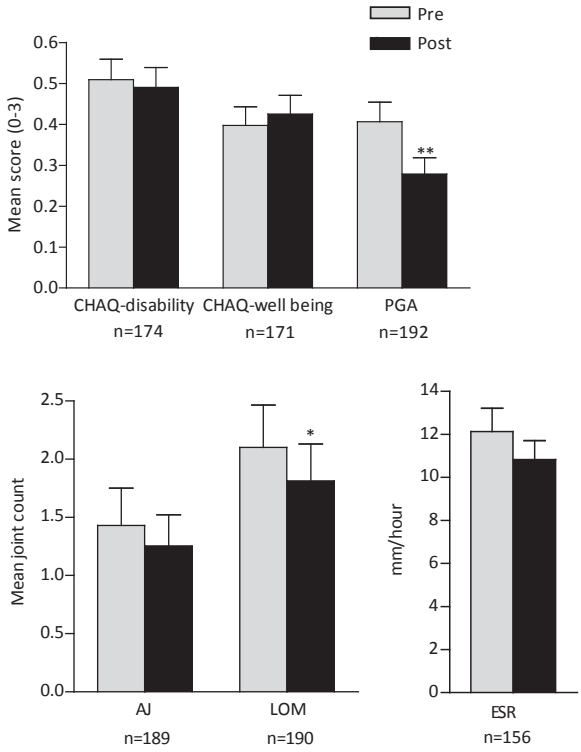


Figure 2 Core set criteria scores 6 months before (Pre) and 6 months after (Post) vaccination with meningococcal serogroup C conjugate in 234 juvenile idiopathic arthritis patients. Values are the mean and SEM. C-HAQ = Childhood Health Assessment Questionnaire; PGA = physician's global assessment; AJ = arthritic joints; LOM = limited range of motion; ESR = erythrocyte sedimentation rate. *= p <0.05; **= p <0.005, versus before vaccination.

Relapses

We further analysed data from 747 visits (373 prevaccination and 374 postvaccination) in a single-center subgroup of 166 patients. A total of 158 relapses were detected in 97 patients (figure 3). Four patients did not visit the outpatient clinic at all during the study period, indicating they did not experience any flares. Ten patients experienced a disease relapse within 1 month after vaccination. The risk of a relapse in the month after vaccination was 6.0%, whereas the risk of a relapse in the remaining 11 months was 8.1%. The resulting RR of relapse in the exposed period was 0.74 (95% CI 0.39;1.41). Relative risks of relapse calculated with an exposed period of 2, 3 or 6 months after MenC vaccination were similar (RR 0.81 [95% CI 0.48;1.38], RR 0.76 [95% CI 0.52;1.12] and RR 0.52 [95% CI 0.37;0.72], respectively). Additional statistical analysis using a program for logistic regres-

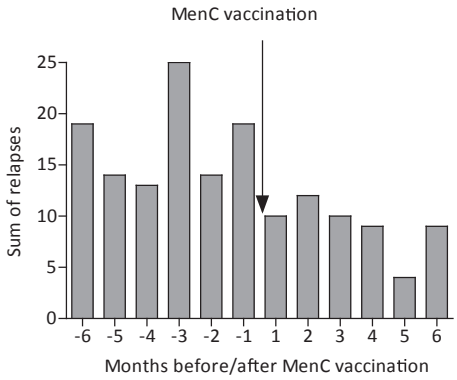


Figure 3 Distribution of relapses in 97 juvenile idiopathic arthritis patients before and after meningococcal serogroup C (MenC) vaccination.

sion of longitudinal data did not detect any increase in risk of relapse after vaccination. No seasonal influence on relapse frequency was seen (p = 0.09).

Efficacy of vaccination

Before vaccination, anti-MenC IgG geometric mean concentrations were comparable among medication groups (table 2). The group as a whole (n=157) showed a significant rise in anti-MenC IgG geometric mean concentration, from 0.4 µg/ml before vaccination to 28.9 µg/ml after vaccination (range 1.0-1,820.5 µg/ml)(p<0.0005). Anti-MenC IgG geometric mean concentrations were significantly lower in patients in medication groups 3 and 4 compared with those in groups 1 and 2 (table 2). Four of 157 tested patients (2.5%) had anti-MenC IgG levels ≤ 2 µg/ml after vaccination. Three of these low responders took low-dose MTX, 2 of them in combination with etanercept. The other low responder was being treated with sulfasalazine. None of the low responders took steroids during the study. JIA was inactive in 2 low responders on the vaccination date, while the other 2 had mild and

Table 2 Anti-MenC IgG geometric mean concentrations and frequency in JIA patients**,** categorized by medication group*

	Group 1	**Group 2**	**Group 3**	**Group 4**
Anti-MenC IgG geometric mean concentration, µg/ml				
Before vaccination	0.38	0.41	0.32	0.36
After vaccination	41.00	46.93	17.53	16.28
Low responders (anti-MenC IgG ≤ 2 mg/ml), no. (%)	0	0	2 (4.7)	2 (7.7)

*For between-group differences in postvaccination anti-MenC IgG geometric mean concentrations, p = 0.002, group 1 versus group 3; p= 0.01, group 1 versus group 4; p = 0.003, group 2 versus group 3; p = 0.012, group 2 versus group 4. There were no significant differences before vaccination. See table 1 for definitions and description of the medication groups.

moderately active disease. Since the expected relapse frequency in low responders was <5 in each medication group, anti-MenC IgG response ≤ 2 µg/ml does not appear to be associated with an increased risk of relapse.

The 4 patients who took oral steroids all took MTX as well, and thus belonged to medication groups 3 and 4. Their mean anti-MenC IgG level did not differ from that of the other patients in groups 3 and 4 (p=0.63 and p=0.73, respectively). Anti-MenC IgG levels in patients with systemic-onset JIA were comparable with levels measured in all other patients tested.

Patients with an anti-MenC response of > 2 µg/ml (high responders) showed a significant mean 17-fold rise in anti-TT antibody titers after MenC vaccination (postvaccination geometric mean titer 14.95, p<0.001), whereas low responders (anti-MenC ≤ 2 µg/ml) had only a 1.5-fold rise in anti-TT antibodies (postvaccination geometric mean titer 3.19, p=0.72).

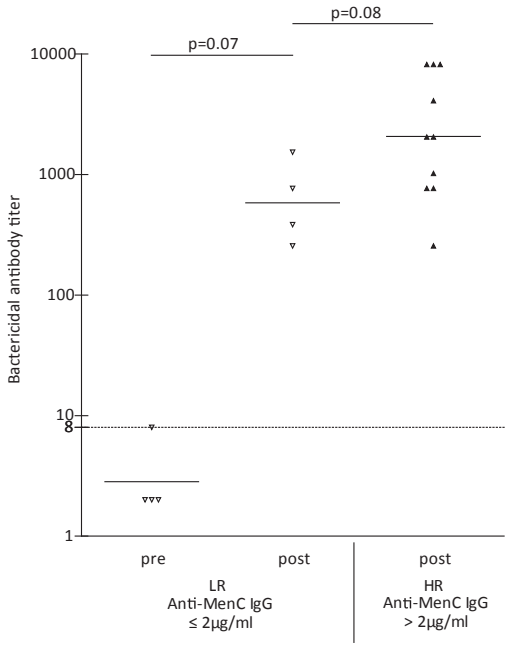


Figure 4 Serum bactericidal antibody titers in patients with juvenile idiopathic arthritis before and after vaccination with meningococcal serogroup C (MenC). Low responders (LR) had postvaccination anti-MenC IgG levels ≤2 µg/ml; high responders (HR) had postvaccination anti-MenC IgG levels >2 µg/ml. Broken line indicates the lower threshold of protection against susceptibility to meningococcal C infection. Each triangle represents an individual patient (all patients in the low responder group, and a random sample of 10 patients from the high responder group). Horizontal lines indicate means.

All tested JIA patients including the 4 with a low anti-MenC IgG response were able to mount SBA titers ≥ 8 (figure 4). Although after vaccination a mean 206-fold rise in SBA geometric mean titer was observed in the 4 low responders, the difference from prevaccination titers did not reach significance (p=0.07) due to low numbers. The postvaccination SBA titer in the low responders was not significantly different from that in the high responders (p=0.08).

DISCUSSION

This study shows that MenC vaccination is safe and effective for use in JIA. In theory, molecular mimicry of components of the vaccine with self antigens, combined with bystander activation as well as a loss of regulatory mechanisms could account for aggravation of autoimmunity after vaccination.[1,41] The occurrence of arthritis in children and adults after natural infection with *Neisseria meningitidis* indeed suggests cross-reactivity between nonself- and self-antigens.[42-44]

In earlier studies, patients with nephrotic syndrome seemed to have increased frequency of relapse after MenC vaccination.[22] Patients with idiopathic thrombocytopenic purpura also are at a particular risk of relapse after administration of life-attenuated measles, mumps, and rubella vaccines.[45] Our study in a large group of JIA patients however, did not detect any worsening of disease activity within six months after MenC vaccination. Moreover, the risk of a relapse in the month after vaccination did not differ from the risk in the unexposed period. Results remained stable when the length of the exposed period was varied from 1 to 6 months. This is consistent with previous studies in which RA and JIA patients tolerated influenza and Hepatitis B vaccinations with no ill effects.[8-10,46,47]

Because children could not be included in a double-blind placebo-controlled study for ethical reasons, the Dutch vaccination campaign against MenC yielded a unique study cohort. Because JIA is the most prevalent autoimmune disease in children, we selected this patient group. Even though it was possible to investigate one of the largest cohorts of vaccinated JIA patients, we realize that statistical power for comparison of treatment groups is limited.

A large proportion (48.5%) of invited patients did not reply to the invitation to participate. A low number of patients agreeing to participate is common in other vaccination studies. Studies on MenC or influenza vaccination have yielded participant rates of 47%, 66% and 53%.[22,48,49] Possible explanations for the low participant rate in this study were the lack of information provided by the authorities concerning vaccination of patients with an

autoimmune disease, parental concerns about a newly introduced vaccine and the need for 2 blood draws from the children participating in this study. As baseline characteristics of nonreplying and replying patients were the same, selection bias is likely to have been minimal.

Since the majority of patients were vaccinated during the summer, there is a potential influence of seasonal variability on relapse frequency. In our patient group, though, a clear seasonality of relapses was absent, as previously established in other large studies.[50,51]

The second aim of our study was to assess the influence of immunosuppressive medication taken by JIA patients on the efficacy of MenC vaccines. The anti-MenC IgG geometric mean concentrations in our JIA patients overall (28.9 μg/ml) was consistent with anti-MenC IgG values of 29.1-51.6 μg/ml observed in 2 separate school cohorts (primary school [age 4.3 years]) and secondary school [age 15.1 years]) and even higher than those observed by others in healthy adults (17.0 μg/ml) or in healthy children aged 12-18 months (13.3 μg/ml) 4 weeks after a single dose of the NeisVac-C vaccine.[52-54] Because of this difference in anti-MenC IgG geometric mean concentrations measured between our JIA patients and healthy controls in the literature, we tested 12 healthy volunteers (age 21-50 years) before and 6 weeks after vaccination. Their anti-MenC IgG geometric mean concentrations (29.6 μg/ml [range 2.1-112.4]) was no different from that in the total group of JIA patients reported here (p=0.631 by Mann-Whitney U test). Like JIA patients, all healthy controls showed protective serum bactericidal activity. This further supports our conclusion that MenC vaccination in JIA patients leads to adequate serum antibody levels.

We did notice lower postvaccination mean anti-MenC IgG geometric mean concentrations in patients using DMARDs. MTX and, less consistently, tumor necrosis factor α (TNFa) blockade and prednisone have earlier been associated with lower pneumococcal antibody responses in adults, but in studies in children with JIA or asthma this has not been shown.[8-10,55-57] Because in our study only 1 patient received anti-TNFa monotherapy (and this patient did have an antibody response >2 μg/ml) and oral steroids were always taken in combination with MTX, we could not assess the effect of these drugs separately. No correlation was found between total IgG levels and anti-MenC IgG levels (r=0.17), indicating that lower anti-MenC levels could not be explained by other immunodeficiencies.

The low response to the MenC part of the vaccine was associated with a low response to the conjugate protein TT. Using an SBA, we measured the ability of these low responders to raise sufficient bactericidal activity after a single dose of the vaccine.[58] Postvaccination SBA titers in low responders (mean SBA titer 736) were as high as in high responders and well above the earlier reported SBA titer in healthy children aged 12-18 months (mean

564).[53] Importantly, all SBA titers after vaccination in patients with a low anti-MenC response were above the level that predicts protection. Therefore, all tested JIA patients seem to be adequately protected against meningococcal serogroup C disease after MenC vaccination, irrespective of the immunosuppressive treatment given.[59]

ACKNOWLEDGEMENTS

We thank Erica Roks, Eline van Amerongen, Mark Klein, Wilco de Jager, Irina Tcherniaeva, Corine Nellestijn, Renée van Soesbergen, Koert Dolman, Rebecca ten Cate, Yvonne Koopman and Vincent Zonneveld for patient referral and technical assistance.

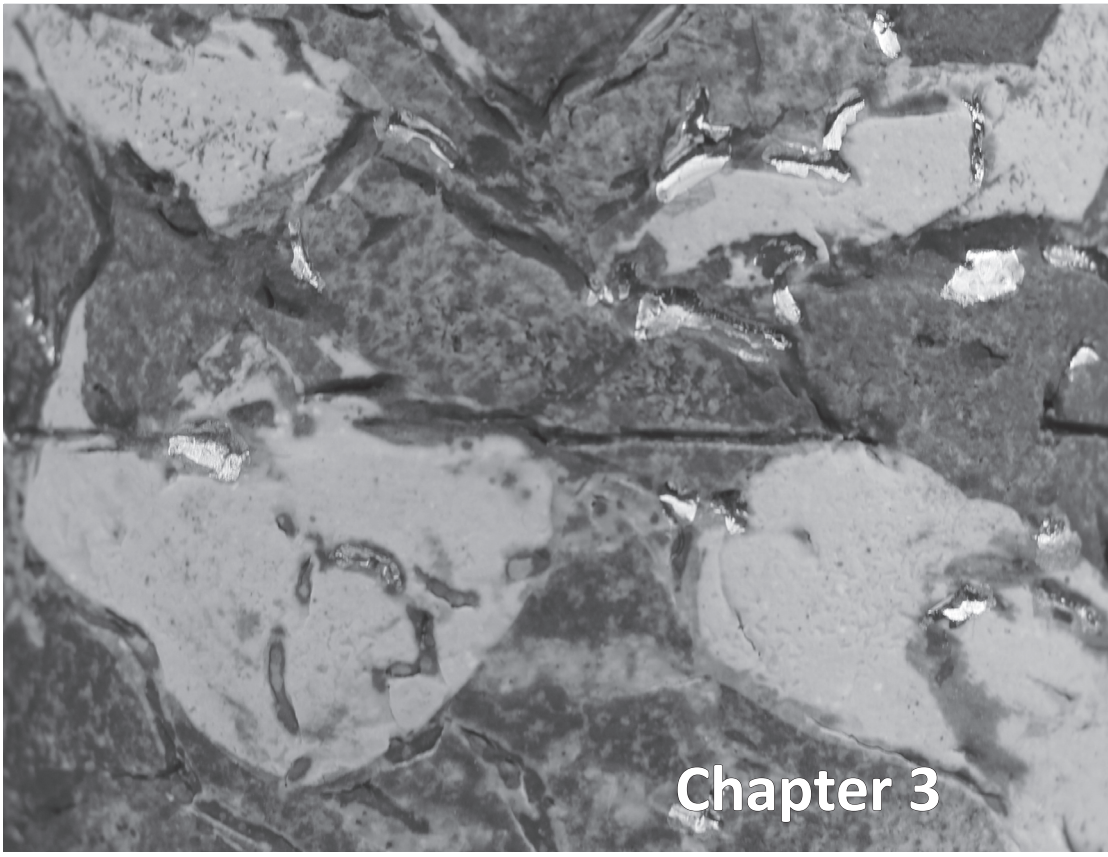
REFERENCE LIST

1. Wraith DC, Goldman M, Lambert PH. Vaccination and autoimmune disease: what is the evidence? *Lancet.* 2003;362:1659-1666.
2. Ravel G, Christ M, Horand F, Descotes J. Autoimmunity, environmental exposure and vaccination: is there a link? *Toxicology.* 2004;196:211-216.
3. Schattner A. Consequence or coincidence? The occurrence, pathogenesis and significance of autoimmune manifestations after viral vaccines. *Vaccine.* 2005;23:3876-3886.
4. Gellin BG, Schaffner W. The risk of vaccination--the importance of "negative" studies. *N Engl J Med.* 2001;344:372-373.
5. Confavreux C, Suissa S, Saddier P, Bourdes V, Vukusic S. Vaccinations and the risk of relapse in multiple sclerosis. Vaccines in Multiple Sclerosis Study Group. *N Engl J Med.* 2001;344:319-326.
6. Ascherio A, Zhang SM, Hernan MA, Olek MJ, Coplan PM, Brodovicz K, et al. Hepatitis B vaccination and the risk of multiple sclerosis. *N Engl J Med.* 2001;344:327-332.
7. Graves PM, Barriga KJ, Norris JM, Hoffman MR, Yu L, Eisenbarth GS, et al. Lack of association between early childhood immunizations and beta-cell autoimmunity. *Diabetes Care.* 1999;22:1694-1697.
8. Kanakoudi-Tsakalidou F, Trachana M, Pratsidou-Gertsi P, Tsitsami E, Kyriazopoulou-Dalaina V. Influenza vaccination in children with chronic rheumatic diseases and long-term immunosuppressive therapy. *Clin Exp Rheumatol.* 2001;19:589-594.
9. Malleson PN, Tekano JL, Scheifele DW, Weber JM. Influenza immunization in children with chronic arthritis: a prospective study. *J Rheumatol.* 1993;20:1769-1773.
10. Kasapcopur O, Cullu F, Kamburoglu-Goksel A, Cam H, Akdenizli E, Calykan S, et al. Hepatitis B vaccination in children with juvenile idiopathic arthritis. *Ann Rheum Dis.* 2004;63:1128-1130.
11. Offit PA, Hackett CJ. Addressing parents' concerns: do vaccines cause allergic or autoimmune diseases? *Pediatrics.* 2003;111:653-659.
12. Hak E, Schonbeck Y, De Melker H, Van Essen GA, Sanders EA. Negative attitude of highly educated parents and health care workers towards future vaccinations in the Dutch childhood vaccination program. *Vaccine.* 2005;23:3103-3107.
13. Inspectorate for Dutch Health Care. Vaccination situation in the Netherlands per January 2002 [with abstract in English]. The Hague; 2004 Aug 1.
14. Doran MF, Crowson CS, Pond GR, O'Fallon WM, Gabriel SE. Frequency of infection in patients with rheumatoid arthritis compared with controls: a population-based study. *Arthritis Rheum.* 2002;46:2287-2293.
15. Davies K, Woo P. Immunization in rheumatic diseases of childhood: an audit of the clinical practice of British Paediatric Rheumatology Group members and a review of the evidence. *Rheumatology (Oxford).* 2002;41:937-941.
16. BSR Clinical Affairs Committee. Vaccinations in the immunocompromised person: guidelines for the patient taking immunosuppressants, steroids and the new biologic therapies. 1-2. 28-2-0002. British Society for Rheumatology.
17. Conyn-van Spaendonck MA, Reintjes R, Spanjaard L, van Kregten E, Kraaijeveld AG, Jacobs PH. Meningococcal carriage in relation to an outbreak of invasive disease due to Neisseria meningitidis serogroup C in the Netherlands. *J Infect.* 1999;39:42-48.
18. van Furth AM, Vandenbroucke-Grauls CM. [Universal vaccination against group C meningococci and pneumococci; advice from the Health Council of the Netherlands]. *Ned Tijdschr Geneeskd.* 2002;146:932-933.

19. WHO. Causality assessment of adverse events following immunization. *Wkly Epidemiol Rec.* 2001;76:85-89.
20. Miller E, Waight P, Farrington CP, Andrews N, Stowe J, Taylor B. Idiopathic thrombocytopenic purpura and MMR vaccine. *Arch Dis Child.* 2001;84:227-229.
21. Benjamin CM, Chew GC, Silman AJ. Joint and limb symptoms in children after immunisation with measles, mumps, and rubella vaccine. *BMJ.* 1992;304:1075-1078.
22. Abeyagunawardena AS, Goldblatt D, Andrews N, Trompeter RS. Risk of relapse after meningococcal C conjugate vaccine in nephrotic syndrome. *Lancet.* 2003;362:449-450.
23. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. *J Rheumatol.* 2004;31:390-392.
24. Giannini EH, Ruperto N, Ravelli A, Lovell DJ, Felson DT, Martini A. Preliminary definition of improvement in juvenile arthritis. *Arthritis Rheum.* 1997;40:1202-1209.
25. Ruperto N, Ravelli A, Pistorio A, Malattia C, Cavuto S, Gado-West L, et al. Cross-cultural adaptation and psychometric evaluation of the Childhood Health Assessment Questionnaire (CHAQ) and the Child Health Questionnaire (CHQ) in 32 countries. Review of the general methodology. *Clin Exp Rheumatol.* 2001;19:S1-S9.
26. Wulffraat N, van der Net JJ, Ruperto N, Kamphuis S, Prakken BJ, Ten Cate R, et al. The Dutch version of the Childhood Health Assessment Questionnaire (CHAQ) and the Child Health Questionnaire (CHQ). *Clin Exp Rheumatol.* 2001;19:S111-S115.
27. Ritchie DM, Boyle JA, McInnes JM, Jasani MK, Dalakos TG, Grieveson P, et al. Clinical studies with an articular index for the assessment of joint tenderness in patients with rheumatoid arthritis. *Q J Med.* 1968;37:393-406.
28. Brunner HI, Lovell DJ, Finck BK, Giannini EH. Preliminary definition of disease flare in juvenile rheumatoid arthritis. *J Rheumatol.* 2002;29:1058-1064.
29. Gheesling LL, Carlone GM, Pais LB, Holder PF, Maslanka SE, Plikaytis BD, et al. Multicenter comparison of Neisseria meningitidis serogroup C anti-capsular polysaccharide antibody levels measured by a standardized enzyme-linked immunosorbent assay. *J Clin Microbiol.* 1994;32:1475-1482.
30. Holder PK, Maslanka SE, Pais LB, Dykes J, Plikaytis BD, Carlone GM. Assignment of Neisseria meningitidis serogroup A and C class-specific anticapsular antibody concentrations to the new standard reference serum CDC1992. *Clin Diagn Lab Immunol.* 1995;2:132-137.
31. Maslanka SE, Tappero JW, Plikaytis BD, Brumberg RS, Dykes JK, Gheesling LL, et al. Age-dependent Neisseria meningitidis serogroup C class-specific antibody concentrations and bactericidal titers in sera from young children from Montana immunized with a licensed polysaccharide vaccine. *Infect Immun.* 1998;66:2453-2459.
32. Gold R, Lepow ML, Goldschneider I, Draper TF, Gotshlich EC. Kinetics of antibody production to group A and group C meningococcal polysaccharide vaccines administered during the first six years of life: prospects for routine immunization of infants and children. *J Infect Dis.* 1979;140:690-697.
33. Hendriksen CF, van der Gun JW, Kreeftenberg JG. Combined estimation of tetanus and diphtheria antitoxin in human sera by the in vitro Toxin-Binding Inhibition (ToBI) test. *J Biol Stand.* 1989;17:191-200.
34. Maslanka SE, Gheesling LL, Libutti DE, Donaldson KB, Harakeh HS, Dykes JK, et al. Standardization and a multilaboratory comparison of Neisseria meningitidis serogroup A and C serum bactericidal assays. The Multilaboratory Study Group. *Clin Diagn Lab Immunol.* 1997;4:156-167.

35. Borrow R, Andrews N, Goldblatt D, Miller E. Serological basis for use of meningococcal serogroup C conjugate vaccines in the United Kingdom: reevaluation of correlates of protection. *Infect Immun.* 2001;69:1568-1573.
36. Fairley CK, Begg N, Borrow R, Fox AJ, Jones DM, Cartwright K. Conjugate meningococcal serogroup A and C vaccine: reactogenicity and immunogenicity in United Kingdom infants. *J Infect Dis.* 1996;174:1360-1363.
37. King WJ, MacDonald NE, Wells G, Huang J, Allen U, Chan F, et al. Total and functional antibody response to a quadrivalent meningococcal polysaccharide vaccine among children. *J Pediatr.* 1996;128:196-202.
38. Mitchell LA, Ochnio JJ, Glover C, Lee AY, Ho MK, Bell A. Analysis of meningococcal serogroup C-specific antibody levels in British Columbian children and adolescents. *J Infect Dis.* 1996;173:1009-1013.
39. Lepow ML, Beeler J, Randolph M, Samuelson JS, Hankins WA. Reactogenicity and immunogenicity of a quadrivalent combined meningococcal polysaccharide vaccine in children. *J Infect Dis.* 1986;154:1033-1036.
40. Hedeker D, Gibbons RD. MIXOR: a computer program for mixed-effects ordinal regression analysis. *Comput Methods Programs Biomed.* 1996;49:157-176.
41. Wucherpfennig KW. Mechanisms for the induction of autoimmunity by infectious agents. *J Clin Invest.* 2001;108:1097-1104.
42. Schaad UB. Arthritis in disease due to Neisseria meningitidis. *Rev Infect Dis.* 1980;2:880-888.
43. Likitnukul S, McCracken GH, Jr., Nelson JD. Arthritis in children with bacterial meningitis. *Am J Dis Child.* 1986;140:424-427.
44. Edwards MS, Baker CJ. Complications and sequelae of meningococcal infections in children. *J Pediatr.* 1981;99:540-545.
45. Vlacha V, Forman EN, Miron D, Peter G. Recurrent thrombocytopenic purpura after repeated measles-mumps-rubella vaccination. *Pediatrics.* 1996;97:738-739.
46. Chalmers A, Scheifele D, Patterson C, Williams D, Weber J, Shuckett R, et al. Immunization of patients with rheumatoid arthritis against influenza: a study of vaccine safety and immunogenicity. *J Rheumatol.* 1994;21:1203-1206.
47. Elkayam O, Yaron M, Caspi D. Safety and efficacy of vaccination against hepatitis B in patients with rheumatoid arthritis. *Ann Rheum Dis.* 2002;61:623-625.
48. Lasky T, Terracciano GJ, Magder L, Koski CL, Ballesteros M, Nash D, et al. The Guillain-Barre syndrome and the 1992-1993 and 1993-1994 influenza vaccines. *N Engl J Med.* 1998;339:1797-1802.
49. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N Engl J Med.* 2004;350:896-903.
50. Uziel Y, Pomeranz A, Brik R, Navon P, Mukamel M, Press J, et al. Seasonal variation in systemic onset juvenile rheumatoid arthritis in Israel. *J Rheumatol.* 1999;26:1187-1189.
51. Feldman BM, Birdi N, Boone JE, Dent PB, Duffy CM, Ellsworth JE, et al. Seasonal onset of systemic-onset juvenile rheumatoid arthritis. *J Pediatr.* 1996;129:513-518.
52. Burrage M, Robinson A, Borrow R, Andrews N, Southern J, Findlow J, et al. Effect of vaccination with carrier protein on response to meningococcal C conjugate vaccines and value of different immunoassays as predictors of protection. *Infect Immun.* 2002;70:4946-4954.
53. Richmond P, Borrow R, Goldblatt D, Findlow J, Martin S, Morris R, et al. Ability of 3 different meningococcal C conjugate vaccines to induce immunologic memory after a single dose in UK toddlers. *J Infect Dis.* 2001;183:160-163.

54. Richmond P, Goldblatt D, Fusco PC, Fusco JD, Heron I, Clark S, et al. Safety and immunogenicity of a new Neisseria meningitidis serogroup C-tetanus toxoid conjugate vaccine in healthy adults. *Vaccine.* 1999;18:641-646.
55. Kapetanovic MC, Saxne T, Sjoholm A, Truedsson L, Jonsson G, Geborek P. Influence of methotrexate, TNF blockers and prednisolone on antibody responses to pneumococcal polysaccharide vaccine in patients with rheumatoid arthritis. *Rheumatology (Oxford).* 2006;45:106-111.
56. Mease PJ, Ritchlin CT, Martin RW, Gottlieb AB, Baumgartner SW, Burge DJ, et al. Pneumococcal vaccine response in psoriatic arthritis patients during treatment with etanercept. *J Rheumatol.* 2004;31:1356-1361.
57. Park CL, Frank AL, Sullivan M, Jindal P, Baxter BD. Influenza vaccination of children during acute asthma exacerbation and concurrent prednisone therapy. *Pediatrics.* 1996;98:196-200.
58. Nicholson A, Lepow IH. Host defense against Neisseria meningitidis requires a complement-dependent bactericidal activity. *Science.* 1979;205:298-299.
59. Andrews N, Borrow R, Miller E. Validation of serological correlate of protection for meningococcal C conjugate vaccine by using efficacy estimates from postlicensure surveillance in England. *Clin Diagn Lab Immunol.* 2003;10:780-786.



Vaccination leads to an aberrant FOXP3 T cell response in non-remitting Juvenile Idiopathic Arthritis

Arash Ronaghy, Wilco de Jager, Evelien Zonneveld-Huijssoon, Mark R Klein, Femke van Wijk, Ger T Rijkers, Wietse Kuis, Nico M Wulffraat, Berent J Prakken

Annals of the Rheumatic Diseases 2011;70:2037-2043

ABSTRACT

Objective

To investigate how Meningococcal C vaccination in patients with remitting (oligoarticular) or progressive (polyarticular) Juvenile Idiopathic Arthritis (JIA) influences the specific T cell response to both the vaccine and heat shock protein (HSP) 60, a regulatory auto-antigen in JIA.

Methods

Twenty-six oligoarticular (OA), 28 polyarticular (PA) JIA patients, and 20 healthy adults were studied before and after MenC vaccination in a prospective follow up study. T cell proliferation assay, flow cytometry, carboxyfluorescin diacetate succinimidyl ester (CFSE) staining and multiplex immunoassay were performed to quantify and qualify the antigen-specific immune responses.

Results

Peripheral blood mononuclear cells (PBMC) from polyarticular JIA patients exemplified higher antigen-specific CD4+ T cell proliferation, IL-2, and TNFα production when compared with oligoarticular JIA or healthy individuals after vaccination. Furthermore, in polyarticular JIA antigen-induced CD4+CD25^{bright} or CD4+FOXP3+ T cells did not increase upon vaccination.

Conclusion

Polyarticular JIA CD4+FOXP3+ T cells did not respond to vaccination and demonstrated a higher percentage of cells irrespective of vaccination when compared with the oligoarticular JIA. These cells are either activated T cells and/or regulatory cells unable to regulate the antigen-specific immune response after vaccination. When compared with oligoarticular JIA, the increased IL-2 and TNFα production underline the immune hyperresponsiveness of polyarticular JIA PBMC to an antigenic trigger. As this may hold a risk for derailment, these findings could provide a cellular basis for the presumed relationship between environmental triggers and disease in human autoimmune diseases.

INTRODUCTION

Vaccinations have reduced morbidity and mortality.[1,2] Yet, concerns exist regarding a correlation between vaccines and autoimmunity.[3-5] Despite a few cases of association, epidemiological reports attest to their safety.[6-11] However, concerns remain about the susceptibility of those genetically predisposed to developing or aggravating autoimmunity without an overall increase of population incidence.[12] For example, in juvenile idiopathic arthritis (JIA) a temporal relationship between disease onset, childhood vaccination, remissions and flares[13] hint at a possible relation of JIA disease activity and vaccinations or infections.[14-17] A nationwide Meningococcal C (MenC) immunisation campaign in children (1-19 years) in The Netherlands did not reveal an aggravation of disease or decreased efficacy of the vaccine in JIA patients.[18-21] The possibility of differing cellular and molecular responses to MenC vaccination in those susceptible led us to investigate the specific T (regulatory) cell immune response induced by MenC vaccination in JIA patients (versus healthy controls). Two subtypes of JIA were used for this study: the relatively benign (oligoarticular JIA) and the more progressive (polyarticular JIA) course. The aim was to investigate how a specific environmental trigger (vaccination with MenC) influences the T cell response towards both the MenC vaccine antigen, and a well-studied arthritis-related auto-antigen (heat shock protein (HSP) 60).

MATERIALS AND METHODS

Patients and healthy controls

JIA patients (1-18 years) diagnosed according to the criteria of the International League of Associations for Rheumatology, were eligible.[13] Twenty-six persistent oligoarticular JIA and 28 polyarticular JIA patients (including three with extended oligoarticular JIA) were included in this prospective follow-up study (table 1) and approved by the local ethical committee. Twenty healthy adults (HC, average age 29.9 $\pm$ 5.5 years) voluntarily received MenC vaccinations and were included as controls. Ethical considerations precluded aged matched control children. Informed consent was obtained from both the HC and either from the parents, the guardians, or directly from the JIA patients if aged 12 years or older.

Disease activity

The physician global assessment measures the disease activity by a pediatric rheumatologist on a 10 cm visual analogue scale and was converted to 0-3 scores.

Table 1 Clinical characteristics of the patient populations at the time of vaccination.

JIA patients enrolled	Oligoarticular JIA	Polyarticular JIA
Number (%)	26 (48%)	23 (47%)
Female/Male	17/9	15/8
Age		
Mean in years (±SD)(Range)	10.9 (±3.9)(3.1-18.0)	11.1 (±2.4)(7.6-14.0)
RF positive	0 (0%)	2 (10%)*
Disease duration		
Mean in years (±SD)(Range)	6.6 (±3.6)(0.7-14.5)	6.6 (±3.5)(0.2-12.4)
Age at onset JIA		
Mean in years (±SD)(Range)	4.4 (±3.5)(0.9-14.5)	4.5 (±1.2)(1.1-10.6)
ESR (± SD)	7.3 (± 2.0)	21.7 (± 12.0)
Active joint count (± SD)	0.6 (+ 1.0)	2.1 (+ 1.0)
PGA (± SD)(Range)	0.7 (±0.9)(0-2.8)	1.0 (±1.1)(0-2.5)
Medication		
Group 1	10 (38.5 %)	5 (22%)
Group 2	9 (34%)	5 (22%)
Group 3	6 (23%)	6 (26%)
Group 4	1 (4%)	7 (30%)
Oral steroids (low dose)	0 (0%)	1 (4%)

* only 20 polyarticular JIA patients were tested for RF positivity.
Medication: group 1, no medication; group2, non-steroidal anti-inflammatory drugs; group 3, low-dose methotrexate (≤10 mg/m2 per week) or sulfasalazine; group 4, high-dose methotrexate (>10 mg/m2 per week) and/or anti-tumour necrosis factor alpha therapy (etanercept or infliximab).
ESR, erythrocyte sedimentation rate; JIA, juvenile idiopathic arthritis; RF, rheumatoid factor; PGA, physician global assessment (0=inactive, 1=mildly active, 2=moderately active, 3=severely active).

Definition of medication groups

During MenC vaccination, patient medication regimens were classified into (table 1): no medication (group 1), non-steroidal anti-inflammatory drugs (NSAID; group 2), low-dose methotrexate (≤10 mg/m^{2}per week) or sulfasalazine with or without NSAID (group 3), high-dose methotrexate (> 10 mg/m^2 per week), or anti-tumour necrosis factor (TNF) therapies (infliximab or etanercept) with or without NSAID (group 4).

MenC conjugate vaccine

The MenC vaccine (NeisVac-C vaccine; Baxter, Vienna, Austria) consists of the *Neisseria Meningitidis* serogroup C polysaccharide (20 µg/ml) conjugated to tetanus toxoid (TT, 20-40 µg/ml). All participants received one intramuscular dose of 0.5 ml MenC vaccine during the Dutch national vaccination campaign. For in-vitro studies, the vaccine was dissolved in 0.7% $NaCl_2$ (MenC antigen; gift of Baxter, Beltsville, Maryland, USA).

Cell culture conditions

Peripheral blood mononuclear cells (PBMC) from 54 JIA and 20 healthy adult controls were isolated from heparinised blood using Ficoll density gradient centrifugation (Pharmacia, Uppsala, Sweden). Cells were cultured (2×10^6 cells/ml in 100 µL per well) in RPMI 1640 supplemented with 2 mmol/l glutamine, 100 U/ml penicillin and streptomycin (Gibco BRL; Gaithersburg, Maryland, USA), and 10 v/v% heat-inactivated human AB-positive serum (Sanquin Blood Bank, Amsterdam, The Netherlands) in round-bottomed 96-well plates (Nunc, Roskilde, Denmark) at 37°C in 5% carbon dioxide with 100% relative humidity.

T cell proliferation

To quantify antigen-specific T cell proliferation, T cells were cultured in triplicate for 120 h, with or without 1 or 10 µg/ml MenC antigen, 1.5 µg/ml TT (National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands), 10 µg/ml human HSP60 (Stressgen, Victoria, Canada) or control antigens: diphtheria-toxoid (DT, 1.5 µg/ml; RIVM) and 10 µg/ml ovalbumin. The lipopolysaccharide content of HSP60 was below 3 EU/ml (Cambreex Bioscience, Verviers, Belgium). The last 16 h were cultured with thymidine (^{3}H, 1 µCi per well; ICN Biomedicals, Amsterdam, The Netherlands). Liquid scintillation measured incorporated radioactivity expressed as stimulation index (SI), calculated as the mean counts per minute of cells cultured with divided by cells cultured without antigen.

Flowcytometry: cytokines and FOXP3 staining

Seventy-two-hour cultured PBMC were stained with diluted phycoerythrin, fluorescein isothiocyanate, cychrome-labeled antihuman monoclonal antibodies CD4 (RPA-T4; BD Biosciences, San Jose, California, USA) and CD25 (clone 2A3; BD Biosciences). For intracellular cytokine staining, PBMC were cultured in medium with or without antigens. Except for FOXP3 staining, in the last 4 h of culture, monensin (BD Biosciences) was added. After staining, the cells were fixed, permeabilised and stained with antihuman monoclonal antibodies as described by BD Biosciences: interleukin (IL)-4 (8D4-8), IL-10 (JES3-19F1), TNFα (Mab11), and interferon (IFN)-γ (4S.133). Finally, cells were analyzed on a FACS-calibur using software (BD Biosciences).

CFSE staining

PBMC were mixed with 0.5 µM carboxyfluorescin diacetate succinimidyl ester (CFSE; Molecular Probes, Eugene, Oregon, USA) for 5 min at 37°C, washed, and cultured for 7 days with and without the above antigens. For the analysis of the proliferative response of PBMC CD4 T cells, cells were stained and analysed by FACS (BD Biosciences). Cell Quest software (BD Biosciences) was used for analysis.

Data analysis

Paired sample t tests compared pre and post-vaccination for T-cell proliferation, expressed as SI or a percentage, extra or intracellular cytokine production, percentage and function of CD4+CD25^{bright} T cells, as well as the percentage of CD4+FOXP3+ T cells. For comparison between HC, JIA, oligoarticular JIA, or polyarticular JIA populations, independent samples t test were utilized. A probability less than 0.05 was considered significantly different. Statistical analyses performed used the statistical package for the social sciences (SPSS) software version 12.0.1. All data are presented as individual points with median and/or means±SD values.

RESULTS

Vaccination induces T-cell proliferation to MenC vaccine antigen and TT

Twenty-six (oligoarticular) JIA and 28 (polyarticular) JIA patients (including three patients with extended oligoarticular JIA) and 20 healthy adults (controls) visited the Pediatric Rheumatology Clinic in Wilhelmina Children's Hospital, University Medical Centre, Utrecht, The Netherlands. Each received a single MenC vaccination, and donated peripheral blood before and one month after vaccination (table 1). PBMC T-cell proliferative responses to vaccine antigens, measured by thymidine incorporation and SI, increased after vaccination in the HC (1.9 $\pm$ 1.8 rose to 6.8 $\pm$ 6.7, P=0.001, N=13) and the JIA patients (4.2 $\pm$ 1.9 to 15.3 $\pm$ 8.9, P=0.005, N=16, figure 1A). The latter was mainly attributed to the polyarticular JIA subgroup (6.1 $\pm$ 5.0 to 23.4 $\pm$ 18.2, P=0.02, N=8, figure 1B) and not the oligoarticular JIA (2.3 $\pm$ 1.3 to 7.2 $\pm$ 4.6, P=0.066, N=8). Similar responses were found towards TT (see supplementary figure). After vaccination, no increase in T cell proliferative responses to the control antigen ovalbumin was found in the HC (1.0 $\pm$ 0.1.0 to 1.1 $\pm$ 1.0, P=0.55) or in the JIA patients (1.0 $\pm$ 1.0 to 1.2 $\pm$ 1.2, P=0.36).

In summary, post-vaccination proliferative T cell responses in both HC and JIA were significantly higher, yet in PBMC of the polyarticular JIA patients they were more vigorous than the HC (P=0.005) or the oligoarticular JIA (P=0.006).

Vaccination induces vaccine-specific IL-2 and TNFα production in polyarticular JIA patients

Before and after vaccination, the PBMC of HC and JIA were stimulated in-vitro for 72 h with MenC vaccine antigen with subsequent quantification of supernatant IL-2, TNFα, and IL-10 in pg/ml. After vaccination, the JIA (pre 11.2 ± 2.0, post 52.1 ± 13.6, P=0.078, N=13), the polyarticular JIA (pre 20.3 ± 22.8, post 109.8 ± 71.3, P=0.065, N=6), the oligoarticular JIA (3.4 ± 1.2 pre to 2.7 ± 2.8 post, P=0.788, N=7, figure 1C, right) and the HC (pre 3.2 ±

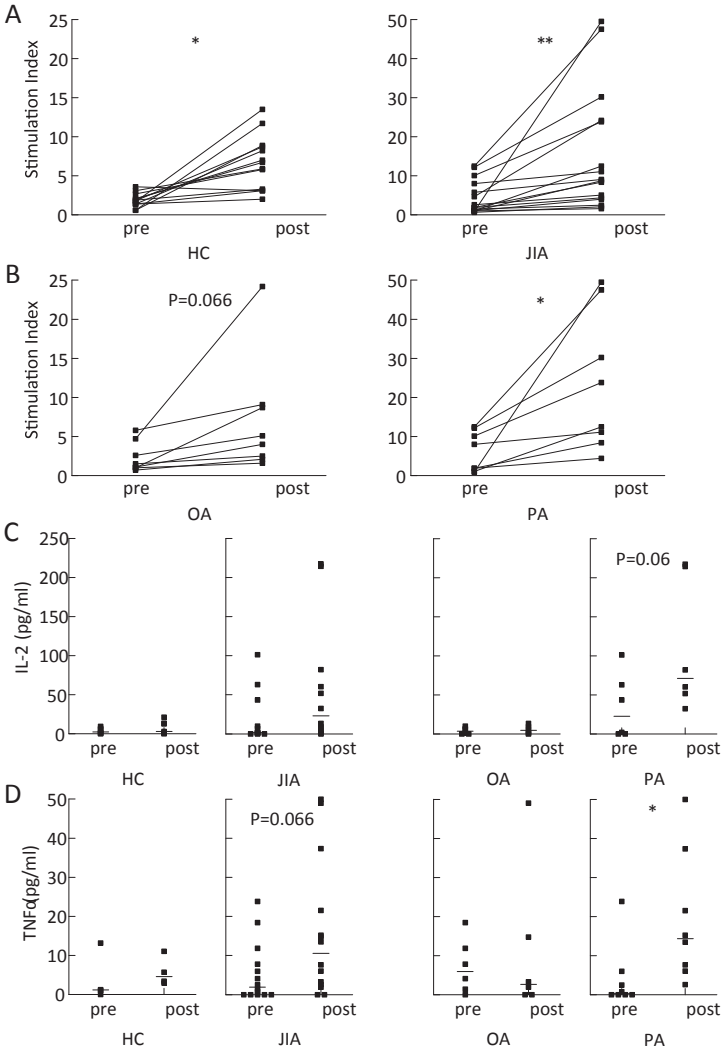


Figure 1 T-cell proliferative responses and qualitative difference in the immune response of the healthy volunteers (HC) and polyarticular (PA) juvenile idiopathic arthritis (JIA) patients against the meningococcal C (MenC) vaccine.

T cell proliferation responses (SI) to MenC Antigen in healthy volunteers ((A) N=13, left) and JIA patients as a whole ((A) N=16, right) as well as oliogoarticular (OA) ((B) N=8, left) and polyarticular JIA patients ((B) N=8, right) using line plots before and after vaccination. Pre, before vaccination; Post, after vaccination. Peripheral blood mononuclear cells of healthy volunteers and JIA patients were taken before (pre) and after (post) MenC vaccination followed by in-vitro re-stimulation with MenC Antigen. Interleukin (IL)-2 (HC N=6, JIA N=13, oligoarticular N=7, polyarticular N=6, (C)) and TNFα (HC N=4, JIA N=14, oligoarticular N=6, polyarticular N=8, (D)) cytokines in supernatants were quantified using a multiplex immunoassay and expressed as pg/ml after subtracting media values. Data are expressed as individual points with medians. Significant differences are indicated as follows: *=p<0.05, **=p<0.01.

1.7, post 7.6 ± 7.9, P=0.306, N=6, figure 1C) did not increase in IL-2 production. However, when comparing the different groups, post-immunization PBMC of the polyarticular JIA produced more IL-2 than the oligoarticular JIA (P=0.026) or the HC (P=0.03).

Similarly, post-vaccine TNFα production demonstrated an increasing trend in JIA patients (pre -0.9 ± 1.9, post 11.8 ± 6.8, P=0.066, N=14, figure 1D) attributed again to polyarticular JIA (pre -6.9± -1.0, post 19.5 ± 14.3,N=8, P=0.012, figure 1D, right), but not to the oligoarticular JIA subgroup (pre 7.2 ± 5.9, post 1.7 ±1.2, P=0.174, N=6, Figure 1D, right) nor the HC (pre 3.9 ± 1.2, post 5.8 ± 4.6, P=0.627, N=4, figure 1D). Post-vaccination PBMC of the polyarticular JIA produced more TNFα than the oligoarticular JIA (P=0.019). No differences were found in supernatant IL-10 concentrations of PBMC of the HC or JIA patients cultured with the vaccine (data not shown). Therefore, after vaccination, the PBMC of the polyarticular JIA patients showed higher vaccine-induced proliferation and production of IL-2 and TNFα compared with either the HC or oligoarticular JIA patients.

Increased HSP60-specific CD4 T-cell proliferation in JIA patients after vaccination with MenC

Next, we investigated whether vaccination could also influence the immune response to a disease-related auto-antigen in JIA patients, namely self-HSP60.[22] Our group and others earlier described that PBMC from JIA patients can proliferate in response to self-HSP60.[23-26] Indeed, also in this study CD4 and CD8 T cells of JIA patients showed low but specific proliferation as measured by CFSE dilution. After MenC vaccination, human HSP60-specific CD4 T cell proliferation in JIA patients increased (pre 0.8 + 0.6%, post 2.4 + 1.8 %, P=0.017, N=14, Figure 2A) without significant difference between the oligoarticular (pre 0.4 + 0.4%, post 2.2 + 1.3 %, P=0.08, N=7) and polyarticular (pre 1.1 + 1.2%, post 2.5 + 2.3 %, P=0.16, N=7, figure 2A) JIA subtypes. The HSP60 specific CD4 T-cell proliferation of the healthy controls after vaccination had an increasing but non-significant trend (pre 3.6 + 1.1, post 23.1+ 18.4, P=0.054, N=7).

HSP60-induced CD8 T-cell-specific proliferation did not change significantly in the JIA group after vaccination (pre 0.7 $\pm$ 0.4%, post 1.3 $\pm$ 0.8%, P=0.184, N=14, figure2A). The increase in post-vaccine proliferative T-cell responses as measured by CFSE towards both MenC vaccine and a foreign (*Escherichia coli*) HSP60 were similar to human HSP60 (data not shown). There were no significant differences in the proliferative response of CD4 or CD8 T cells to the control antigen DT before and after vaccination. These results show that in JIA patients, vaccination with MenC not only induces increased CD4 T-cell proliferation towards the MenC antigen but also towards the arthritis related auto-antigen (human HSP60).

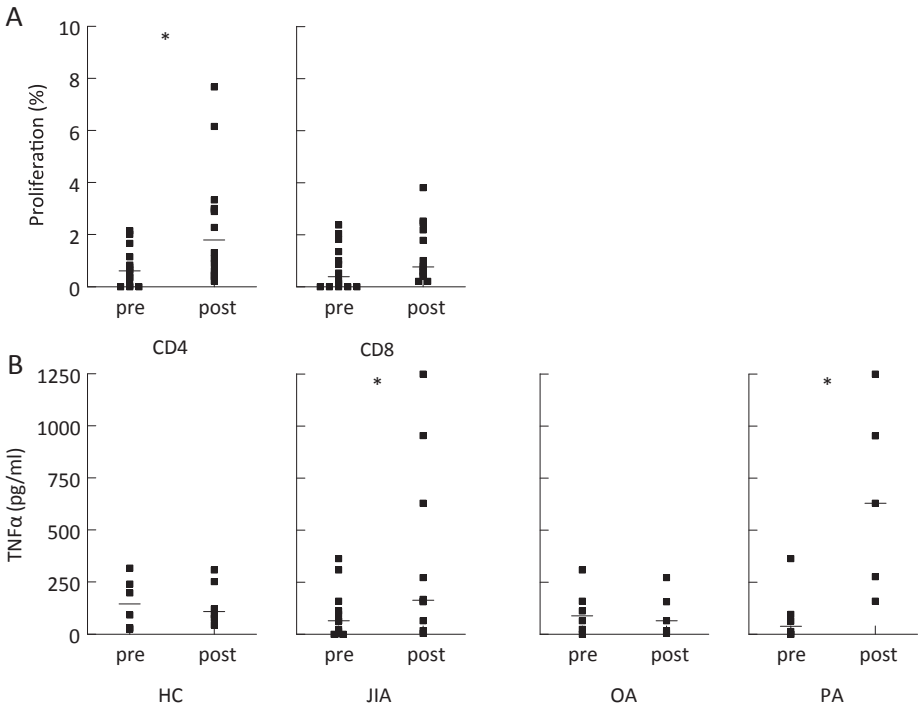


Figure 2 T cell proliferative response and tumour necrosis factor (TNF) alpha production to human heat shock protein (HSP) 60 from peripheral blood mononuclear cells (PBMC) of healthy volunteers (HC) and juvenile idiopathic arthritis (JIA) patients.

CD4 and CD8 T-cell proliferative responses of the JIA patients (N=14,(A)) as well as TNFα cytokine production against human HSP60 from PBMC of healthy volunteers and JIA patients (HC N=6, JIA N=10, oligoarticular (OA) N=5, polyarticular (PA) N=5, (B)). PBMC were isolated before (pre) and after (post) meningococcal C (MenC) vaccination and subsequently in-vitro re-stimulated with human HSP60 before assessing antigen-specific proliferation and TNFα levels in the supernatants. Data are expressed as individual points and means ± median. Significant differences are indicated as follows: *p<0.05.

Increased self-HSP60 induced production of TNFα in polyarticular JIA after MenC vaccination

To characterise the HSP60 immune response, we measured the antigen-specific cytokine production in supernatants of cultured PBMC from HC or JIA patients using multiplex immunoassay before and after MenC vaccination with in-vitro re-stimulation with human HSP60. No significant differences in cytokine production were detected for IL-4, IL-5, IL-10, IL-17, and INFγ. However, vaccination and in-vitro re-stimulation with human HSP60 did increase TNFα production in the supernatant of the PBMC from JIA patients (pre 85.2 ± 64.1 rose to 368.2 ± 163.5 post-vaccination, P=0.039, N=10, figure 2B) largely due to an increase in the polyarticular JIA (pre 104.5 ± 63.8 rose to 633.5 ± 629.8 post-vaccination, P=0.041, N=5) but not in the HC (pre 150.4 ± 145.1 and 149.4 ± 107.6 post-vaccination,

P=0.98, N=6) or in the oligoarticular JIA population (pre 65.9 ± 64.4 rose to 102.9 ± 64.3 post vac, P=0.153, N=5). Also, post-immunisation levels of the HSP60-induced TNFα production of the polyarticular JIA patients were not significantly higher than either the oligoarticular JIA (P=0.069) or the HC (P=0.088). Therefore, following MenC vaccination, the PBMC from polyarticular JIA patients show not only increased T-cell proliferation but also an increased TNFα production towards HSP60, while such an increase in TNFα production is not seen in HC or oligoarticular JIA patients.

Vaccination leads to an increase of MenC Antigen and HSP60-induced CD4+CD25^{bright} T cells and CD4+FOXP3+ T cells in healthy controls but not in polyarticular JIA patients

We next questioned whether the higher antigen-specific T-cell proliferation and TNFα production from the PBMC of the polyarticular JIA induced by MenC vaccination might correlate with a potential aberrant control by regulatory (CD4+FOXP3+) T cells. As CD4+CD25^{bright} T cells count as surrogates for CD4+FOXP3+ T cells we first investigated differences in CD25 expression in the different groups and with respect to vaccination before addressing functionality. We thus measured the number of antigen-induced CD4+CD25^{bright} T cells (expressed as a percentage of CD4 T cells) as a surrogate marker for regulatory T cells.

In-vitro re-stimulation with MenC Antigen after vaccination induced a higher percentage of CD4+CD25^{bright} T cells in the HC (pre 0.8 $\pm$ 0.7 rose to 4.6 $\pm$ 4.3 post, P=0.02, N=6) and JIA patients (pre 1.0 $\pm$ 1.0 rose to 1.8 $\pm$ 1.8 post, P=0.02, N=12) with the latter mainly attributed to the oligoarticular JIA subgroup (pre 0.9 $\pm$ 0.9 rose to 1.8 $\pm$ 1.7 post, P=0.034, N=7) but not the polyarticular JIA(pre 1.1 $\pm$ 1.1 and 1.7 $\pm$ 1.9 post, P=0.291, N=5, figure 3A). MenC vaccination and in-vitro human HSP60 re-stimulation demonstrated a similar pattern of an increased percentage of CD4+CD25^{bright} T cells in the HC (pre 0.4 $\pm$ 0.2 rose to 2.1 $\pm$ 2.2 post, P< 0.0005, N=6), the oligoarticular JIA (pre 1.0 $\pm$ 1.0 rose to 2.1 $\pm$ 2.0 post, P=0.02, N=9), but not in the polyarticular JIA (pre 1.5 $\pm$ 1.2 and 1.5 $\pm$ 1.2 post, P=0.94, N=7, figure 3B).

As CD25 expression is not confined to regulatory T cells, we measured the numbers of FOXP3+CD4+ T cells. Vaccination and MenC antigen in-vitro re-stimulation only moderately increased these cell numbers in the HC (pre 1.6 $\pm$ 1.3 rose to 2.2 $\pm$ 1.7 post, P=0.06, N=7). In contrast to HC, the JIA group (pre 2.1 $\pm$ 1.8 and 2.1 $\pm$ 1.7 post, P=0.9, N=17), the oligoarticular JIA (pre 1.5 $\pm$ 1.1 and 1.5 $\pm$ 1.5 post, P=0.8, N=9), and the polyarticular JIA subgroups (pre 2.9 $\pm$ 2.2 and 2.9 $\pm$ 2.6 post, P=0.9, N=8) failed to respond to vaccination. Remarkably, despite the lack of response to vaccination, the percentage of induced CD4+FOXP3+ T cells in the polyarticular JIA subgroup before and after vaccination was the highest and significantly higher when compared to the oligoarticular JIA (P=0.008, figure 3C). In addi-

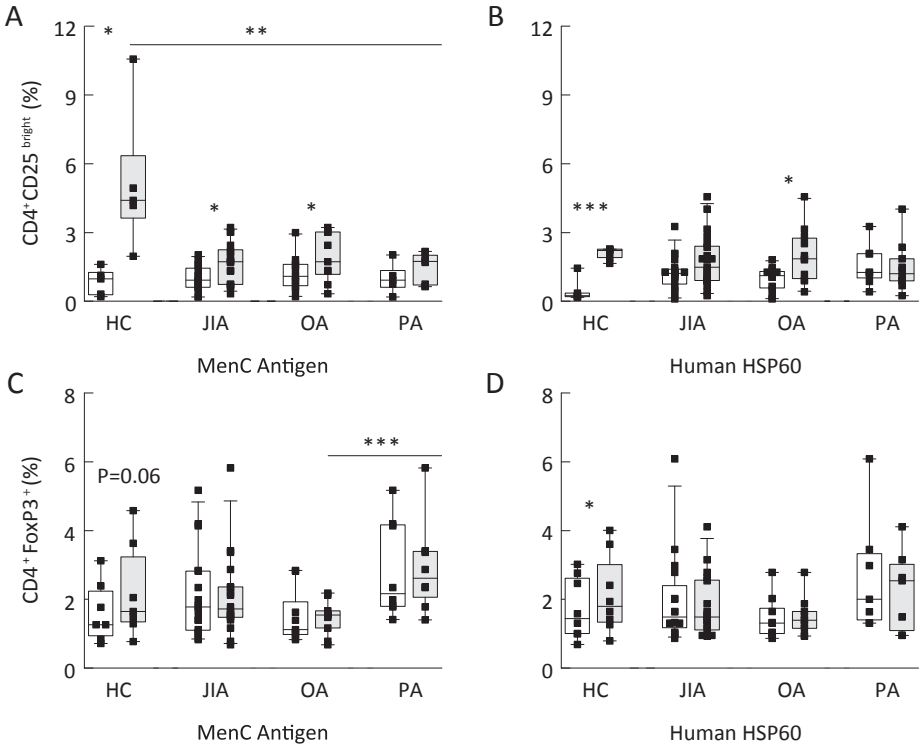


Figure 3 Percentage of CD4+CD25^{bright} T cells and CD4+FOXP3+ T cells induced after vaccination and in-vitro re-stimulation with meningococcal C (MenC) antigen as well as human heat shock protein (HSP) 60. The number of CD4+CD25bright T cells and CD4+FOXP3+T cells expressed as a percentage of the total CD4 T cells are measured in the healthy volunteers (HC) and juvenile idiopathic arthritis (JIA) patients before vaccination (left, white bars) as well as after (right, grey bars). A: HC N=8, JIA N=17, oligoarticular (OA) N=9, polyarticular (PA) N=8. B: HC N=6, JIA N=16, OA N=9, PA N=7. C: HC N=7, JIA N=17, OA N=9, PA N=8. D: HC N=8, JIA N=16, OA N=9, PA N=7. Data are expressed as individual points and means +/- median. Significant differences are indicated as follows: *p<0.05, ***p<0.001.

tion, the polyarticular JIA patients also failed to respond to vaccination with an increase of antigen-induced CD4+CD25^{bright} T cells (figure 3A).

MenC vaccination and in-vitro re-stimulation with self-HSP60 again led to a very moderate increase in CD4+FOXP3+ T cells only in the HC (pre 1.7 $\pm$ 1.4 rose to 2.1 $\pm$ 1.8 post, P=0.04, N=8), but not in the JIA group (pre 2.0 $\pm$ 1.5 and 1.8 $\pm$ 1.5 post, P=0.56, N=16), oligoarticular JIA (pre 1.4 $\pm$ 1.3 and 1.5 $\pm$ 1.4 post, P=0.75, N=9), or polyarticular JIA subgroup (pre 2.7 $\pm$ 2.0 and 2.3 $\pm$ 2.5 post, P=0.47, N=7, figure 3D). Similar results with respect to the percentage of CD4+FOXP3+ T cells and CD4+CD25^{bright} T cells were obtained following in-vitro activation with *E. coli* HSP60 as well as TT (included in the vaccine, data not shown). However, after vaccination of the HC or the JIA we could not find significant changes in

numbers of CD4+CD25^{bright} T cells or CD4+FOXP3+ T cells following in-vitro stimulation with the control antigens ovalbumin or DT (data not shown). It has to be noted, however, that the incubation period of these in-vitro assays may not be sufficient to develop functional FOXP3 and CD25 ^{bright} CD4+ T cells regulatory T cells in vitro.[27]

DISCUSSION

In those genetically predisposed, environmental triggers (infections or vaccinations) may lead to continuous inflammation and culminate in human autoimmunity. After antigen encounter a specialized subset of murine T cells called natural or adaptive T regulatory cells derived from the thymus or periphery respectively may suppress ongoing T cell responses. To date, most describe differences in absolute numbers and function[28-31] instead of the how FOXP3+T cells respond in vivo to an environmental antigen in humans. This "plasticity" may be more important than mere cell numbers. This is the first study that demonstrates how the immune system of patients with a human autoimmune disease (JIA) responds to an in-vivo microbial trigger such as vaccination with MenC. Remarkably, when compared with the HC and oligoarticular JIA, the polyarticular JIA demonstrated a higher risk of arthritis by responding to vaccination with higher levels of vaccine and self-HSP60-induced T-cell proliferation with an accompanied increase in IL-2 and TNFα. Collectively, vaccination of the polyarticular JIA patients induced T cells with a more risky phenotype (high T cell proliferation and TNFα production), as their supposed regulatory arm of the specific immune response displayed less plasticity to vaccination. During inflammation, for example, as triggered by an infection or vaccination (as in this study), tissue damage may up-regulate HSP60 expression activating more vaccine and HSP60-specific CD4+FOXP3+ T cells, subsequently down regulating the immune response. HSP60-specific CD4+FOXP3+ T cells have been previously described as being anti-inflammatory. Our data suggest that in the severe polyarticular JIA population these cells may demonstrate a reduced plasticity and diminished in-vitro suppression, suggesting in an inherited deficiency to mount a regulatory response to environmental triggers. Furthermore, the chronicity and intensity of inflammation in polyarticular JIA may overcome the regulatory response as high levels of TNFα negatively affect T regulatory function through the up-regulation of TNF receptor II in unstimulated T regulatory cells.[32,33] This mechanism may play a role in the polyarticular JIA as this study demonstrated significantly higher production of antigen-induced TNFα by their PBMC in vitro.

It needs to be recognized that differences in treatment and disease activity may have influenced the results. We could not detect such an effect in this study, but it is possible that after subdividing the oligoarticular or polyarticular JIA into no medication (group 1)

versus high medication groups (group 3 and 4) the resulting samples sizes were probably too small to draw definite conclusions.

In summary, following vaccination, the antigen-induced CD4+FOXP3+ T cellular response of the polyarticular JIA, unlike the oligoarticular JIA or HC, may demonstrate a deficiency in suppressive capacity to counter a pro-inflammatory response if prolonged and skewed towards a T-helper type 1 phenotype (measles, mumps and rubella (MMR) vaccination or prolonged infections) ultimately exacerbating disease. This increased risk may not be detected in epidemiological studies on direct relationships between infections/vaccinations and clinical symptoms. Obviously, this by no means should lead to the conclusion that polyarticular JIA patients must not be vaccinated. However, the study does emphasize the need for further investigations of the safety of immunizations in polyarticular JIA patients in general, especially with regard to life vaccines. As human autoimmunity is thought to be complex multi-hit disease, an environmental trigger could lower the threshold for an amplified inflammatory response, without directly leading to clinical symptoms. This is the first study describing the flexibility of CD4+FOXP3+ and CD4+ $CD25^{bright}$ T cells following an in-vivo environmental challenge in a human autoimmune disease**.** It underscores the premises that environmental triggers may contribute to a dysregulation of the immune system in chronic inflammatory diseases.

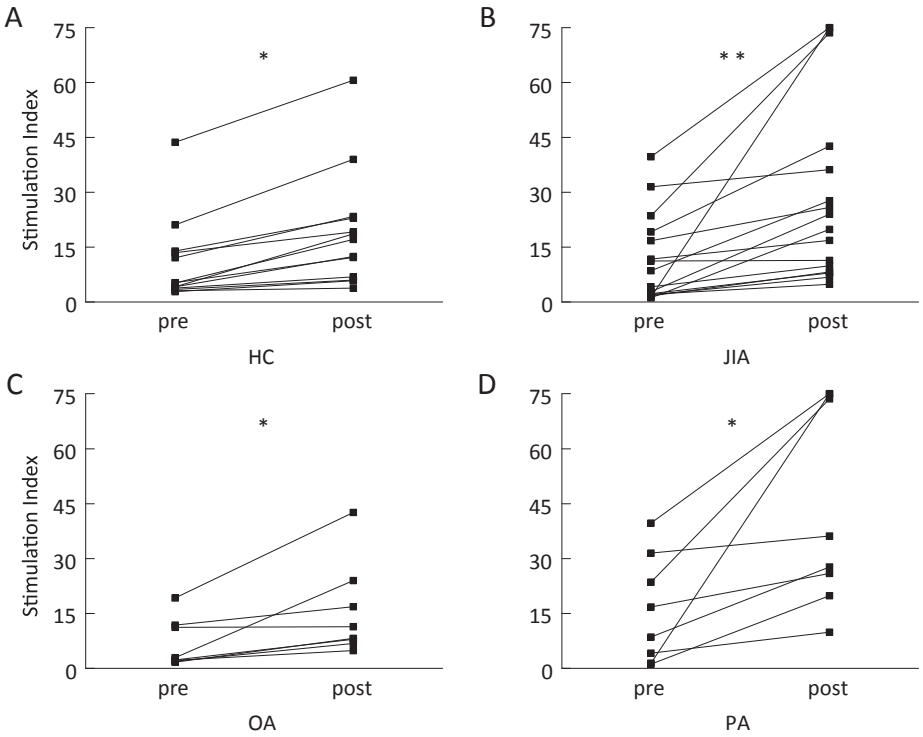
ACKNOWLEDGEMENTS

The authors wish to thank Dr. Bas Vastert, Dr. Sarah T. A. Roord, and Erica Roks for their help in preparation of this manuscript. Furthermore, thanks to the Boston University School of Medicine advisors Paul M. O'Bryan and Deborah W. Vaughan for their support and guidance.

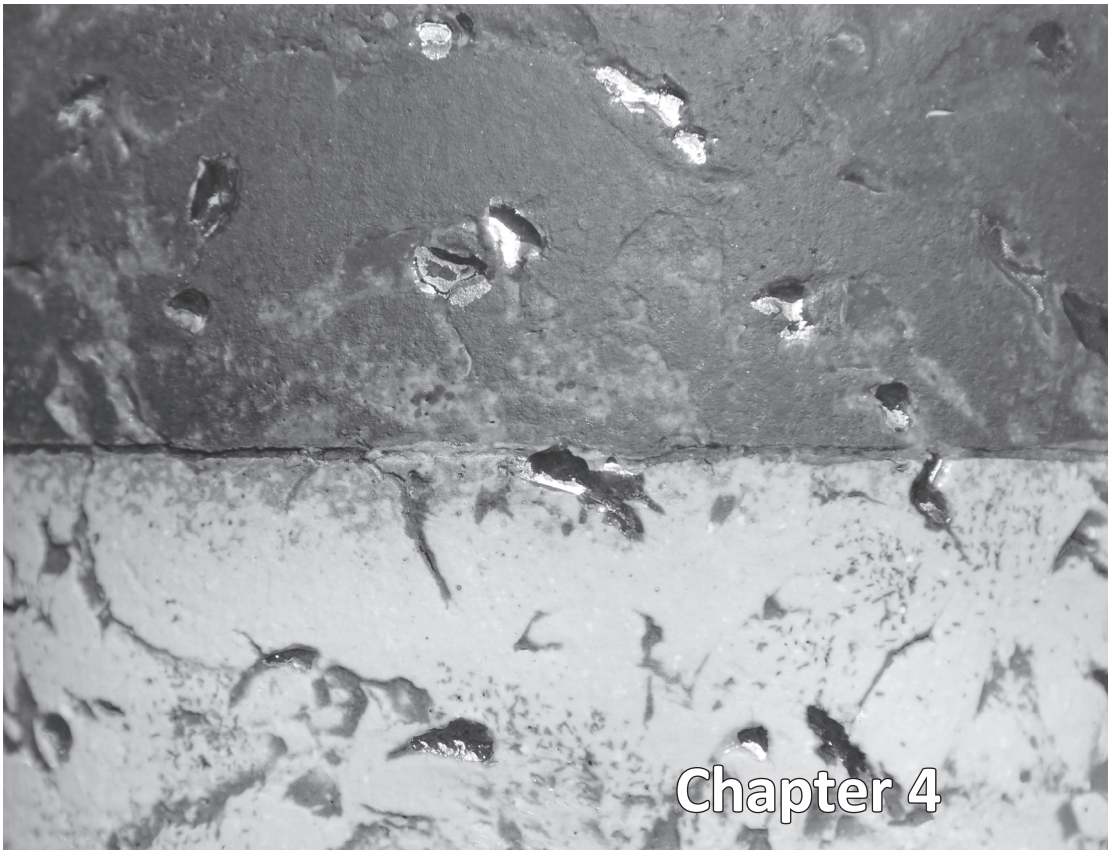
REFERENCE LIST

1. Vaccine preventable deaths and the Global Immunization Vision and Strategy, 2006-2015. *MMWR Morb Mortal Wkly Rep.* 2006;55:511-515.
2. Christenson B, Lundbergh P, Hedlund J, Ortqvist A. Effects of a large-scale intervention with influenza and 23-valent pneumococcal vaccines in adults aged 65 years or older: a prospective study. *Lancet.* 2001;357:1008-1011.
3. Wraith DC, Goldman M, Lambert PH. Vaccination and autoimmune disease: what is the evidence? *Lancet.* 2003;362:1659-1666.
4. Gout O, Lyon-Caen O. [Sclerotic plaques and vaccination against hepatitis B]. *Rev Neurol (Paris).* 1998;154:205-207.
5. Zingg W. [Does vaccination cause disease?]. *Ther Umsch.* 2005;62:665-674.
6. Confavreux C, Suissa S, Saddier P, Bourdes V, Vukusic S. Vaccinations and the risk of relapse in multiple sclerosis. Vaccines in Multiple Sclerosis Study Group. *N Engl J Med.* 2001;344:319-326.
7. Lasky T, Terracciano GJ, Magder L, Koski CL, Ballesteros M, Nash D, et al. The Guillain-Barre syndrome and the 1992-1993 and 1993-1994 influenza vaccines. *N Engl J Med.* 1998;339:1797-1802.
8. Graves PM, Barriga KJ, Norris JM, Hoffman MR, Yu L, Eisenbarth GS, et al. Lack of association between early childhood immunizations and beta-cell autoimmunity. *Diabetes Care.* 1999;22:1694-1697.
9. Ascherio A, Zhang SM, Hernan MA, Olek MJ, Coplan PM, Brodovicz K, et al. Hepatitis B vaccination and the risk of multiple sclerosis. *N Engl J Med.* 2001;344:327-332.
10. DeStefano F, Mullooly JP, Okoro CA, Chen RT, Marcy SM, Ward JI, et al. Childhood vaccinations, vaccination timing, and risk of type 1 diabetes mellitus. *Pediatrics.* 2001;108:E112.
11. Ascherio A, Zhang S, Hernan M, Olek M, Coplan P, Brodovicz K. [Hepatitis B vaccination and the risk of multiple sclerosis: case-control studies]. *Gastroenterol Clin Biol.* 2001;25:927-929.
12. Vial T, Descotes J. Autoimmune diseases and vaccinations. *Eur J Dermatol.* 2004;14:86-90.
13. Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. *J Rheumatol.* 2004;31:390-392.
14. Olson NY, Lindsley CB. Influenza immunization in children with chronic arthritis. *J Rheumatol.* 1994;21:1581-1582.
15. Sebag O, Bolla G, Bebin B, Sebag F. [Exacerbation of chronic juvenile arthritis induced by hepatitis B vaccination]. *Arch Pediatr.* 1998;5:1046.
16. Maillefert JF, Sibilia J, Toussirot E, Vignon E, Eschard JP, Lorcerie B, et al. Rheumatic disorders developed after hepatitis B vaccination. *Rheumatology (Oxford).* 1999;38:978-983.
17. Postepski J, Opoka-Winiarska V, Koziol-Montewka M, Korobowicz A, Tuszkiewicz-Misztal E. [Role of mycoplasma pneumoniae infection in aetiopathogenesis of juvenile idiopatic arthritis]. *Med Wieku Rozwoj.* 2003;7:271-277.
18. Conyn-van Spaendonck MA, Reintjes R, Spanjaard L, van Kregten E, Kraaijeveld AG, Jacobs PH. Meningococcal carriage in relation to an outbreak of invasive disease due to Neisseria meningitidis serogroup C in the Netherlands. *J Infect.* 1999;39:42-48.
19. van Furth AM, Vandenbroucke-Grauls CM. [Universal vaccination against group C meningococci and pneumococci; advice from the Health Council of the Netherlands]. *Ned Tijdschr Geneeskd.* 2002;146:932-933.
20. Kasapcopur O, Cullu F, Kamburoglu-Goksel A, Cam H, Akdenizli E, Calykan S, et al. Hepatitis B vaccination in children with juvenile idiopathic arthritis. *Ann Rheum Dis.* 2004;63:1128-1130.

21. Zonneveld-Huijssoon E, Ronaghy A, Van Rossum MA, Rijkers GT, van der Klis FR, Sanders EA, et al. Safety and efficacy of meningococcal c vaccination in juvenile idiopathic arthritis. *Arthritis Rheum.* 2007;56:639-646.
22. van Eden W, van der Zee R, Prakken B. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol.* 2005;5:318-330.
23. de Kleer I, Kamphuis SM, Rijkers GT, Scholtens L, Gordon G, de Jager W, et al. The spontaneous remission of juvenile idiopathic arthritis is characterized by CD30+ T cells directed to human heat-shock protein 60 capable of producing the regulatory cytokine interleukin-10. *Arthritis Rheum.* 2003;48:2001-2010.
24. de Graeff-Meeder ER, van der Zee R, Rijkers GT, Schuurman HJ, Kuis W, Bijlsma JW, et al. Recognition of human 60 kD heat shock protein by mononuclear cells from patients with juvenile chronic arthritis. *Lancet.* 1991;337:1368-1372.
25. Prakken AB, van Eden W, Rijkers GT, Kuis W, Toebes EA, Graeff-Meeder ER, et al. Autoreactivity to human heat-shock protein 60 predicts disease remission in oligoarticular juvenile rheumatoid arthritis. *Arthritis Rheum.* 1996;39:1826-1832.
26. de Graeff-Meeder ER, van Eden W, Rijkers GT, Prakken BJ, Kuis W, Voorhorst-Ogink MM, et al. Juvenile chronic arthritis: T cell reactivity to human HSP60 in patients with a favorable course of arthritis. *J Clin Invest.* 1995;95:934-940.
27. Gregori S, Bacchetta R, Passerini L, Levings MK, Roncarolo MG. Isolation, expansion, and characterization of human natural and adaptive regulatory T cells. *Methods Mol Biol.* 2007;380:83-105.:83-105.
28. Kekalainen E, Tuovinen H, Joensuu J, Gylling M, Franssila R, Pontynen N, et al. A defect of regulatory T cells in patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy. *J Immunol.* 2007;178:1208-1215.
29. Caudy AA, Reddy ST, Chatila T, Atkinson JP, Verbsky JW. CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes. *J Allergy Clin Immunol.* 2007;119:482-487.
30. Longhi MS, Hussain MJ, Mitry RR, Arora SK, Mieli-Vergani G, Vergani D, et al. Functional study of CD4+CD25+ regulatory T cells in health and autoimmune hepatitis. *J Immunol.* 2006;176:4484-4491.
31. de Kleer IM, Wedderburn LR, Taams LS, Patel A, Varsani H, Klein M, et al. CD4+CD25bright regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. *J Immunol.* 2004;172:6435-6443.
32. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood.* 2006;108:253-261.
33. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med.* 2004;200:277-285.



Supplementary figure Proliferation of peripheral blood mononuclear cells from (A) healthy volunteers (HC) or (B) juvenile idiopathic arthritis (JIA) patients after restimulation with tetanus toxoid (TT) before (pre) or after (post) meningococcal C (MenC) vaccination. JIA patients have been divided in (C) oligoarticular (OA) and (D) polyarticular (PA) subgroups in the lower panel



Chapter 4

Heat shock protein bystander antigens for peptide immunotherapy in autoimmune disease

Evelien Zonneveld-Huijssoon, Salvatore Albani, Berent J Prakken, Femke van Wijk

Clinical and Experimental Immunology, review under revision

KEY POINTS

- Effective antigen specific immunotherapy for autoimmune diseases can be achieved by (mucosal) administration of an immunogenic antigen eliciting bystander suppression at the site of inflammation.
- Heat shock proteins are bystander antigens with immunoregulatory properties that can be used for peptide specific immunotherapy in both experimental and human autoimmune disease.
- Efficacy of peptide immunotherapy may be increased by enhancing the peptide-specific immune response by proinflammatory agents.
- For a therapeutic effect of peptide immunotherapy, combination treatment with short duration generalized immune modulators may be indispensable.

INTRODUCTION

The fascinating issue of how to restore the immune balance in a deranged immune system that attacks self tissues in autoimmune diseases is a continuing focus of research. Current treatment of autoimmunity still mainly depends on conventional life-long general immune suppression. Although a step forward has been made in clinical efficiency by the introduction of biologics that can block pro-inflammatory cytokines, autoimmunity revives as soon as therapy is discontinued. Moreover, the considerable immune depression evoked by cytokine blockade has been associated with severe side-effects like tuberculosis, serious opportunistic infections and malignancies like lymphoma.[1-6]

A more directed approach could overcome back draws of non-specific immune suppressive therapy. By specific targeting of auto-aggressive T cells in autoimmunity, side effects may be reduced and possibly, a longer lasting effect may be achieved. Antigen specific immunotherapy has been shown effective in multiple animal models of autoimmunity, without severe side-effects (reviewed by [7]). Translation of these findings into humans had promising results but efficacy has been less than expected (table 1). To improve the efficacy of antigen specific therapy in clinical autoimmune diseases, three issues concerning the choice of antigen, route of administration and timing of therapy need to be considered.

The choice of antigen in animal models is facilitated by the fact that the disease-inducing antigen is known. The identification of such an antigen in human autoimmunity is more challenging as the disease inducing antigen in many autoimmune diseases remains unknown and it is unsure whether this one disease-inducing agent really exists. Therefore new targets for antigen specific therapy are needed.

The second issue concerns the route of antigen administration. In the majority of clinical trials the antigen was administered by injection while a more effective option to restore immune tolerance would be the administration of the antigen in a tolerogenic environment, like the gut or nasal mucosa.[7,8] The tolerogenic presentation of the antigen is thought to convert the antigen-specific pro-inflammatory immune response that is present in autoimmune disease into a peptide specific regulatory response (reviewed by [9]). Even non-self antigens derived from commensal bacteria have recently been shown to facilitate colonic Treg development in the gut.[10] However, the major backdraw to overcome is the limited immunogenicity of peptides administered via the mucosal route, indicating a need for enhancement of peptide recognition.[11,12]

Third, another possible cause for the limited efficacy of the clinical trials could be the timing of therapy at a time point where the inflammatory process is ongoing and epitope

Table 1 Major clinical trials of peptide immunotherapy in autoimmune disease.

	trial name	design	type of therapy	peptide*	route†	patient group‡	immunomodulatory effects	clinical efficacy	future	reference
Diabetes		phase I	parenteral peptide	pro-insulin peptide C19-A3	i.d.	longstanding DM type I	increase of IL-10 producing peptide specific T cells	none measured		Thrower Clin Exp Imm 2009
		phase I	parenteral peptide	human insulin B chain peptide	i.m.	new onset DM type I	insulin-specific B and T cell responses	none measured		Orban J Autoimm 2010
		phase I	parenteral peptide	NBI-6024 (altered peptide of insulin B epitope B9-23)	s.c.	new onset DM type I	shift from Th1 to Th2 immunoregulatory phenotype	none measured		Alleva Scand J Immunol 2006
	DIAMYD	phase II	parenteral peptide with adjuvant	GAD65-Alum	s.c.	new onset DM type I	increased percentage of total Treg and GAD65 specific Treg cells, increased secretion IL-5, IL-10, IL-13	preservation of residual insulin secretion	ongoing phase II NCT00529399	Ludvigsson NEJM 2008, Hjorth Clin Imm 2011
MS	PreCISe	phase I/II	parenteral peptide	Copaxone (Glatiramer-acetaat, peptide mixture)	s.c.	early MS	none measured	risk reduction for developing clinically definite MS, delayed onset, decreased relapse rate		Comi Lancet 2009, Cohen Neurol 2007
		phase II	parenteral peptide	altered peptide of MBP83-99	s.c.	MS		increased disease activity		Bielekova, Kappos Nat Med 2000

SLE	phase II	parenteral peptide	spliceosomal peptide p140	s.c.	SLE	anti dsDNA antibody levels reduced			Muller Arthr Rheum 2008
Uveitis	open trial, no control group	oral peptide	B27PD (self-HLA peptide)	p.o.	long lasting therapy refractive uveitis	none measured	discontinuation of steroids possible because of reduced intraocular inflammation	ongoing phase I/II NCT01195948	Thureau Immunol Lett 1999, ANYAS 2004

*MBP: myelin basic protein † i.d.: intradermal, i.m.: intramuscular, s.c.: subcutaneous

‡MS: multiple sclerosis, SLE: systemic lupus erythematosus, DM: diabetes mellitus.

spreading has already taken place, resulting in a widespread immune response elicited by multiple antigens.[7] Treating autoimmunity at an earlier time point therefore seems preferable. In type 1 diabetes (T1D) and multiple sclerosis (MS) efficacy of antigen-specific therapy in phase II trials was indeed most striking when the drug was administered after recent onset (table 1).[13] Unfortunately, very little early markers of autoimmune disease in humans are currently available in the clinic, limiting early intervention to time of diagnosis.

In summary, the results of clinical trials of peptide immunotherapy are promising, but could be enhanced by early mucosal administration of an immunogenic antigen. In this review, we will discuss antigen specific immunotherapy with a special focus on heat shock proteins, and consider how future peptide immunotherapy may be improved.

HEAT SHOCK PROTEINS AS CANDIDATES FOR IMMUNOTHERAPY

Bystander antigens

At the time of diagnosis, human autoimmune diseases are already characterized by a secondary non-specific inflammatory process in which multiple antigens are targets of the immune system (a process known as epitope spreading). These antigens could be candidates for antigen specific immunotherapy if they are immunogenic and upregulated at the site of inflammation (bystander antigens). Induced tolerogenic immune responses to these bystander antigens could lead to a local down regulation of the ongoing immune response (bystander suppression).

Heat shock protein as a bystander antigen

Heat shock proteins (HSPs), which are highly conserved intracellular molecular chaperones important for cell survival under stressful conditions, fulfil both above mentioned criteria for bystander antigens (reviewed in [14]).

First, HSPs (also known as 'cell stress proteins') are up-regulated upon cell stress en therefore present at sites of inflammation. HSPs are indeed abundantly present in muscle cells of juvenile dermatomyositis patients,[15] in synovial fluid and synovial tissue of JIA[16] and RA patients[17] and in inflamed bowel of Crohn patients.[18] There is supporting evidence that HSPs are secreted from stressed cells, for example free HSP60 is found in the blood during various inflammatory conditions.[19]

Second, HSPs are very immunogenic.[20,21] HSP-specific responses have shown to be immunodominant in infection, but also in autoimmune disease.[14] For example, certain HSP60 peptide-specific T cell clones play a significant role in the perpetuation of Crohn's disease.[18] Furthermore, tissue-specific T cell clones from diabetic children recognized human HSP60 as an auto-antigen.[22,23] Humoral responses to HSPs have been observed as well,

as antibody responses to multiple HSP families were detected in sera from RA and JIA patients.[24,25]

Altogether, HSPs seem to be suitable candidates for induction of bystander suppression by antigen specific immunotherapy.

Immunoregulatory properties of HSPs

As mentioned above, HSPs are also known for their strong evolutionary conservation, resulting in a high level of homology between bacterial and mammalian HSPs.[26] Theoretically, this high homology in combination with their up-regulation during stress and immunodominancy could be dangerous, putting the host at risk for autoimmunity through antigenic mimicry.[27] However, T and B cell responses to self-HSP are present in healthy individuals (and even in cord blood) without consequent widespread inflammation or autoimmunity.[28-30] Therefore, some regulatory immune response preventing autoimmunity has to be present in healthy individuals. HSP specific immune responses have been suggested to have a driving factor in the generation of this regulatory action.[28,31] The regulation-inducing capacity of HSPs could be mediated by innate or adaptive effects.[14]

Innate effects of HSP

The innate immune system was originally thought to only recognize pathogen associated molecular patterns (PAMPs) via their pathogen recognition receptors (PRR), also known as the 'infectious non-self model'.[32] Matzinger proposed that the innate immune system rather responds to endogenous danger signals (danger associated molecular patterns, DAMPs), released by damaged or stressed cells with the tissue playing an important role in determining the quality of the immune response.[33,34] As HSPs are upregulated and excreted during stress, these proteins have long been implicated in triggering innate immune responses. Currently, a debate is going whether these innate effects of HSP (reviewed in [19]) could have been the result of contamination by other TLR agonists (reviewed in [35]). Although the debate is ongoing, considerable evidence exists to support the innate effects of HSP. Properly controlled research revealed that self-HSP60 (and not microbial HSP60) has a direct, LPS-independent innate effect on T cells mediated through TLR2 and on monocytes and macrophages through TLR 4.[36]

Adaptive effects of HSP

The induction of regulation by HSPs could also be mediated by adaptive immune responses to self-HSP. Due to the homology between bacterial and mammalian HSPs, the presence of self-HSP specific T and B cell responses in healthy individuals has been hypothesized to be the result of stimulation by HSPs from commensal bacteria in the gut.[20] To safely contain these autoreactive T cells that escaped central tolerance, peripheral tolerance results in mainly regulatory properties of self-HSP reactive T cells. Data from animal

models indicate that cross reactive immunoregulatory T cell responses to self-HSP may also play a role in disease protection.[31,37-39] In line with this hypothesis, the presence of self-HSP60-specific T cell responses in Juvenile idiopathic arthritis (JIA) patients correlated with a benign disease course.[40-42] Self-HSP-specific T cell responses have been reported to be immunoregulatory in various other autoimmune diseases like RA[43] and JDM[15] by the production of anti-inflammatory cytokines like IL-10, IL-4 and TGFβ.[40,44] A recent study revealed that self-HSP60 could directly induce highly suppressive FoxP3+ Tregs in vitro.[45] Also, low concentrations of human HSP60 or p277 (a synthetic human HSP60 derived peptide) have shown to be able to enhance the regulatory function of CD25+ Treg from human PBMC.[21,46] Theoretically, the stimulation of innate immune receptors by HSP might enhance a regulatory response by the adaptive arm of the immune system. This would be in line with observations that activation of TLRs by commensal bacteria plays an important role in the maintenance of intestinal homeostasis.[47]

In conclusion, HSPs seem to be suitable bystander antigens that can be targeted by antigen specific immunotherapy as they induce protection in experimental autoimmune models and elicit in vitro regulatory responses in human autoimmune disease.

Peptide immunotherapy

Antigen specific immunotherapy with proteins has been hampered by side effects like mast cell activation or cytotoxic T cell responses.[48-50] Peptide immunotherapy can be an attractive alternative, as it increases specificity and thereby reduces side effects. Moreover, synthetic peptides are free of microbial products.

Peptide selection

In human disease, selection of appropriate peptides for immunotherapy is a major challenge. The selection process is helped by focussing on desirable characteristics of the peptide.

First, the peptide should be recognized by the human immune system and thus be able to bind disease-associated HLA molecules. For this purpose, multiple prediction models of peptide binding to HLA have been shown helpful.[51-54] Second, the peptide should mimic the naturally processed epitope as altered peptides may behave unpredictably.[55,56] To fulfil this criterion, selection can be based on elution studies of HLA-peptide complexes. Third, as self-crossreactive responses have been shown to be important in the disease-protecting effect of peptides, a peptide with high homology to self that still is immunogenic is desirable.

HSP-peptide immunotherapy

HSP-peptides have been shown to prevent autoimmune disease in multiple experimental animal models (table 2). Most peptides used in these models were not primarily selected on binding capacity of disease associated human HLA molecules, a feature desired for translation of the experimental results into peptide immunotherapy in humans.

In two recent studies, HSP60-derived HLA-binding peptides were tested in an experimental arthritis model.[57,58] In one of the two studies, the identified human HSP60 epitope was artificially modified to increase the HLA binding affinity and to skew toward a regulatory T cell response.[57] Intradermal administration of this altered peptide suppressed experimental arthritis (AA) in vivo by the induction of regulatory T cells (Treg) and increased Treg frequency in ex vivo assays with peripheral blood mononuclear cells (PBMC) from RA patients in contrast with the native peptide.[57] However, as mentioned, native peptides that mimic the naturally processed epitope are preferred for safe antigen specific immunotherapy and intradermal administration is not the optimal route for tolerance induction.

The other study performed by our group identified native HLA-binding T cell epitopes of human and mycobacterial HSP60 that are recognized by the majority of arthritis patients and evoke a tolerogenic immune response in PBMCs of arthritis patients without the need for modulation.[43,44] Nasal administration of one of these HLA binding mycobacterial HSP60-peptides was effective in experimental arthritis and induced a CD4+ T cell population with reduced TNFα production at the site of inflammation. The induced T cell population expressed FoxP3 and had potent suppressive capacity, which upon transfer protected against arthritis.[58]

These specific experimental results have not been translated into clinical trials yet. So far, clinical trials have been performed with two other interesting HSP-epitopes (table 3).

DnaJP1

The first clinical trial with a HSP-peptide in human arthritis was performed with dnaJP1. DnaJP1 is a 15-mer peptide derived from *E. Coli* HSP40, containing a sequence of five amino acids found in the majority of HLA-DR alleles linked with RA ('shared epitope'). In a phase I trial, patients with early active RA received oral dnaJP1 during 6 months. After treatment, in vitro responses to dnaJP1 changed from pro-inflammatory to anti-inflammatory, with increased IL-10 production and augmented FoxP3 expression in Treg cells.[59]

In a following phase II trial, patients with active RA with proven immunological reactivity to dnaJP1 received the same mucosal DnaJ treatment. Clinical improvement was achieved at multiple time points and was accompanied again by anti-inflammatory in-vitro responses to dnaJP1 with reduced production of TNFα and a trend towards increased production of IL-10.[60]

Table 2 Protective heat shock protein (HSP)-peptide treatment in experimental models of autoimmunity.

	model*	route†	adjuvant‡	regimen¥	peptides	HSP	references
Arthritis	DIA	i.d.	IFA	p	mixture of 120-134 and 213-277	self	Moudgil J Imm 2005
	PIA	i.p.	none	p and t	261-271	non-self	Thompson J Imm 1998, Francis Imm 2000
	AA and CPIA	i.d.	DDA	p	256-270	non-self	Anderton J Exp Med 1995
	AA and AIA	i.n.	none	p	176-190	non-self	Prakken PNAS 1997
	AA	i.d.	IFA	p	180-188	non-self	Golden Agents Actions 1991
	AA	i.n.	none	t	180-188	non-self	Roord Plos 2006
	AA	i.d.	IFA	p	234-252	non-self	Tanaka J Imm 1999
	AA	i.n.	none	p	111-125	non-self	Wendling J Imm 2000
	AA	i.p.	none	p	61-80 (mHSP65), 31-46, 37-52 (self)HSP60	both	Ulmansky J Imm 2002
	AA	i.n.	none	p	254-268	non-self	Zonneveld-Huijssoon Ann Rheum Dis 2011
	AA	s.c.	DDA	p	mixture of 417-431, 441-455, 465-479, 513-527, 521-535 (BCTD)	non-self	Moudgil J Exp Med 1997, Durai J Imm 2004
	AA	s.c.	IFA	p	177-191	non-self	Durai J Rheumatol 2007
DM	NOD	s.c.	IFA	t	437-460 (p277)	self	Cohen Lancet 1994
	NOD	i.p.	IFA	p and t	437-460 (p277)	self	Elias Diabetes 1995, Diabetes 1997, Ablamunits J Autoimm 1998, Tian J Imm 1998, Elias PNAS 1991
	STZ	i.p.	mineral oil	t	437-460 (p277)	self	Elias Diabetes 1996
	NOD	s.c.	IFA	p	166-185 (p12)	self	Elias J Autoimm 1997
	BB-DP	p.o.	none	p	peptide analogue of p277 (Diapep277)	self	Brugman Diabetologia 2004
Sjogren	SS	s.c.	IFA	p	437-460	self	Delaleu Arthr Rheum 2008

Adapted from [14] and [24]

*AA: adjuvant arthritis, DIA: dimethyl dioctadecyl ammoniumbromide induced arthritis, PIA: pristane induced arthritis, AIA: avridine-induced arthritis, CPIA: CP20961 induced arthritis, STZ: STZ toxin induced diabetes, BB-DP: BioBreeding-Diabetes Prone rat, SS: spontaneous sjogren syndrome

†i.d.: intradermal, i.p.: intraperitoneal, i.n.: intra nasal, s.c.: subcutaneous, p.o.: per os, oral

‡IFA: incomplete Freund's adjuvant, DDA: dimethyl dioctadecyl ammoniumbromide

¥p: preventive regimen, t: therapeutic regimen

Table 3 Clinical trials of peptide immunotherapy with heat shock protein (HSP) peptides in autoimmune diseases.

	trial name	design	type of therapy	peptide	route	patient group*	immunomodulatory effects	clinical efficacy†	ref
Diabetes	DiaPep277	Phase II	parenteral peptide	p277 (HSP60 437-460)	s.c.	new onset T1D	increased IL-10 production by T cells is associated with preservation of C-peptide up to 12-18 months	lower need for exogenous insulin	64
RA		Phase II	oral peptide	DnaJP1 peptide (HSP40)	oral	RA	immune diviation from TNFa to IL-10, peptide induced FoxP3 expression on CD25bright cells	ACR20 and ACR50 score reduced	60

*T1D: type 1 diabetes, RA: rheumatoid arthritis

†ACR 20, ACR50: measurements of improvement in rheumatoid arthritis.

Diapep277

A vaccination strategy based on HSP60 as diabetes autoantigen was performed with p277 (DiaPep277), a 24-amino-acid peptide of mouse HSP60 (437-460) that had preventive and therapeutic effects in experimental diabetes.[61,62]

Multiple phase II trials have been performed with subcutaneous p277.[63-66] In adults newly diagnosed with T1D, residual C-peptide levels (reflecting the amount of insulin production) could be preserved, without a reduction in the amount of exogenous insulin needed.[66,67] A recent immunological study revealed that the preservation of C-peptide was associated with peptide specific tolerance.[64] Phase II trials are currently ongoing aiming at the maintenance of insulin secretion as measured by C-peptide levels.[68]

Now good candidates for immunotherapy have been proven effective in multiple animal models of autoimmune diseases, and translation into humans has provided encouraging results, the next step will be to improve therapeutic efficiency in human autoimmune disease. Enhancement of peptide immunogenicity when delivered via the mucosal route and combination of peptide therapy with immune modulating agents could be interesting options in this perspective.

IMPROVING ANTIGEN SPECIFIC THERAPY

Enhancing peptide immunogenicity

The peptide signal delivered via the tolerogenic mucosal route may be too small to induce immune deviation due to the intrinsic weakness of the peptide signal alone, directed at

stimulation of the adaptive immune system only.[11,12] In a healthy immune system, activation of the innate immune system leads to better presentation of the peptide and thereby enhances peptide-triggered adaptive immune responses. The combination of enhancing both adaptive and innate immunity may therefore be an attractive option for the enhancement of mucosal immunotherapy in autoimmune disease. This makes sense when considered that control of the effector class is an active process in which immune activation is needed for optimal control.[34]

Route of administration

It is conceivable that subcutaneous administration can enhance immunogenicity by eliciting a local inflammatory (innate) response. This route has shown to be effective in allergy desensitisation and in T1D.[64,69] Nevertheless, subcutaneous injection of a peptide holds the risk of exaggerated pro-inflammatory immune responses as anaphylactic responses to soluble antigen have been observed after subcutaneous administration.[55,56,70]

Other strategies to increase efficiency of antigen delivery like peptide presented by artificial APC (ethylcarbodiimide (ECDI) fixed splenocytes), plasmid DNA encoding antigen, peptide-MHC complexes or fusion proteins with tandem repeats of the peptide, have been effective in animal models, but were not tested in patients.[70-73]

Adjuvant

To stimulate the innate immune system and thereby enhance peptide presentation, the use of adjuvants has been successful in mucosal vaccination strategies for infectious diseases including polio and influenza.[74-79] Although adjuvants have been used in autoimmunity for subcutaneous or intradermal vaccination strategies, the concept of enhancing mucosal vaccination with an adjuvant for preventive peptide therapy in autoimmune disease is novel (figure 1).

In clinical diabetes, alum as an adjuvant for antigen specific immunotherapy led to preservation of residual insulin secretion in adults and children with early onset T1D.[80-83] The increased effectiveness of alum-adjuvanted peptide immunotherapy has been shown to depend on activation of innate immunity by DNA released from dying cells.[84] However, alum as an adjuvant could not restore euglycemia in type I diabetes patients, indicating a need to explore other adjuvants.

An adjuvant with promising results in NOD mice is the non-toxic B subunit of the cholera enterotoxin (CTB). Oral administration of islet auto-antigens linked to CTB, significantly improved suppression of hyperglycemia and pancreatic inflammation.[85,86] As CTB has been shown to induce enhanced antigen capture by dendritic cells, this therapeutic efficacy could be due to enhanced presentation of the linked peptide.[87,88]

An agent that can be considered an innate-activating adjuvant for mucosal peptide therapy is HSP itself. For example, HSP can upregulate adaptive immune responses by stimulat-

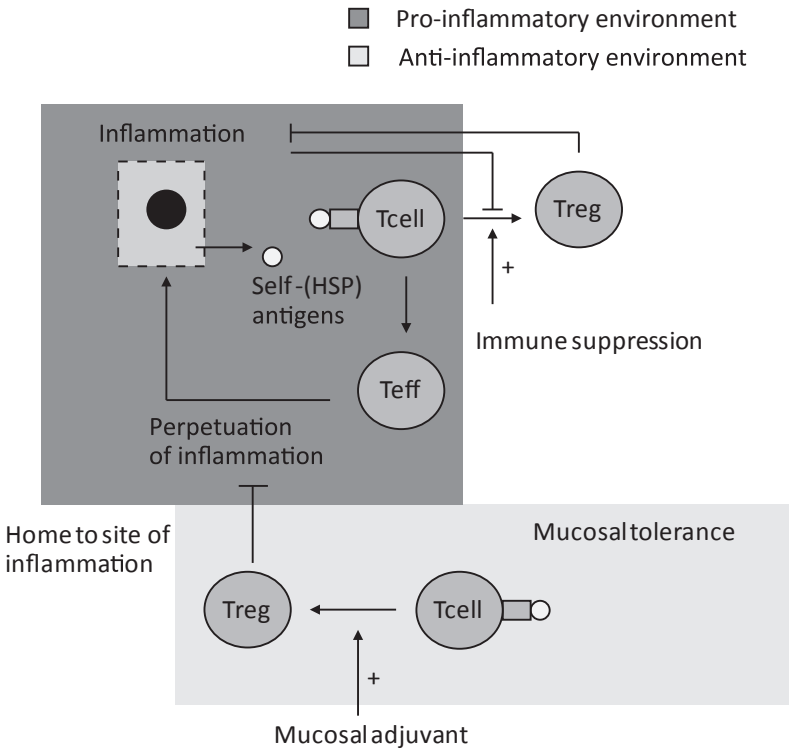


Figure 1 The dual role of heat shock proteins (HSPs) (pro- and anti-inflammatory) in modulating the immune system is influenced by multiple factors. The cytokine profile of HSP reactive cells, phase of the immune response or the tissue in which the recognition takes place may determine whether HSP60 autoreactivity is noxious or beneficial. T cells induced via the mucosal route can thus be directed by the anti-inflammatory environment towards a predominantly tolerogenic response. The mucosally induced antigen specific T cells consist of multiple kinds of regulatory cells and are thought to migrate to the site of inflammation as their cognate antigen (e.g. HSP) is expressed there. At the site of inflammation, these antigen specific Tregs skew the pro-inflammatory T cell response toward an anti-inflammatory phenotype by cytokine release like IL-10 or cell-cell contact.
Mucosal adjuvant could enhance peptide presentation by APCs at the site of tolerance induction, enlarging the pool of Treg formed after mucosal tolerance induction.
The inflammatory environment hampers the development of (self-HSP specific) Tregs. Combination therapy of antigen specific mucosal tolerance induction with immune suppression could therefore reduce systemic inflammation, creating a more favourable environment for the development of these Tregs.

ing innate receptors like TLR 2 and 4.[36] HSP60 indeed enhanced immunogenicity of CMV peptide vaccines[89] and increased efficacy of p277 therapy in diabetic mice.[73] HSP60 seems to function as the body's natural adjuvant or immunogenic carrier, maybe as a result of its ability to activate both the innate and adaptive response.[21]

Adjuvants of particular interest that can be administered mucosally are CpG-oligodeoxy-nucleotides (CpG-ODN), which consist of a nucleotide sequence common in bacterial DNA.

CpG-ODN stimulates TLR9 on antigen presenting cells and has been successfully used as vaccine adjuvant in AVA vaccination in healthy volunteers.[90,91] Preliminary data from our group indicate that CpG-ODN may enhance antigen-specific immunotherapy in an experimental arthritis model (unpublished data).

In conclusion, enhancing immunogenicity of a peptide in a preventive regimen seems very efficient in improving peptide-specific immunotherapy. However, caution should be taken in the addition of pro-inflammatory agents to a peptide in a therapeutic setting, as it could lead to overactivation of an already deranged immune system.

Combination therapy with general immune modulators

In addition to the risks of presenting an antigen in an inflammatory setting, the inflamed environment could also mask the peptide signal, thereby decreasing the efficacy of the therapy. This may explain why peptide specific therapy is only partially effective in established autoimmune disease with widespread immune activation. To artificially create a more anti-inflammatory environment, combination of antigen specific immunotherapy with general immune modulators could be of value (figure 1).
Next to the reduction of inflammatory background 'noise', creating an environment in which the antigen specific response can be detected and modulated, dampening the inflammation may also be crucial for adequate functioning of Tregs (reviewed by [92]). A chronic inflammatory environment can cause local dysfunction of Tregs or convert them into pro-inflammatory Th17 cells.[93-98] Generalized immune suppression by TNFα blockade[99,100] or immune modulation by anti CD3[101-103] monotherapy have been described to favour the development of Treg cells. However, it is conceivable that after these non-antigen specific immune therapies only a small number of these Tregs will be specific for antigens expressed in the target autoimmune organ. Therefore, the enhanced peptide recognition due to the combination of immune suppression with antigen specific peptide therapy could expand the antigen specific Tregs that can migrate to the place where their cognate antigen is expressed: the site of inflammation.

Several successful combination therapy strategies in autoimmune diseases have been reported in literature. For example, combination therapy of anti-CD3 with disease related peptides has shown to be successful in experimental models of new onset diabetes. The combined therapy approach was more efficient than peptide or anti-CD3 monotherapy and induced antigen specific Tregs that could transfer protection.[104,105] Combined anti-CD3 therapy with disease related peptides has so far not been tested in human T1D. Maybe such a combined approach could improve recent results of anti-CD3 monotherapy in human T1DM.[103]

Another proven effective strategy is to combine antigen specific immunotherapy with TNFα blockade. In the rat adjuvant arthritis model, a low dose of anti-TNFα (Etanercept) combined with nasal administration of a HSP60 peptide could induce clinical control in a therapeutic setting to a larger extent than peptide treatment alone. The clinical response was accompanied by an increase in peptide specific FoxP3 expressing T cells to a degree comparable to full dose Etanercept. The combination treatment was a stronger peptide specific IL-10 inducer than Etanercept monotherapy.[106]
An interesting finding regarding combination therapy of a peptide with immune modulation in humans has been done in the earlier described dnaJP1 clinical trial in rheumatoid arthritis. A post hoc analysis revealed that best clinical results were obtained in a subgroup of patients taking hydroxychloroquine (HCQ). HCQ is an immune modulating agent enhancing the availability of the exogenous administered peptide by blocking the intracellular peptide presentation.[107] Next to the earlier mentioned combination strategies in animal models, these findings indicate the potential therapeutic efficacy of combination treatment in humans as well.[60]

In summary, combination therapy of antigen specific therapy with low-dose general immune modulators shows promising results in experimental autoimmunity but in human autoimmune disease, evidence is yet limited. The efficacy of this combination therapy is worth exploring while the possibility of lowering the dose of immune modulators reduces side effects associated with life-long drug administration.

CONCLUSION

In this review, we discussed strategies to improve the clinical outcome of antigen specific immunotherapy in human autoimmune disease. Three major issues concerning the choice of antigen, route of administration and timing of therapy were under discussion in this paper, but other issues remain before safe and effective antigen specific immunotherapy can be applied in human autoimmune disease.
For example, dosing is important for oral tolerance therapy to be effective[8] and dose finding studies are needed to further improve therapeutic results.
Furthermore, the selection of patients in clinical trials of peptide specific immunotherapy seems to be crucial. Prerequisites for treatment response have been identified in experimental and human studies. First, genetic factors play a role as the availability of antigen specific T cells needed for a beneficial response after treatment varied between different genetic backgrounds in mice models of diabetes.[105] Second, a high representation of tolerogenic and anergic immune pathways at baseline has been associated with clinical responsiveness to peptide immunotherapy.[60] Third, the presence and quality of peptide

specific responses before start of treatment seem to play a role in the eventual efficacy of peptide immunotherapy.[60,64] These results could be of help in patient selection for future studies.

In conclusion, peptide immunotherapy with bystander antigens such as HSPs shows promising results in experimental models and the first positive results from clinical trials are currently emerging. New approaches aiming for enhanced peptide recognition in a controlled immune environment by the use of combination therapies may hold promise for a successful future for peptide specific immunotherapy in autoimmune diseases.

REFERENCE LIST

1. Dekker L, Armbrust W, Rademaker CM, Prakken B, Kuis W, Wulffraat NM. Safety of anti-TNFalpha therapy in children with juvenile idiopathic arthritis. *Clin Exp Rheumatol.* 2004;22:252-258.
2. Maini RN, Taylor PC. Anti-cytokine therapy for rheumatoid arthritis. *Annu Rev Med.* 2000;51:207-229.
3. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med.* 2001;345:1098-1104.
4. Bloom BJ. Development of diabetes mellitus during etanercept therapy in a child with systemic-onset juvenile rheumatoid arthritis. *Arthritis Rheum.* 2000;43:2606-2608.
5. Lepore L, Marchetti F, Facchini S, Leone V, Ventura A. Drug-induced systemic lupus erythematosus associated with etanercept therapy in a child with juvenile idiopathic arthritis. *Clin Exp Rheumatol.* 2003;21:276-277.
6. FDA Drug Safety Communication: Safety Review update on reports of Hepatosplenic T-Cell Lymphoma in adolescents and young adults receiving tumor necrosis factor (TNF) blockers, azathioprine and/or mercaptopurine. 2011.
7. Sabatos-Peyton CA, Verhagen J, Wraith DC. Antigen-specific immunotherapy of autoimmune and allergic diseases. *Curr Opin Immunol.* 2010;22:609-615.
8. Faria AM, Weiner HL. Oral tolerance. *Immunol Rev.* 2005;206:232-259.
9. Wraith DC. Therapeutic peptide vaccines for treatment of autoimmune diseases. *Immunol Lett.* 2009;122:134-136.
10. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, Santacruz N, et al. Peripheral education of the immune system by colonic commensal microbiota. *Nature.* 2011;478:250-254.
11. Decrausaz L, Domingos-Pereira S, Duc M, Bobst M, Romero P, Schiller JT, et al. Parenteral is more efficient than mucosal immunization to induce regression of human papillomavirus-associated genital tumors. *Int J Cancer.* 2011;129:762-772.
12. Bouvet JP, Decroix N, Pamonsinlapatham P. Stimulation of local antibody production: parenteral or mucosal vaccination? *Trends Immunol.* 2002;23:209-213.
13. Bresson D, von Herrath M. Moving towards efficient therapies in type 1 diabetes: to combine or not to combine? *Autoimmun Rev.* 2007;6:315-322.
14. van Eden W, van der Zee R, Prakken B. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol.* 2005;5:318-330.
15. Elst EF, Klein M, de Jager W, Kamphuis S, Wedderburn LR, van der Zee R, et al. Hsp60 in inflamed muscle tissue is the target of regulatory autoreactive T cells in patients with juvenile dermatomyositis. *Arthritis Rheum.* 2008;58:547-555.
16. Boog CJ, de Graeff-Meeder ER, Lucassen MA, van der Zee R, Voorhorst-Ogink MM, van Kooten PJ, et al. Two monoclonal antibodies generated against human hsp60 show reactivity with synovial membranes of patients with juvenile chronic arthritis. *J Exp Med.* 1992;175:1805-1810.
17. Huang QQ, Sobkoviak R, Jockheck-Clark AR, Shi B, Mandelin AM, Tak PP, et al. Heat shock protein 96 is elevated in rheumatoid arthritis and activates macrophages primarily via TLR2 signaling. *J Immunol.* 2009;182:4965-4973.
18. Puga Yung GL, Fidler M, Albani E, Spermon N, Teklenburg G, Newbury R, et al. Heat shock protein-derived T-cell epitopes contribute to autoimmune inflammation in pediatric Crohn's disease. *PLoS ONE.* 2009;4:e7714.

19. Henderson B, Pockley AG. Molecular chaperones and protein-folding catalysts as intercellular signaling regulators in immunity and inflammation. *J Leukoc Biol.* 2010;88:445-462.
20. Cohen IR. Autoimmunity to chaperonins in the pathogenesis of arthritis and diabetes. *Annu Rev Immunol.* 1991;9:567-589.
21. Quintana FJ, Cohen IR. The HSP60 immune system network. *Trends Immunol.* 2011;32:89-95.
22. Sobel DO, Creswell K. Characterization of anti-islet cytotoxic human T-cell clones from patients with type 1 diabetes mellitus. *Autoimmunity.* 2006;39:323-332.
23. Jiang H, Canfield SM, Gallagher MP, Jiang HH, Jiang Y, Zheng Z, et al. HLA-E-restricted regulatory CD8(+) T cells are involved in development and control of human autoimmune type 1 diabetes. *J Clin Invest.* 2010;120:3641-3650.
24. Huang MN, Yu H, Moudgil KD. The involvement of heat-shock proteins in the pathogenesis of autoimmune arthritis: a critical appraisal. *Semin Arthritis Rheum.* 2010;40:164-175.
25. Wu CT, Ou LS, Yeh KW, Lee WI, Huang JL. Serum heat shock protein 60 can predict remission of flare-up in juvenile idiopathic arthritis. *Clin Rheumatol.* 2011;30:959-965.
26. Karlin S, Brocchieri L. Heat shock protein 60 sequence comparisons: duplications, lateral transfer, and mitochondrial evolution. *Proc Natl Acad Sci U S A.* 2000;97:11348-11353.
27. van Wijk F, Prakken B. Heat shock proteins: Darwinistic immune modulation on dangerous grounds. *J Leukoc Biol.* 2010;88:431-434.
28. Cohen IR. Peptide therapy for Type I diabetes: the immunological homunculus and the rationale for vaccination. *Diabetologia.* 2002;45:1468-1474.
29. Fischer HP, Sharrock CE, Panayi GS. High frequency of cord blood lymphocytes against mycobacterial 65-kDa heat-shock protein. *Eur J Immunol.* 1992;22:1667-1669.
30. Aalberse JA, Kapitein B, de Roock S, Klein MR, de Jager W, van der Zee R, et al. Cord blood CD4+ T cells respond to self heat shock protein 60 (HSP60). *PLoS ONE.* 2011;6:e24119.
31. Anderton SM, van der Zee R, Prakken B, Noordzij A, van Eden W. Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *J Exp Med.* 1995;181:943-952.
32. Janeway CA, Jr., Dianzani U, Portoles P, Rath S, Reich EP, Rojo J, et al. Cross-linking and conformational change in T-cell receptors: role in activation and in repertoire selection. *Cold Spring Harb Symp Quant Biol.* 1989;54 Pt 2:657-666.
33. Matzinger P. The danger model: a renewed sense of self. *Science.* 2002;296:301-305.
34. Matzinger P, Kamala T. Tissue-based class control: the other side of tolerance. *Nat Rev Immunol.* 2011;11:221-230.
35. Tsan MF. Heat shock proteins and high mobility group box 1 protein lack cytokine function. *J Leukoc Biol.* 2011;89:847-853.
36. Zanin-Zhorov A, Nussbaum G, Franitza S, Cohen IR, Lider O. T cells respond to heat shock protein 60 via TLR2: activation of adhesion and inhibition of chemokine receptors. *FASEB J.* 2003;17:1567-1569.
37. Durai M, Kim HR, Moudgil KD. The regulatory C-terminal determinants within mycobacterial heat shock protein 65 are cryptic and cross-reactive with the dominant self homologs: implications for the pathogenesis of autoimmune arthritis. *J Immunol.* 2004;173:181-188.
38. Durai M, Kim HR, Bala KK, Moudgil KD. T cells against the pathogenic and protective epitopes of heat-shock protein 65 are crossreactive and display functional similarity: novel aspect of regulation of autoimmune arthritis. *J Rheumatol.* 2007;34:2134-2143.
39. Wendling U, Paul L, van der Zee R, Prakken B, Singh M, van Eden W. A conserved mycobacterial heat shock protein (hsp) 70 sequence prevents adjuvant arthritis upon nasal administration and induces

IL-10-producing T cells that cross-react with the mammalian self-hsp70 homologue. *J Immunol.* 2000;164:2711-2717.

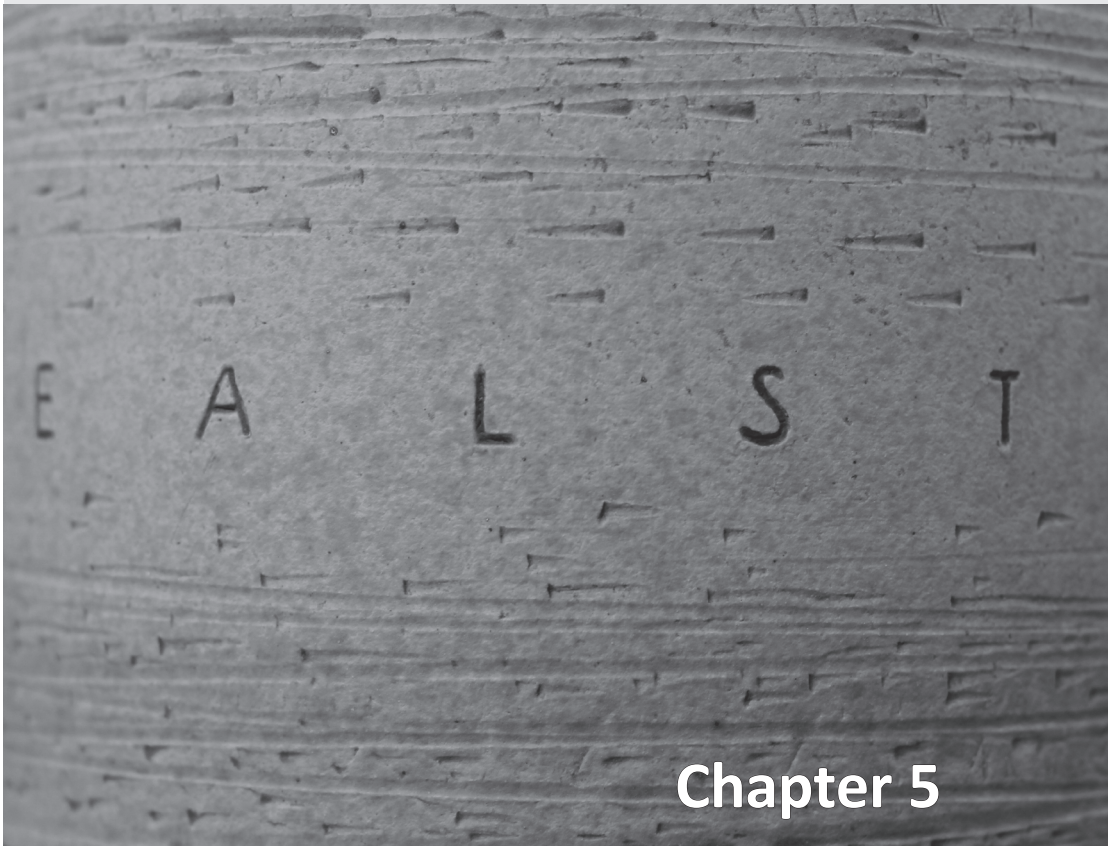
40. Prakken AB, van Eden W, Rijkers GT, Kuis W, Toebes EA, Graeff-Meeder ER, et al. Autoreactivity to human heat-shock protein 60 predicts disease remission in oligoarticular juvenile rheumatoid arthritis. *Arthritis Rheum.* 1996;39:1826-1832.
41. Albani S, Ravelli A, Massa M, De Benedetti F, Andree G, Roudier J, et al. Immune responses to the Escherichia coli dnaJ heat shock protein in juvenile rheumatoid arthritis and their correlation with disease activity. *J Pediatr.* 1994;124:561-565.
42. de Graeff-Meeder ER, van der Zee R, Rijkers GT, Schuurman HJ, Kuis W, Bijlsma JW, et al. Recognition of human 60 kD heat shock protein by mononuclear cells from patients with juvenile chronic arthritis. *Lancet.* 1991;337:1368-1372.
43. de Jong H, Lafeber FF, de Jager W, Haverkamp MH, Kuis W, Bijlsma JW, et al. PAN-DR-Binding Hsp60 self epitopes induce an interleukin-10-mediated immune response in rheumatoid arthritis. *Arthritis Rheum.* 2009;60:1966-1976.
44. Kamphuis S, Kuis W, de Jager W, Teklenburg G, Massa M, Gordon G, et al. Tolerogenic immune responses to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis. *Lancet.* 2005;366:50-56.
45. de Kleer I, Vercoulen Y, Klein M, Meerding J, Albani S, van der Zee R, et al. CD30 discriminates heat shock protein 60-induced FOXP3+ CD4+ T cells with a regulatory phenotype. *J Immunol.* 2010;185:2071-2079.
46. Zanin-Zhorov A, Cahalon L, Tal G, Margalit R, Lider O, Cohen IR. Heat shock protein 60 enhances CD4 CD25 regulatory T cell function via innate TLR2 signaling. *J Clin Invest.* 2006;116:2022-2032.
47. Rakoff-Nahoum S, Paglino J, Eslami-Varzaneh F, Edberg S, Medzhitov R. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell.* 2004;118:229-241.
48. Genain CP, Abel K, Belmar N, Villinger F, Rosenberg DP, Linington C, et al. Late complications of immune deviation therapy in a nonhuman primate. *Science.* 1996;274:2054-2057.
49. Blanas E, Carbone FR, Allison J, Miller JF, Heath WR. Induction of autoimmune diabetes by oral administration of autoantigen. *Science.* 1996;274:1707-1709.
50. Hanninen A, Braakhuis A, Heath WR, Harrison LC. Mucosal antigen primes diabetogenic cytotoxic T-lymphocytes regardless of dose or delivery route. *Diabetes.* 2001;50:771-775.
51. Sette A. Tools of the trade in vaccine design. *Science.* 2000;290:2074b-2075b.
52. Singh H, Raghava GP. ProPred: prediction of HLA-DR binding sites. *Bioinformatics.* 2001;17:1236-1237.
53. Bian H, Hammer J. Discovery of promiscuous HLA-II-restricted T cell epitopes with TEPITOPE. *Methods.* 2004;34:468-475.
54. Mohanapriya A, Lulu S, Kayathri R, Kangueane P. Class II HLA-peptide binding prediction using structural principles. *Hum Immunol.* 2009;70:159-169.
55. Kappos L, Comi G, Panitch H, Oger J, Antel J, Conlon P, et al. Induction of a non-encephalitogenic type 2 T helper-cell autoimmune response in multiple sclerosis after administration of an altered peptide ligand in a placebo-controlled, randomized phase II trial. The Altered Peptide Ligand in Relapsing MS Study Group. *Nat Med.* 2000;6:1176-1182.
56. Bielekova B, Goodwin B, Richert N, Cortese I, Kondo T, Afshar G, et al. Encephalitogenic potential of the myelin basic protein peptide (amino acids 83-99) in multiple sclerosis: results of a phase II clinical trial with an altered peptide ligand. *Nat Med.* 2000;6:1167-1175.
57. Dominguez MC, Lorenzo N, Barbera A, Darrasse-Jeze G, Hernandez MV, Torres A, et al. An altered peptide ligand corresponding to a novel epitope from heat-shock protein 60 induces regulatory T

cells and suppresses pathogenic response in an animal model of adjuvant-induced arthritis. *Autoimmunity.* 2011;44:471-482.

58. Zonneveld-Huijssoon E, Roord ST, de Jager W, Klein M, Albani S, Anderton SM, et al. Bystander suppression of experimental arthritis by nasal administration of a heat shock protein peptide. *Ann Rheum Dis.* 2011.
59. Prakken BJ, Samodal R, Le TD, Giannoni F, Yung GP, Scavulli J, et al. Epitope-specific immunotherapy induces immune deviation of proinflammatory T cells in rheumatoid arthritis. *Proc Natl Acad Sci U S A.* 2004;101:4228-4233.
60. Koffeman EC, Genovese M, Amox D, Keogh E, Santana E, Matteson EL, et al. Epitope-specific immunotherapy of rheumatoid arthritis: clinical responsiveness occurs with immune deviation and relies on the expression of a cluster of molecules associated with T cell tolerance in a double-blind, placebo-controlled, pilot phase II trial. *Arthritis Rheum.* 2009;60:3207-3216.
61. Birk OS, Elias D, Weiss AS, Rosen A, van der Zee R, Walker MD, et al. NOD mouse diabetes: the ubiquitous mouse hsp60 is a beta-cell target antigen of autoimmune T cells. *J Autoimmun.* 1996;9:159-166.
62. Elias D, Cohen IR. Peptide therapy for diabetes in NOD mice. *Lancet.* 1994;343:704-706.
63. Raz I, Elias D, Avron A, Tamir M, Metzger M, Cohen IR. Beta-cell function in new-onset type 1 diabetes and immunomodulation with a heat-shock protein peptide (DiaPep277): a randomised, double-blind, phase II trial. *Lancet.* 2001;358:1749-1753.
64. Huurman VA, van der Meide PE, Duinkerken G, Willemen S, Cohen IR, Elias D, et al. Immunological efficacy of heat shock protein 60 peptide DiaPep277 therapy in clinical type I diabetes. *Clin Exp Immunol.* 2008;152:488-497.
65. Raz I, Avron A, Tamir M, Metzger M, Symer L, Eldor R, et al. Treatment of new-onset type 1 diabetes with peptide DiaPep277 is safe and associated with preserved beta-cell function: extension of a randomized, double-blind, phase II trial. *Diabetes Metab Res Rev.* 2007;23:292-298.
66. Lazar L, Ofan R, Weintrob N, Avron A, Tamir M, Elias D, et al. Heat-shock protein peptide DiaPep277 treatment in children with newly diagnosed type 1 diabetes: a randomised, double-blind phase II study. *Diabetes Metab Res Rev.* 2007;23:286-291.
67. Huurman VA, Decochez K, Mathieu C, Cohen IR, Roep BO. Therapy with the hsp60 peptide DiaPep277 in C-peptide positive type 1 diabetes patients. *Diabetes Metab Res Rev.* 2007;23:269-275.
68. Eldor R, Kassem S, Raz I. Immune modulation in type 1 diabetes mellitus using DiaPep277: a short review and update of recent clinical trial results. *Diabetes Metab Res Rev.* 2009;25:316-320.
69. Akdis CA, Akdis M. Mechanisms of allergen-specific immunotherapy. *J Allergy Clin Immunol.* 2011;127:18-27.
70. Miller SD, Turley DM, Podojil JR. Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. *Nat Rev Immunol.* 2007;7:665-677.
71. Casares S, Hurtado A, McEvoy RC, Sarukhan A, von Boehmer H, Brumeanu TD. Down-regulation of diabetogenic CD4+ T cells by a soluble dimeric peptide-MHC class II chimera. *Nat Immunol.* 2002;3:383-391.
72. Lin M, Stoica-Nazarov C, Surls J, Kehl M, Bona C, Olsen C, et al. Reversal of type 1 diabetes by a new MHC II-peptide chimera: “Single-epitope-mediated suppression” to stabilize a polyclonal autoimmune T-cell process. *Eur J Immunol.* 2010;40:2277-2288.
73. Liang J, Aihua Z, Yu W, Yong L, Jingjing L. HSP65 serves as an immunogenic carrier for a diabetogenic peptide P277 inducing anti-inflammatory immune response in NOD mice by nasal administration. *Vaccine.* 2010;28:3312-3317.

74. Dietrich G, Griot-Wenk M, Metcalfe IC, Lang AB, Viret JF. Experience with registered mucosal vaccines. *Vaccine.* 2003;21:678-683.
75. Ivanov AP, Dragunsky EM, Chumakov KM. 1,25-dihydroxyvitamin d3 enhances systemic and mucosal immune responses to inactivated poliovirus vaccine in mice. *J Infect Dis.* 2006;193:598-600.
76. Ichinohe T, Ainai A, Tashiro M, Sata T, Hasegawa H. PolyI:polyC12U adjuvant-combined intranasal vaccine protects mice against highly pathogenic H5N1 influenza virus variants. *Vaccine.* 2009;27:6276-6279.
77. Ivory CP, Chadee K. Intranasal immunization with Gal-inhibitable lectin plus an adjuvant of CpG oligodeoxynucleotides protects against Entamoeba histolytica challenge. *Infect Immun.* 2007;75:4917-4922.
78. Mariotti S, Teloni R, von Hunolstein C, Romagnoli G, Orefici G, Nisini R. Immunogenicity of anti-Haemophilus influenzae type b CRM197 conjugate following mucosal vaccination with oligodeoxynucleotide containing immunostimulatory sequences as adjuvant. *Vaccine.* 2002;20:2229-2239.
79. Holmgren J, Adamsson J, Anjuere F, Clemens J, Czerkinsky C, Eriksson K, et al. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. *Immunol Lett.* 2005;97:181-188.
80. Ludvigsson J, Hjorth M, Cheramy M, Axelsson S, Pihl M, Forsander G, et al. Extended evaluation of the safety and efficacy of GAD treatment of children and adolescents with recent-onset type 1 diabetes: a randomised controlled trial. *Diabetologia.* 2011;54:634-640.
81. Ludvigsson J, Faresjo M, Hjorth M, Axelsson S, Cheramy M, Pihl M, et al. GAD treatment and insulin secretion in recent-onset type 1 diabetes. *N Engl J Med.* 2008;359:1909-1920.
82. Agardh CD, Cilio CM, Lethagen A, Lynch K, Leslie RD, Palmer M, et al. Clinical evidence for the safety of GAD65 immunomodulation in adult-onset autoimmune diabetes. *J Diabetes Complications.* 2005;19:238-246.
83. Agardh CD, Lynch KF, Palmer M, Link K, Lernmark A. GAD65 vaccination: 5 years of follow-up in a randomised dose-escalating study in adult-onset autoimmune diabetes. *Diabetologia.* 2009;52:1363-1368.
84. Marichal T, Ohata K, Bedoret D, Mesnil C, Sabatel C, Kobiyama K, et al. DNA released from dying host cells mediates aluminum adjuvant activity. *Nat Med.* 2011;17:996-1002.
85. Arakawa T, Yu J, Langridge WH. Food plant-delivered cholera toxin B subunit for vaccination and immunotolerization. *Adv Exp Med Biol.* 1999;464:161-178.
86. Arakawa T, Yu J, Chong DK, Hough J, Engen PC, Langridge WH. A plant-based cholera toxin B subunit-insulin fusion protein protects against the development of autoimmune diabetes. *Nat Biotechnol.* 1998;16:934-938.
87. Anosova NG, Chabot S, Shreedhar V, Borawski JA, Dickinson BL, Neutra MR. Cholera toxin, E. coli heat-labile toxin, and non-toxic derivatives induce dendritic cell migration into the follicle-associated epithelium of Peyer's patches. *Mucosal Immunol.* 2008;1:59-67.
88. Shreedhar VK, Kelsall BL, Neutra MR. Cholera toxin induces migration of dendritic cells from the subepithelial dome region to T- and B-cell areas of Peyer's patches. *Infect Immun.* 2003;71:504-509.
89. Rouvio O, Dvorkin T, mir-Kroll H, Atias D, Cohen IR, Rager-Zisman B, et al. Self HSP60 peptide serves as an immunogenic carrier for a CTL epitope against persistence of murine cytomegalovirus in the salivary gland. *Vaccine.* 2005;23:3508-3518.
90. Krieg AM. CpG motifs: the active ingredient in bacterial extracts? *Nat Med.* 2003;9:831-835.
91. Klinman D, Shirota H, Tross D, Sato T, Klaschik S. Synthetic oligonucleotides as modulators of inflammation. *J Leukoc Biol.* 2008;84:958-964.

92. Wehrens EJ, van Wijk F, Roord ST, Albani S, Prakken BJ. Treating arthritis by immunomodulation: is there a role for regulatory T cells? *Rheumatology (Oxford).* 2010;49:1632-1644.
93. Wehrens EJ, Mijnheer G, Duurland CL, Klein M, Meerding J, van Loosdregt J, et al. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. *Blood.* 2011.
94. Ruprecht CR, Gattorno M, Ferlito F, Gregorio A, Martini A, Lanzavecchia A, et al. Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. *J Exp Med.* 2005;201:1793-1803.
95. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science.* 2003;299:1033-1036.
96. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. *Blood.* 2008;112:2340-2352.
97. Radhakrishnan S, Cabrera R, Schenk EL, Nava-Parada P, Bell MP, Van K, V, et al. Reprogrammed FoxP3+ T regulatory cells become IL-17+ antigen-specific autoimmune effectors in vitro and in vivo. *J Immunol.* 2008;181:3137-3147.
98. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood.* 2006;108:253-261.
99. Ricciardelli I, Lindley KJ, Londei M, Quaratino S. Anti tumour necrosis-alpha therapy increases the number of FOXP3 regulatory T cells in children affected by Crohn's disease. *Immunology.* 2008;125:178-183.
100. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med.* 2004;200:277-285.
101. Boettler T, von Herrath M. Immunotherapy of type 1 diabetes--how to rationally prioritize combination therapies in T1D. *Int Immunopharmacol.* 2010;10:1491-1495.
102. Penaranda C, Tang Q, Bluestone JA. Anti-CD3 Therapy Promotes Tolerance by Selectively Depleting Pathogenic Cells while Preserving Regulatory T Cells. *J Immunol.* 2011;187:2015-2022.
103. Sherry N, Hagopian W, Ludvigsson J, Jain SM, Wahlen J, Ferry RJ, Jr., et al. Teplizumab for treatment of type 1 diabetes (Protege study): 1-year results from a randomised, placebo-controlled trial. *Lancet.* 2011;378:487-497.
104. Bresson D, Togher L, Rodrigo E, Chen Y, Bluestone JA, Herold KC, et al. Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs. *J Clin Invest.* 2006;116:1371-1381.
105. Bresson D, Fradkin M, Manenkova Y, Rottembourg D, von Herrath M. Genetic-induced variations in the GAD65 T-cell repertoire governs efficacy of anti-CD3/GAD65 combination therapy in new-onset type 1 diabetes. *Mol Ther.* 2010;18:307-316.
106. Roord ST, Zonneveld-Huijssoon E, Le T, Yung GP, Koffeman E, Ronaghy A, et al. Modulation of T cell function by combination of epitope specific and low dose anticytokine therapy controls autoimmune arthritis. *PLoS ONE.* 2006;1:e87.
107. Fox RI, Kang HI. Mechanism of action of antimalarial drugs: inhibition of antigen processing and presentation. *Lupus.* 1993;2 Suppl 1:S9-12.



Chapter 5

Bystander suppression of experimental arthritis by nasal administration of a heat shock protein peptide

Evelien Zonneveld-Huijssoon, Sarah TA Roord, Wilco de Jager, Mark Klein, Salvatore Albani, Stephen M Anderton, Wietse Kuis, Femke van Wijk, Berent J Prakken

Annals of the Rheumatic Diseases 2011;70(12):2199-206

ABSTRACT

Objective

Mucosal immune therapy with disease-inducing antigens is an effective way to prevent experimental arthritis, but in humans these antigens are unknown. In juvenile idiopathic arthritis however, T cell recognition of a so-called bystander antigen heat shock protein 60 (HSP60), is associated with a good prognosis. Recently epitopes derived from HSP60, a microbial peptide (p1) and its self-homologue (p2) were reported to induce tolerogenic T cell responses in vitro in patients with arthritis. A study was undertaken to determine whether mucosal administration of these bystander epitopes can be similarly effective in suppressing arthritis.

Methods

Rats were treated nasally with p1, p2 or phosphate-buffered saline before arthritis induction. Arthritis scores were assessed and peptide-specific proliferative responses, phenotypic analysis, cytokine production and in vitro suppressive capacity of cells were measured in lymph nodes and spleens. CD4 spleen T cells from p1 or p2 treated rats were adoptively transferred into naïve rats that were subsequently injected with complete Freund's adjuvant for arthritis induction.

Results

Nasal administration of p1 prevented experimental arthritis whereas treatment with the self-homologue p2 did not. Adoptive transfer of CD4 T cells protected against experimental arthritis. Treatment with p1 increased peptide-specific and self-crossreactive interferon g (IFNg) production. Tumour necrosis factor α (TNFα) levels were reduced at the site of inflammation. Forkhead box P3 (Foxp3) expression remained stable but the suppressive capacity of T regulatory cells in p1-treated rats was enhanced.

Conclusion

P1 immune therapy induces a population of CD4 T cells with reduced TNFα and increased peptide-specific IFNg production at the site of inflammation. This population expresses FoxP3 and has potent suppressive capacity which, upon transfer, protects against arthritis. The bystander epitope p1 may therefore be a suitable candidate for antigen-specific immunotherapy in arthritis.

INTRODUCTION

Arthritis is one of the most prevalent chronic health problems, affecting all age groups. Current treatment of chronic arthritis is based on suppression of inflammation mostly through non-specific immune suppression. In particular, blockade of cytokine pathways with biological agents such as etanercept is very effective.[1,2] However, such treatment does not induce long-term remission of the disease, warranting long-term treatment with increasing risks of side effects.[1] There is therefore a need for alternative and/or complementary strategies aiming at a lasting immune deviation instead of general immune suppression.

Mucosally-introduced antigens induce protection in multiple experimental models of autoimmune disease.[3-9] In most autoimmune models, including arthritis models such as collagen-induced arthritis and proteoglycan-induced arthritis, the disease-inducing antigen is used for mucosal therapy, often in preventive protocols.[4-8] Although important, this is obviously difficult to translate to human autoimmune diseases such as rheumatoid arthritis (RA) and juvenile idiopathic arthritis (JIA) in which the disease-inducing antigens are not known. We questioned whether mucosal administration of bystander antigens that are recognised by the immune system of patients with arthritis can be similarly effective in inducing T cell regulation and suppressing arthritis and, if so, what their mechanism of action could be.

Preferably, a bystander antigen for effective and site-specific immune therapy should be immune dominant, recognised by T cells of the patient, specifically upregulated at the site of inflammation but unable to induce disease. Heat shock proteins (HSP) fulfil these criteria.[10] Indeed, based both on data in experimental models and in vitro studies in patients with JIA and RA, several families of HSPs, such as HSP10, HSP60, HSP70 and binding protein (BiP) have been proposed as suitable target antigens for treatment in arthritis.[10-13] Recently, a Phase II placebo-controlled trial with a molecular mimicry epitope derived from HSP DnaJ was reported to show encouraging clinical effects in humans, with an earlier study suggesting that immune therapy with this peptide induces immune deviation.[11,12,14] We previously identified two potential bystander HSP60 epitopes: mycobacterial HSP65 254-268 (p1) and its self-homologue HSP60 280-294 (p2).[15] These epitopes, originally selected based on a pan-DR binding motif, induce tolerogenic T cell responses in peripheral blood mononuclear cells (PBMC) from patients with chronic arthritis.[15,16] We hypothesised that these epitopes are potential candidates for specific immune therapy in arthritis as they fulfil the major prerequisites for a bystander epitope. 1) HSP60 is expressed in inflamed synovial tissue[17]; 2) the selection based on pan-DR binding ensures possible recognition in patients independent of their human leucocyte antigen (HLA) background; and 3) closely

related peptides are unable to induce arthritis alone or in combination with adjuvant in earlier rat experiments (Ronaghy/Prakken, unpublished observations).[16]

To determine whether mucosal immune therapy with these bystander epitopes in principle could interfere in arthritis, we used adjuvant arthritis (AA), a well-defined experimental arthritis model with a close immunological and histopathological resemblance to RA and JIA.[18] Earlier studies showed that, in AA, pre-immunization with HSP60 or a peptide derived from this protein, when administered subcutaneously in dimethyl dioctadecyl ammonium bromide, protected animals from the development of arthritis.[16,19] We now set out to test whether mucosal administration of two other bystander epitopes from HSP60 that are recognized by the immune system of patients with arthritis could influence the course of AA and, if so, the possible mechanism of action. Though these peptides were identified in humans, we were hopeful that we could translate these findings and apply the peptides in AA as, based on RT1Bl binding models, both peptides were expected to bind rat major histocompatibility complex (MHC).[20,21] Also, a peptide that is only a 2-amino frame shift from peptide p1 was earlier shown to be immunogenic in rat AA.[16]

In this study, we provide the first in vivo evidence of effective immune therapy with a bystander epitope that also is recognized by T cells of patients with arthritis. Our data can be the first step towards mucosal therapy with bystander HSP60 epitopes in patients with arthritis.

MATERIALS AND METHODS

Animals

Male inbred Lewis rats (RT1.Bl) were obtained from Maastricht University (Maastricht, The Netherlands). Adjuvant Arthritis was induced in 7-10 week-old rats of mean body weight 210±33 grams. All experiments were performed according to the guidelines of the Dutch Animal Welfare Committee and were approved by the responsible authorities.

Antigens and adjuvants

Heat-killed *Mycobacterium tuberculosis* (strain H37Ra) and Incomplete Freund's adjuvant (IFA) were purchased from Difco (Lawrence, Kansas, USA). Purified recombinant HSP65 of *Mycobacterium bovis* BCG (identical to *M tuberculosis* HSP65) and human HSP60 was generously provided by Dr. R. van der Zee (Institute of Infectious diseases and Immunology, Faculty of Veterinary Medicine, Utrecht, The Netherlands). Human HSP60 (P10809) has 95% identities with rat HSP60 (P63039) as computed at the SIB using the Basic Logical

Alignment Search Tool network service. Concanavalin A was obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).
Peptides were synthesized as 15-mers by automated simultaneous multiple peptide synthesis as described previously and checked by high-performance liquid chromatography for a purity of at least 95% (Ansynth, Roosendaal, The Netherlands).
The following peptides were used: *M tuberculosis* HSP65 254-268 (GEALSTLVVNKIRGT) (p1) and its self-HSP60 homologue 280-294 (GEALSTLVLNRLKVG) (p2). As the rat sequence of p2 is 100% identical to human HSP60 280-294), the self-human peptide also served as self homologue in this rat model. Ovalbumin (OVA) peptide 323-339 (ISQAVHAAHAEINEAGR) was used as a negative control.

Peptide immunotherapy protocol

Rats were lightly anesthetised using isoflurane during nasal administration by micropipette of 100 µg of a single peptide dissolved in phosphate-buffered saline (PBS) in a total volume of 30 µl (15 µl per nostril).
Peptide was administered 14, 9 and 5 days preceding the induction of arthritis. Control rats were treated with 30 µl PBS nasally. All treatment groups had the same mean weight at the start of experiments. Each experimental and control group consisted of six rats and all experiments were repeated at least once.

Induction and clinical assessment of AA

Adjuvant arthritis (AA) was induced by administering 200 µg *M tuberculosis* suspended in 100 µl IFA as a single intradermal injection in the base of the tail. The rats were examined daily in a blind manner for clinical signs of arthritis. Disease severity was determined by assessing weight loss and by scoring each paw (on a scale of 0-4) based on the degree of swelling, redness and deformation of the joints (maximum arthritis score of 16).[22]

T cell proliferation assay

Single cell suspensions were prepared from mandibular lymph nodes (MLN), separate inguinal lymph nodes (ILN) or pooled inguinal and popliteal lymph nodes (ILN/PLN) and spleens. Mononuclear cells of spleens were isolated using Ficoll isopaque density gradient centrifugation (Ficoll-Paque, Amersham Biosciences, GE Healthcare Europe GmbH, Diegem, Belgium). Cells were cultured in triplicate (200 µl/well) in 96-well, round-bottomed plates (Nalge Nunc, Roskilde, Denmark) at 2 x 10^5 cells/well, with or without antigen. Mycobacterial or human HSP was used at a final concentration of 10 µg/ml and individual HSP peptides or OVA peptide at 20 µg/ml. Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin (all from Invitrogen, Breda, The Netherlands) and 5 x10^{-5} M 2-mercaptoethanol (Biorad, Hercules, California, USA) was used as culture medium. Cultures were incubated

for 96 h at 37°C in a humidified atmosphere of 5% CO_2 and pulsed for the final 16-18 h with ^{3}H thymidine (ICN Radiochemicals, Irvine, USA), 1µCi/well. ^{3}H uptake was measured using a liquid scintillation β-counter. The stimulation index (SI) was computed as the mean counts per minute (cpm) of cells cultured with antigen, divided by the mean cpm of cells cultured with medium alone.

Adoptive transfer of protection

Donor rats were treated nasally with 100 µg p1 or p2 three times at 5-day intervals, or left untreated. Splenocytes of naïve rats were used as control. Splenocytes were harvested 7 days after administration of the last dose and were cultured at 5-6 x10^6/ml for 24 h in medium with 2% normal rat serum and 2.5 µg/ml concanavalin A. After 24 h, viable cells were harvested using a Ficoll gradient and separated into CD4+ and CD4- populations using the magnetic-activated cell sorting (MACS) system. Of CD4+ spleen cells, 10 x 10^6 cells were injected intravenously into naïve recipient rats (purity >88,97% CD4+). Two days after transfer of splenocytes, AA was induced.

Cytokine assays

Supernatants were collected after 72 h of stimulation with each of the antigens tested. Rat interleukin 10 (IL-10) and interferon g (IFNg) were quantitated by ELISA using an OPTEIA kit (BD Pharmingen, San Diego, California, USA). Rat transforming growth factor β (TGFβ) was quantified using TGFβ1-ELISA (R&D, Abingdon, UK). The assays were performed according to the manufacturer's instructions. Cytokine levels were expressed as pg/ml based on calibration curves constructed using recombinant cytokines as standards. Cytokine levels in peptide-stimulated cells corrected for cytokine production in unstimulated cells are shown. The lower limits of detection were 3.9 pg/ml for IL-10, 7.8 pg/ml for IFNg and 15.6 pg/ml for TGFβ.

Forkhead box P3 (FoxP3) and cytokine fluorescent activated cell sorting (FACS) staining

The following monoclonal antibodies were used: anti-rat cluster of differentiation (CD3)-Alexa647 or fluorescein-labelled antibodies (FITC) (clone IF-4); anti-rat CD4-phycoerythrin (PE)-cychrome5.5, PE-Cy7 or allophycocyanin (APC)-Cy7 (clone OX-35); anti-rat CD25-FITC, APC or PE (clone OX39); anti-rat/mouse FoxP3-PE, peridinin chlorophyll protein complex (PerCp)-Cy5.5 (clone FJK-16s); anti-rat IL-10 PE (clone A5-4); anti-rat IFNg FITC or PE (clone DB-1); TNFα-PE (clone TN3-19.12); anti-rat immunoglobulin D (IgD)-biotin (clone *MARD-3*) conjugated to streptavidin-APC-Cy7, anti-rat RT1.Bl-PerCp-Cy5.5 (clone OX-6). FoxP3-specific antibody was obtained from eBioscience (San Diego, California, USA), IgD-specific antibody from Serotec (Dusseldorf, Germany) and all other antibodies were obtained from Becton Dickinson (Erembodegem, Belgium).

Up to 1 x 10^6 cells resuspended in 50 µl PBS with 2% fetal bovine serum and 0.01% azide were surface stained with the extracellular monoclonal antibody (mAb) for 20 min at 4°C. For intracellular staining of cytokines and FoxP3, cells were permeabilised for 30 min (cytofix/cytoperm solution, BD/eBioscience), washed twice with Perm Wash (BD/eBioscience) followed by incubation for 30 min with anti-rat Foxp3, IL-10, IFNg or TNFα mAb. Cells were analysed after a final washing step on a FACS Calibur flow cytometer using Cell Quest software (all Becton Dickinson). All procedures were performed on ice until analysis.

In vitro suppression assays

CD4 T cells were purified from spleens or ILN/PLN by using magnetic bead-activated cell sorting (MACS; Miltenyi Biotec, Bisley, Surrey, Great Britain). In brief, splenocytes were incubated for 20 min at 4°C with CD4-coated magnetic beads (10 µl/10 x 10^6 cells). After washing, cells were passed through lymphocyte-depletion (LD) columns within the MACS magnet. The resulting CD4 T-cell fraction was sorted by FACS into $CD3^+CD4^+CD25^-$ T effector cells (Teff, spleen cells of PBS rats) and $CD3^+CD4^+CD25^{hi}$ T regulatory cells (Treg, ILN/PLN cells of all treatment groups) (FACSVantage, Becton Dickinson, San Jose, California, USA). The buffer used throughout the whole procedure was PBS supplemented with 2% fetal calf serum (FCS) and 2 mM EDTA.

For suppression assays, 25 x 10^3 FACS-sorted Teff cells were labelled with carboxyfluorescein (CFSE) (Invitrogen) at 2µM for 10 min at 37°C, and co-cultured in a 1:1, 2:1, 5:1 or 10:1 ratio with Treg cells in a 96-well plate coated with anti-CD3 (clone G4.18, 5 µg/ml). The MACS-separated $CD4^-$ T cell fraction was irradiated with 3500 rad and used as antigen-presenting cells (APC, 60 x 10^3 per well). After incubation at 37°C for 5 days, cells were stained with anti-rat CD3-Alexa647 and anti-rat CD4 PE-Cy7. Proliferative responses of Teff cells were measured by FACS determining the percentage of dividing cells based on CFSE dilution. The results are expressed as the mean percentage of proliferation, with 25 x 10^3 Teff cells as a standard ±SEM.

Statistical analysis

To detect differences between treatment groups with normally distributed data (area under the curve (AUC) values, T cell proliferation), one way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons was used. If data were not normally distributed (as in cytokine data and FACS data), differences between treatment groups were evaluated using non-parametric Kruskal-Wallis test. When two groups with skewed data were compared (cytokine data, FACS data, suppression assays) the Mann-Whitney U test for unrelated samples was used. Statistical analysis was carried out with SPSS version 17.0 (SPSS inc., Chicago, Illinois, USA). Two-tailed p-values <0.05 were considered significant.

RESULTS

Nasal administration of bystander epitope p1 but not p2 prevents experimental arthritis

The goal of the current study was to evaluate the possible in vivo regulatory effect of two potential bystander epitopes, mycobacterial HSP65 254-268 (p1) and self-HSP60 280-294 (p2). Peptide p1, p2 or PBS was administered nasally 14, 9 and 5 days prior to the induction of AA. Nasal treatment with peptide p1 led to a decrease in arthritis scores throughout disease course, with a significant reduction in the area under the arthritis score curve (AUC) (figure 1A,B). Arthritis scores of the p2-treated group did not differ from the PBS-treated control group. Weight curves (figure 1C) reflected arthritis score curves as p1-treated rats experienced the least weight loss during the experiment. Taken together, these results show that nasal pretreatment with the mycobacterial HSP peptide p1 significantly reduced the severity of AA but that pretreatment with the self-homologue p2 did not.

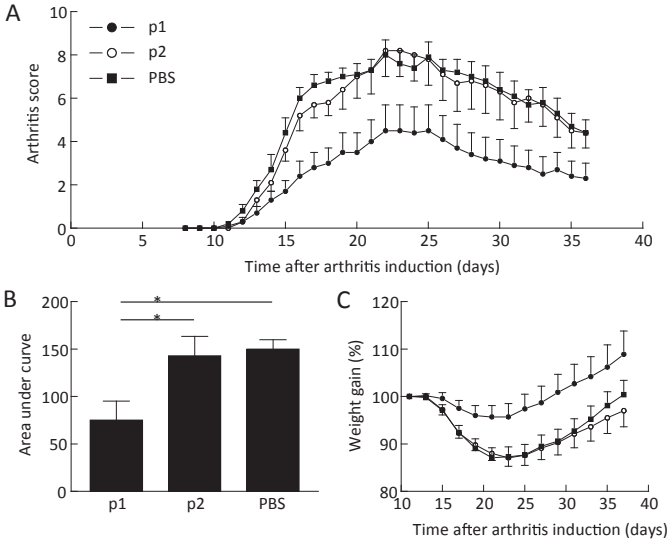


Figure 1 Modulation of adjuvant-induced arthritis (AA) assessed by (A) arthritis score, (B) area under the arthritis score curve (AUC) and (C) body weight change after nasal administration of heat shock protein (HSP) peptides p1 (mycobacterial HSP 254-268), p2 (self-HSP 280-294) or phosphate buffered saline (PBS). Rats were pre treated on days -14, -9 and -5. On day 0 rats were immunised with 0.2 mg *Mycobacterium tuberculosis* in 100 µl Freund's incomplete adjuvant to induce AA. Arthritis scores were assessed daily in a blinded manner from day 8 after AA induction. Rats were killed on days 49-55. Pooled results of two separate experiments are shown (n=12 rats for p1 treatment and n=10 rats for p2 and PBS treatment). Depicted are (A) mean arthritis scores per treatment group per day, (B) mean area under the curves per treatment group and (C) percentage change from maximum body weight per treatment group per day ±SEM (error bars). * $p<0.05$ PBS vs p1 in (A), in (B) as indicated

P1 and p2 are no major epitopes after CFA immunization or peptide-treated arthritis

T cell proliferative responses after CFA

To investigate the potential bystander role of p1 and p2 in AA, we determined whether CFA injection generates peptide-specific T cell proliferative responses. As *M tuberculosis* in CFA contains mHSP65, we observed mycobacterial HSP-specific T cell proliferation (SI>1,8) both systemically (spleen) as in the joint draining lymph nodes (supplement I). Peptide-specific T cell responses to p1, p2 or control peptide OVA 323-339 were not detected indicating that these peptides are not presented during disease induction, which agrees with earlier observations that most prominent T cell responses after CFA are only directed towards mHSP65 180-188, the disease-inducing epitope.[23]

T cell proliferative responses 50 days after peptide-treated AA

To test for peptide-specific T cell responses after peptide-treated arthritis, we harvested MLN, pooled ILN/PLN and spleens after disappearance of arthritic signs 50 days after AA induction. Cells were stimulated in vitro for 96 h with mycHSP65, humHSP60, p1, p2 or the control antigen OVA 323-339. As expected, significant mycobacterial HSP specific T cell proliferation was observed in spleens of all treatment groups and no peptide-specific T cell responses could be detected (supplement II). Remarkably, in p1-treated rats a response to self-HSP60 was generated.

Thus, a clear T cell response to the whole mycobacterial HSP65 protein was elicited systemically by CFA injection, but nasal peptide treatment did not lead to detectable levels of HSP peptide-specific T cell proliferation at treatment (MLN), systemic (spleen) or arthritic sites (ILN/PLN). Moreover, p1 treatment led to T cell recognition of self-HSP60.

Protection induced by p1 against experimental arthritis can be partly transferred by CD4 splenic T cells

As the 15-mers p1 and p2 preferably bind class II MHC, we expected CD4 T cell responses.[15] To test whether peptide-induced CD4 cells play a role in disease reduction, we adoptively transferred $10x10^6$ CD4 splenic T cells of p1-treated, p2-treated or untreated rats. Recipients were injected 2 days after transfer with CFA to induce arthritis. Adoptive transfer of CD4 spleen cells of p1 treated rats partly protected against arthritis, while CD4 cells of naïve rats or p2-treated rats did not (figure 2A). Arthritis scores were reflected by lower AUC values and reduced weight loss in the group receiving CD4 spleen cells from p1-treated rats (figure 2B,C). Similar results were found in a separate experiment comparing adoptive transfer from CD4 T cells of p1-treated rats with transfer of CD4 T cells from untreated rats (difference AUC at day 32, $p<0.05$, see supplement III). Taken together, these data indicate

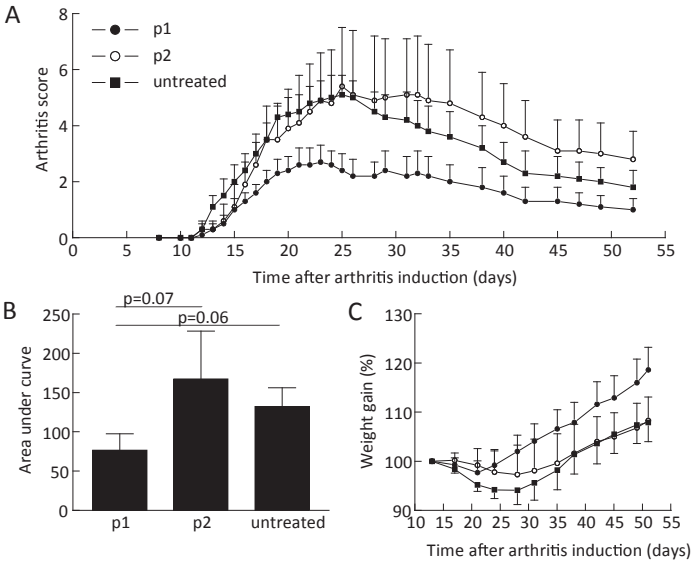


Figure 2 Adoptive transfer of CD4 lymphocytes. Spleen cells were harvested from p1-treated rats, p2-treated rats or untreated rats (n=6 per treatment group) 2 days after the last treatment. Cells were pooled per treatment group and stimulated overnight with concanavalin A. Magnetic-activated cell sorting separated CD4 cells were transferred into recipient rats (n=6 per treatment group, except for p2-treated CD4 cells, n=4) injected with complete Freund's adjuvant immunisation 2 days later. Arthritis scores were assessed daily in a blinded manner from day 8 after AA induction. Shown are (A) mean arthritis scores per treatment group per day, (B) mean area under the curves per treatment group and (C) percentage change from maximum body weight per day per treatment group ±SEM (error bars) of recipient rats. * p <0.05 untreated vs p1 in (A), in (B) as indicated. The p1 and untreated CD4 T cell transfer data are representative of two independent experiments.

that CD4 cells of rats nasally treated with p1 may have a role in disease reduction. We further analysed this T cell subset for cytokine production and Treg content.

Treatment with p1 attenuates the proinflammatory cytokine profile and induces peptide-specific cytokine production at the site of inflammation

To investigate the cytokine profile of CD4 cells at the site of inflammation, we measured intracellular cytokine production of ILN/PLN and spleens 50 days after arthritis induction in peptide treated rats (figure 3A-D). TNFα was still produced by CD4 lymph node cells of PBS- and p2-treated rats. In line with the disease-reducing effect of p1, TNFα was hardly present in p1-treated rats (figure 3A,B). Surprisingly, CD4 cells did produce large amounts of IFNy after p1 treatment in the joint draining lymph nodes whereas, after control treatment, this was restricted to systemic sites (figure 3C,D).

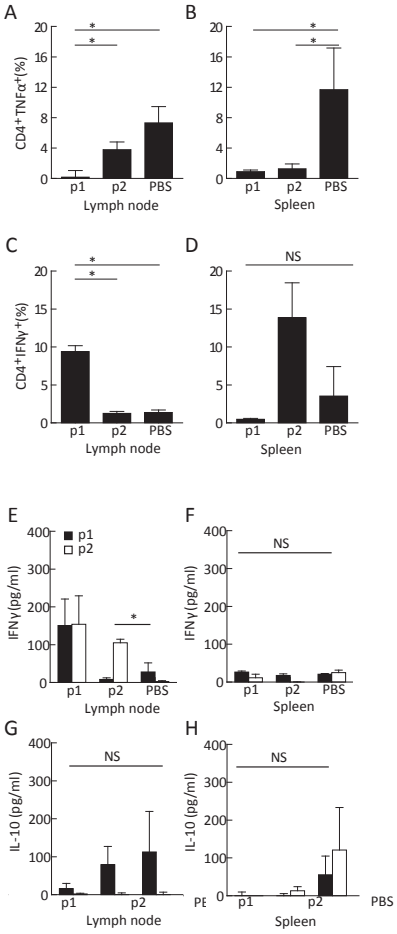


Figure 3 Cytokine production by pooled inguinal and popliteal lymph nodes (A,C,E,G) or spleens (B,D,F,H) after nasal peptide treatment. Lymph nodes and spleens were harvested 49-55 days after AA induction. Intracellular cytokine production of tumour necrosis factor α (TNFα) (A,B) and interferong (IFNg) (C,D) in rats treated nasally with p1, p2 or phosphate buffered saline. Cells were stained directly ex vivo for surface markers and intracellular cytokines. Intracellular cytokine production is expressed as the percentage of double-positive cells for both CD4 and cytokine. Gates for analysis were set on lymphocytes and CD4 cells. Mean ±SEM values are shown. For extracellular cytokine production (E-H), cells were cultured in vitro with p1 (black bars), p2 (white bars) or culture medium alone. After 72 h, supernatants were taken. IFNg (E,F) or IL-10 (G,H) production in supernatants were assessed by ELISA. Cytokine levels above medium values are shown. * $p < 0.05$ (n=4 rats per treatment group).

To investigate whether the detected IFNg production after p1 treatment was peptide specific, we also measured IFNg in supernatants of peptide-stimulated pooled ILN/PLN harvested at the same time point. Treatment with p1 induced large amounts of p1-specific (but also p2-specific) IFNg (figure 3E). Rats treated with p2 did not produce p1-specific IFNg

but comparable amounts of p2-specific IFNg at the site of inflammation. The increased self-crossreactivity after p1 treatment was reflected by increased self-HSP60-specific T cell proliferation as measured by the ^{3}H incorporation assay at the same time point (supplement II). No IFNg was detected in spleen cell cultures (figure 3F).

Intracellular IL-10 levels measured by FACS were <1% (data not shown). Some extracellular p1-specific IL-10 production was detected but the results did not differ between treat-

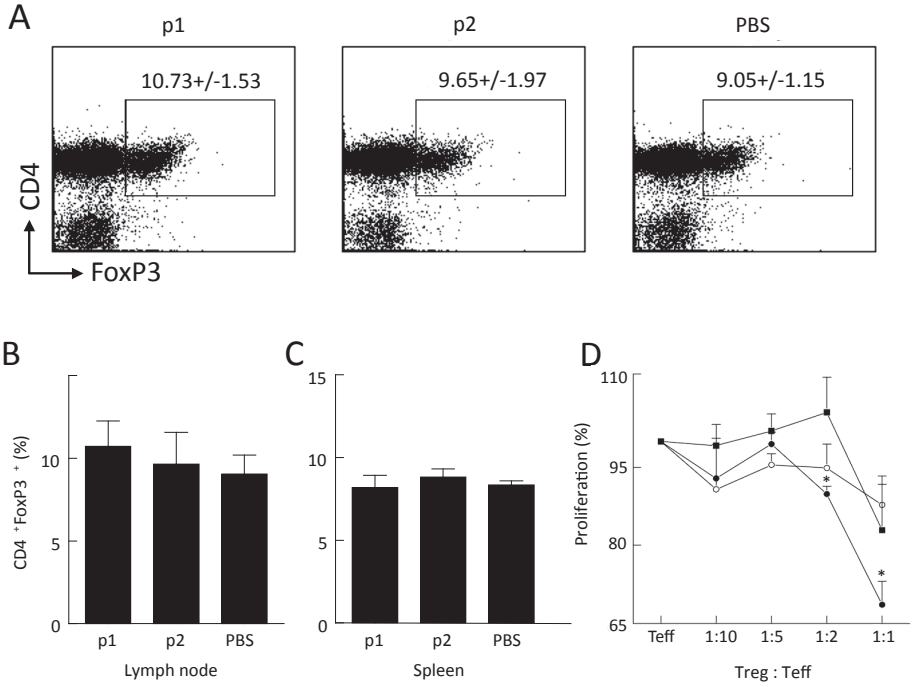


Figure 4 (A) Fluorescent activated cell sorting (FACS) analysis of percentage regulatory T cells in p1-treated (left panel), p2-treated (middle panel) and phosphate-buffered saline (PBS)-treated (right panel) rats 14 days after arthritis induction. Gates for analysis were set on living CD3 cells. Shown is the percentage CD4+FoxP3+ of gated cells ±SEM. Data represent four rats per treatment group. Results have been repeated in two separate experiments. Percentage of CD3+CD4+FoxP3+ cells within gated population of (B) pooled inguinal and politeal lymph nodes or (C) spleens from rats described in (A).
(D) Suppression assay with sorted CD3+CD4+CD25+ cells from PBS-treated (closed squares), p1-treated (closed circles) or p2-treated (open circles) rats. Spleen cells and inguinal and popliteal lymph nodes were harvested 14 days after arthritis induction. 25000 FACS-sorted CD3+CD4+CD25- CFSE labelled effector spleen cells of PBS-treated rats (Teff) were stimulated with anti-CD3 in the presence of 60 000 antigen presenting cells. Increasing amounts (2500, 5000, 12500 and 25000) of CD4+CD25+ T regulatory cells of different treatment groups were added. After 96 h, cells were FACS-stained for CD3, CD4 and proliferation of CD4 T cells was quantified by CFSE dilution. The results are shown as the percentage proliferation of 25000 T effector cells ±SEM. (PBS-treated rats, n=5; p1-treated rats, n=6; p2-treated rats, n=5; results represent two separate experiments). *p<0.05 p1 vs PBS.

ment groups (figure 3G,H). As no reliable FACS antibodies were available for IL-17, we instead measured IL-17 levels by ELISA in supernatants of re-stimulated spleen samples. No differences were found between the treatment groups, and supernatant IL-17 levels after peptide re-stimulation did not exceed values found in cultures with medium alone (data not shown). For its known role in Th3 cells, we also measured TGFβ levels in the above described cells, but these were all below the detection level of 15.6 pg/ml (data not shown).[24]

In summary, p1 treatment was accompanied by attenuation of the proinflammatory cytokine profile. Although peptide-specific T cell proliferation could not be measured after peptide-treated arthritis, we did detect peptide-specific cytokine responses at the site of former inflammation, indicating that p1 recognition may be important for disease reduction.

Treatment with p1 is associated with more suppressive Tregs at the site of inflammation

We next questioned whether the prevention of arthritis by p1 treatment could be due to the induction of CD4+FoxP3+ Treg. We harvested ILN/PLN from inflamed joints and from spleen cells as a systemic control on day 14 after CFA injection (during active disease) and stained them directly for FACS analysis. At this timepoint, no differences in the percentages of CD3+CD4+FoxP3+ cells or their FoxP3 mean fluorescence intensity (MFI) were observed (figure 4A-C).

The functional capacity of the inguinal Tregs was then tested. In rats, when restricted to surface markers for sorting, CD3+CD4+CD25+ T cells represent most accurately the Treg pool.[25] We FACS sorted CD3+CD4+CD25+ Tregs from the site of inflammation (ILN/PLN) of PBS-, p1- or p2-treated rats and co-cultured them with sorted CD3+CD4+CD25- effector cells from spleens of PBS-treated rats at different ratios. Sorted CD3+CD4+CD25+ cells from different treatment groups did not differ in the percentage of FoxP3+ cells (87.7 ±7.7%) or FoxP3 content per cells (MFI 2330±1187). At both 1:2 and 1:1 ratios, Tregs from p1-treated rats showed significantly more suppression than Tregs from PBS- or p2-treated rats (figure 4D). Nasal p1 treatment thus was accompanied by more suppressive Tregs than control treatment.

DISCUSSION

Nasal peptide-specific therapy is a promising approach to the treatment of arthritis.[16,19,26-40] For the clinical application of peptide immune therapy, one of the major challenges is the identification of suitable candidate epitopes. In human autoimmune diseases such as RA,

the disease-triggering auto-antigen is often unknown and, over time, the pool of auto-antigens that fuel the inflammatory immune response has been shown to expand and change.[41] In previous experimental studies pretreatment with the arthritogenic epitope or an altered peptide ligand of the arthritogenic epitope was successful in preventing arthritis.[19,28]

In this study we show that nasal administration of a bystander (HSP60) epitope (p1) can also effectively suppress arthritis. P1 was originally selected based on a pan-DR binding motif and was found to induce a regulatory profile in PBMC of patients with chronic arthritis.[15,42] To our knowledge, p1 is the first bystander epitope that is both recognised by the majority of RA and JIA patients and is effective in experimental arthritis.

It has been shown that peptide treatment can be mediated by several mechanisms including altered cytokine profiles and possibly the induction of Tregs.[7,11,19,43] TGFβ-producing Th3 cells and IL-10-producing Tr1 cells have also been implicated in mucosally-induced tolerance.[24,44] In the present study the cytokine profile at the site of inflammation was changed, with reduced TNFa and p1-specific CD4 T cells producing IFNg, some IL-10 but no TGFβ. Furthermore, Tregs of p1-treated animals retained suppressive capacity that was lower in control groups. This is supported by data demonstrating that CD4+CD25+Foxp3+ T cells can be expanded and even induced in response to peptide dosing.[7,45,46]

The increase of IFNg in animals with reduced disease may be counterintuitive. IFNg secreted by Teff cells has been implicated in the progression of AA,[47] but also has been shown to have beneficial effects in autoimmune disease by counteracting Th17 cells and the stimulation of Treg cell function.[48,49] Treg cells in AA have been reported to secrete IFNg,[50] and IFNg production after peptide treatment has been associated with suppressed disease.[48,49,51] Thus, the combination of increased IFNg production with reduced TNFa in this study could reflect a less inflammatory profile. This seems to reflect a population of activated Tregs that could be of special relevance in human disease. Further research is needed to further specify the CD4+ cells that produce IFNg.

After p1 treatment, a self-crossreactive T cell population appeared at the site of inflammation. This is in line with earlier studies on the closely related mHSP65 epitope 256-270, being the only HSP65 epitope able to induce self-crossreactive T cell responses in rats, associated with reduced arthritis scores.[16,23] A non-self epitope can indeed exert its effect by generating a beneficial crossreactive response to self.[16,52] According to this hypothesis, continuous exposure to microbial antigens enables the maintenance of a population of potential self-crossreactive T cells, controlled by mechanisms of peripheral tolerance. On encountering the self-antigen expressed at sites of inflammation, these cells could expand

and thus exert their bystander effects by antigenic mimicry.[10] From this perspective, nasal therapy with microbial antigens could enhance specifically this population of cells. Indeed, the observation that p1-treated rats induced self-HSP60-specific T cell responses and IFNg production upon stimulation with p1 (non-self) as well as with p2 (self) may fit such a scenario. Thus, although nasal p1 treatment is more effective than p2 treatment, its efficacy could be due to cross-reactivity with self (p2).

Timing of treatment may be crucial. In this study, we tested the effect of peptides administered before the induction of arthritis- that is, in a preventive regimen. To translate these results to the human situation, nasal p1 administration needs to be effective in a therapeutic regimen. The widespread inflammation present in an already established arthritic disease, however, can hamper the recognition of a small peptide like p1 by the immune system and limit its efficacy. Despite this, it is possible to treat established RA with a HSP-derived peptide (DnaJ).[14] In addition, it seems attractive to enhance the efficacy of specific immune therapy further by combining it with non-specific immunosuppressive medication, thus restraining the inflammatory environment. We believe combination therapy is one of the promising future strategies in immune interventions.[40,53]

ACKNOWLEDGEMENTS

The authors thank Wai Ming Liu for technical assistance, Ger Arkesteijn for his help and advice on FACS sorting and Erica Roks for her excellent secretarial assistance.

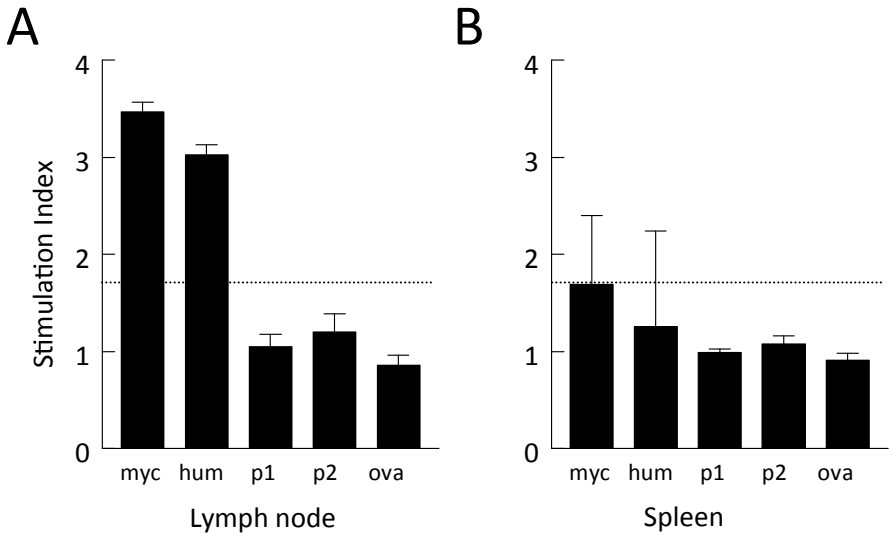
REFERENCE LIST

1. Dekker L, Armbrust W, Rademaker CM, Prakken B, Kuis W, Wulffraat NM. Safety of anti-TNFalpha therapy in children with juvenile idiopathic arthritis. *Clin Exp Rheumatol.* 2004;22:252-258.
2. Quartier P, Taupin P, Bourdeaut F, Lemelle I, Pillet P, Bost M, et al. Efficacy of etanercept for the treatment of juvenile idiopathic arthritis according to the onset type. *Arthritis Rheum.* 2003;48:1093-1101.
3. Prakken BJ, Wendling U, van der Zee R, Rutten VP, Kuis W, van Eden W. Induction of IL-10 and inhibition of experimental arthritis are specific features of microbial heat shock proteins that are absent for other evolutionarily conserved immunodominant proteins. *J Immunol.* 2001;167:4147-4153.
4. Belogurov AA Jr., Zargarova TA, Turobov VI, Novikova NI, Favorova OO, Ponomarenko NA, et al. Suppression of ongoing experimental allergic encephalomyelitis in DA rats by novel peptide drug, structural part of human myelin basic protein 46-62. *Autoimmunity.* 2009;42:362-364.
5. Reynolds J, Abbott DS, Karegli J, Evans DJ, Pusey CD. Mucosal tolerance induced by an immunodominant peptide from rat alpha3(IV)NC1 in established experimental autoimmune glomerulonephritis. *Am J Pathol.* 2009;174:2202-2210.
6. Broere F, Wieten L, Klein Koerkamp EI, van Roon JA, Guichelaar T, Lafeber FP, et al. Oral or nasal antigen induces regulatory T cells that suppress arthritis and proliferation of arthritogenic T cells in joint draining lymph nodes. *J Immunol.* 2008;181:899-906.
7. Zhao J, Li R, He J, Shi J, Long L, Li Z. Mucosal administration of an altered CII263-272 peptide inhibits collagen-induced arthritis by suppression of Th1/Th17 cells and expansion of regulatory T cells. *Rheumatol Int.* 2008;29:9-16.
8. Every AL, Kramer DR, Mannering SI, Lew AM, Harrison LC. Intranasal vaccination with proinsulin DNA induces regulatory CD4+ T cells that prevent experimental autoimmune diabetes. *J Immunol.* 2006;176:4608-4615.
9. Souroujon MC, Maiti PK, Feferman T, Im SH, Raveh L, Fuchs S. Suppression of myasthenia gravis by antigen-specific mucosal tolerance and modulation of cytokines and costimulatory factors. *Ann N Y Acad Sci.* 2003;998:533-536.
10. van Eden W, van der Zee R, Prakken B. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol.* 2005;5:318-330.
11. Prakken BJ, Samodal R, Le TD, Giannoni F, Yung GP, Scavulli J, et al. Epitope-specific immunotherapy induces immune deviation of proinflammatory T cells in rheumatoid arthritis. *Proc Natl Acad Sci U S A.* 2004;101:4228-4233.
12. Massa M, Passalia M, Manzoni SM, Campanelli R, Ciardelli L, Yung GP, et al. Differential recognition of heat-shock protein dnaJ-derived epitopes by effector and Treg cells leads to modulation of inflammation in juvenile idiopathic arthritis. *Arthritis Rheum.* 2007;56:1648-1657.
13. Hauet-Broere F, Wieten L, Guichelaar T, Berlo S, van der Zee R, van Eden W. Heat shock proteins induce T cell regulation of chronic inflammation. *Ann Rheum Dis.* 2006;65 Suppl 3:iii65-iii68.
14. Koffeman EC, Genovese M, Amox D, Keogh E, Santana E, Matteson EL, et al. Epitope-specific immunotherapy of rheumatoid arthritis: clinical responsiveness occurs with immune deviation and relies on the expression of a cluster of molecules associated with T cell tolerance in a double-blind, placebo-controlled, pilot phase II trial. *Arthritis Rheum.* 2009;60:3207-3216.
15. Kamphuis S, Kuis W, de Jager W, Teklenburg G, Massa M, Gordon G, et al. Tolerogenic immune responses to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis. *Lancet.* 2005;366:50-56.

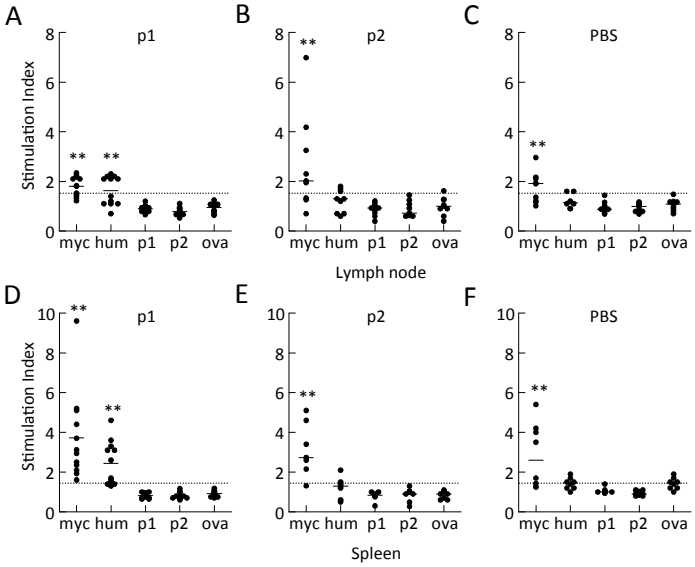
16. Anderton SM, van der Zee R, Prakken B, Noordzij A, van Eden W. Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *J Exp Med.* 1995;181:943-952.
17. de Graeff-Meeder ER, van Eden W, Rijkers GT, Prakken BJ, Kuis W, Voorhorst-Ogink MM, et al. Juvenile chronic arthritis: T cell reactivity to human HSP60 in patients with a favorable course of arthritis. *J Clin Invest.* 1995;95:934-940.
18. Prakken BJ, Roord S, Ronaghy A, Wauben M, Albani S, van Eden W. Heat shock protein 60 and adjuvant arthritis: a model for T cell regulation in human arthritis. *Springer Semin Immunopathol.* 2003;25:47-63.
19. Prakken BJ, Roord S, van Kooten PJ, Wagenaar JP, van Eden W, Albani S, et al. Inhibition of adjuvant-induced arthritis by interleukin-10-driven regulatory cells induced via nasal administration of a peptide analog of an arthritis-related heat-shock protein 60 T cell epitope. *Arthritis Rheum.* 2002;46:1937-1946.
20. Reizis B, Mor F, Eisenstein M, Schild H, Stefanovic S, Rammensee HG, et al. The peptide binding specificity of the MHC class II I-A molecule of the Lewis rat, RT1.BI. *Int Immunol.* 1996;8:1825-1832.
21. Wauben MH, van der Kraan M, Grosfeld-Stulemeyer MC, Joosten I. Definition of an extended MHC class II-peptide binding motif for the autoimmune disease-associated Lewis rat RT1.BL molecule. *Int Immunol.* 1997;9:281-290.
22. Trentham DE, Townes AS, Kang AH. Autoimmunity to type II collagen an experimental model of arthritis. *J Exp Med.* 1977;146:857-868.
23. Anderton SM, van der Zee R, Noordzij A, van Eden W. Differential mycobacterial 65-kDa heat shock protein T cell epitope recognition after adjuvant arthritis-inducing or protective immunization protocols. *J Immunol.* 1994;152:3656-3664.
24. Weiner HL. Induction and mechanism of action of transforming growth factor-beta-secreting Th3 regulatory cells. *Immunol Rev.* 2001;182:207-214.
25. Shevach EM. CD4+ CD25+ suppressor T cells: more questions than answers. *Nat Rev Immunol.* 2002;2:389-400.
26. Durai M, Gupta RS, Moudgil KD. The T cells specific for the carboxyl-terminal determinants of self (rat) heat-shock protein 65 escape tolerance induction and are involved in regulation of autoimmune arthritis. *J Immunol.* 2004;172:2795-2802.
27. Prakken BJ, van der Zee R, Anderton SM, van Kooten PJ, Kuis W, van Eden W. Peptide-induced nasal tolerance for a mycobacterial heat shock protein 60 T cell epitope in rats suppresses both adjuvant arthritis and nonmicrobially induced experimental arthritis. *Proc Natl Acad Sci U S A.* 1997;94:3284-3289.
28. Prakken BJ, van der Zee R, Anderton SM, van Kooten P, Kuis W, van Eden W. Tolerance to an arthritogenic T-cell epitope of HSP65 and the regulation of experimental arthritis. *Ann N Y Acad Sci.* 1996;778:425-426.
29. Wendling U, Paul L, van der Zee R, Prakken B, Singh M, van Eden W. A conserved mycobacterial heat shock protein (hsp) 70 sequence prevents adjuvant arthritis upon nasal administration and induces IL-10-producing T cells that cross-react with the mammalian self-hsp70 homologue. *J Immunol.* 2000;164:2711-2717.
30. van Bilsen JH, Wagenaar-Hilbers JP, van der Cammen MJ, van Dijk ME, van Eden W, Wauben MH. Successful immunotherapy with matrix metalloproteinase-derived peptides in adjuvant arthritis depends on the timing of peptide administration. *Arthritis Res.* 2002;4:R2.
31. Bardos T, Czipri M, Vermes C, Zhang J, Mikecz K, Glant TT. Continuous nasal administration of antigen is critical to maintain tolerance in adoptively transferred autoimmune arthritis in SCID mice. *Clin Exp Immunol.* 2002;129:224-231.

32. Derry CJ, Harper N, Davies DH, Murphy JJ, Staines NA. Importance of dose of type II collagen in suppression of collagen-induced arthritis by nasal tolerance. *Arthritis Rheum.* 2001;44:1917-1927.
33. van Tienhoven EA, Broeren CP, Noordzij A, Wagenaar JP, van Eden W, Wauben MH. Nasal application of a naturally processed and presented T cell epitope derived from TCR AV11 protects against adjuvant arthritis. *Int Immunol.* 2000;12:1715-1721.
34. Higuchi K, Kweon MN, Fujihashi K, McGhee JR, Kiyono H. Comparison of nasal and oral tolerance for the prevention of collagen induced murine arthritis. *J Rheumatol.* 2000;27:1038-1044.
35. Garcia G, Komagata Y, Slavin AJ, Maron R, Weiner HL. Suppression of collagen-induced arthritis by oral or nasal administration of type II collagen. *J Autoimmun.* 1999;13:315-324.
36. Chu CQ, Londei M. Differential activities of immunogenic collagen type II peptides in the induction of nasal tolerance to collagen-induced arthritis. *J Autoimmun.* 1999;12:35-42.
37. Bayrak S, Mitchison NA. Bystander suppression of murine collagen-induced arthritis by long-term nasal administration of a self type II collagen peptide. *Clin Exp Immunol.* 1998;113:92-95.
38. Myers LK, Seyer JM, Stuart JM, Kang AH. Suppression of murine collagen-induced arthritis by nasal administration of collagen. *Immunology.* 1997;90:161-164.
39. Staines NA, Harper N, Ward FJ, Malmstrom V, Holmdahl R, Bansal S. Mucosal tolerance and suppression of collagen-induced arthritis (CIA) induced by nasal inhalation of synthetic peptide 184-198 of bovine type II collagen (CII) expressing a dominant T cell epitope. *Clin Exp Immunol.* 1996;103:368-375.
40. Roord ST, Zonneveld-Huijssoon E, Le T, Yung GP, Koffeman E, Ronaghy A, et al. Modulation of T cell function by combination of epitope specific and low dose anticytokine therapy controls autoimmune arthritis. *PLoS ONE.* 2006;1:e87.
41. Vanderlugt CL, Miller SD. Epitope spreading in immune-mediated diseases: implications for immunotherapy. *Nat Rev Immunol.* 2002;2:85-95.
42. de Jong H, Lafeber FF, de Jager W, Haverkamp MH, Kuis W, Bijlsma JW, et al. PAN-DR-Binding Hsp60 self epitopes induce an interleukin-10-mediated immune response in rheumatoid arthritis. *Arthritis Rheum.* 2009;60:1966-1976.
43. Quintana FJ, Carmi P, Mor F, Cohen IR. DNA fragments of the human 60-kDa heat shock protein (HSP60) vaccinate against adjuvant arthritis: identification of a regulatory HSP60 peptide. *J Immunol.* 2003;171:3533-3541.
44. Roncarolo MG, 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;212:28-50.
45. Apostolou I, von BH. In vivo instruction of suppressor commitment in naive T cells. *J Exp Med.* 2004;199:1401-1408.
46. Bohle B, Kinaciyan T, Gerstmayr M, Radakovics A, Jahn-Schmid B, Ebner C. Sublingual immunotherapy induces IL-10-producing T regulatory cells, allergen-specific T-cell tolerance, and immune deviation. *J Allergy Clin Immunol.* 2007;120:707-713.
47. Schmidt-Weber CB, Pohlers D, Siegling A, Schadlich H, Buchner E, Volk HD, et al. Cytokine gene activation in synovial membrane, regional lymph nodes, and spleen during the course of rat adjuvant arthritis. *Cell Immunol.* 1999;195:53-65.
48. Kelchtermans H, Billiau A, Matthys P. How interferon-gamma keeps autoimmune diseases in check. *Trends Immunol.* 2008;29:479-486.
49. Sawitzki B, Kingsley CI, Oliveira V, Karim M, Herber M, Wood KJ. IFN-gamma production by alloantigen-reactive regulatory T cells is important for their regulatory function in vivo. *J Exp Med.* 2005;201:1925-1935.

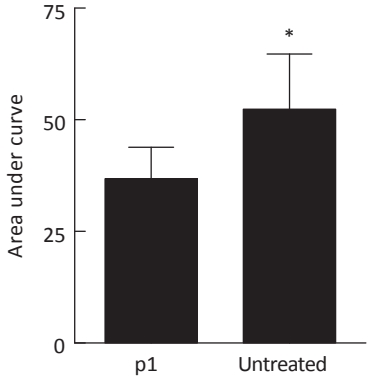
50. Paul AG, van Kooten PJ, van Eden W, van der Zee R. Highly autoproliferative T cells specific for 60-kDa heat shock protein produce IL-4/IL-10 and IFN-gamma and are protective in adjuvant arthritis. *J Immunol.* 2000;165:7270-7277.
51. Boccaccio GL, Mor F, Steinman L. Non-coding plasmid DNA induces IFN-gamma in vivo and suppresses autoimmune encephalomyelitis. *Int Immunol.* 1999;11:289-296.
52. van Eden W, Wendling U, Paul L, Prakken B, van Kooten P, van der Zee R. Arthritis protective regulatory potential of self-heat shock protein cross-reactive T cells. *Cell Stress Chaperones.* 2000;5:452-457.
53. Bresson D, Togher L, Rodrigo E, Chen Y, Bluestone JA, Herold KC, et al. Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs. *J Clin Invest.* 2006;116:1371-1381.



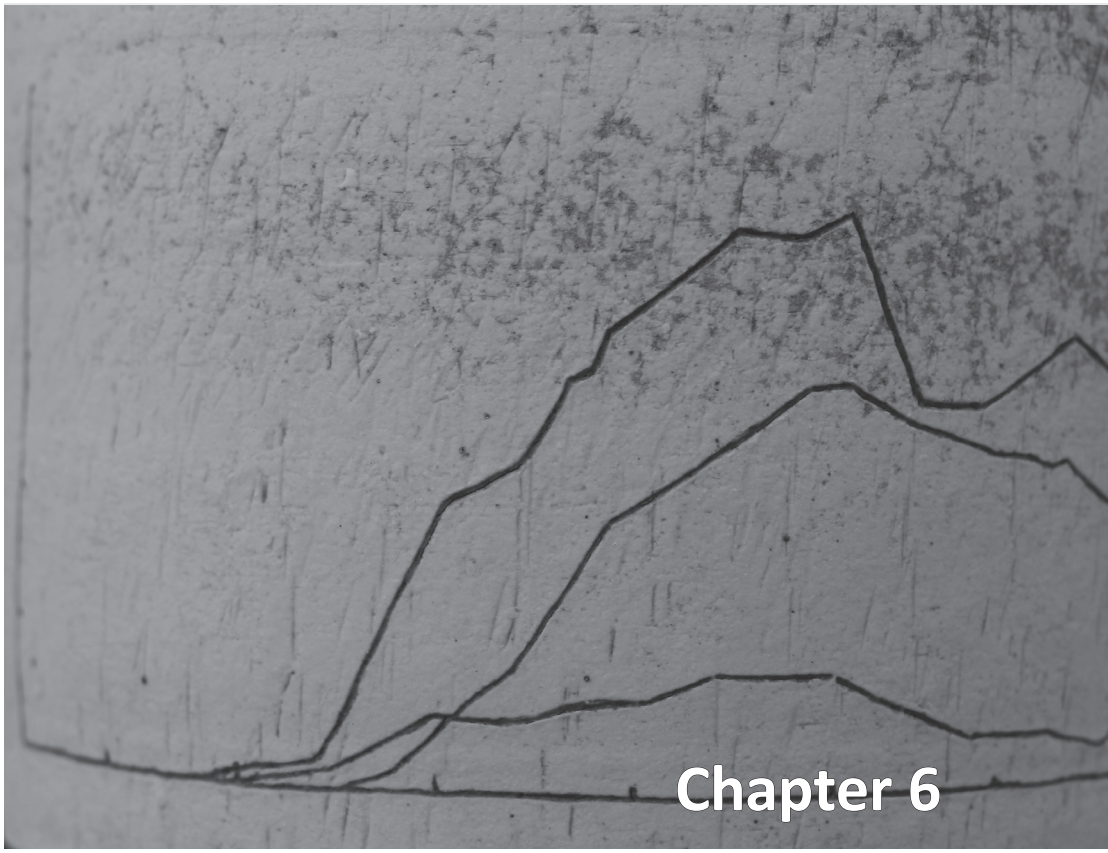
Supplement I T-cell proliferation of (A) inguinal lymph node cells or (B) spleens 10 days after CFA injection. Naïve rats (n=2) were subcutaneously injected with 200µg CFA at the base of the tail. Ten days later, cells were harvested and in vitro stimulated for 96 h with mycHSP65, humanHSP60, p1, p2 or the control antigen OVA 323-339. Stimulation indexes (SI) were computed as mean counts per minute (cpm) of cells cultured with antigen, divided by the mean cpm of cells cultured with medium alone. Shown is the mean SI per in vitro stimulus ±SD. Dotted line indicates SI value of 1,8.



Supplement II Proliferation of (A-C) pooled inguinal and popliteal lymph node cells or (D-F) spleen cells of p1-treated (A,D), p2-treated (B,E) or PBS-treated (C,F) rats 50 days after arthritis induction. Cells were stimulated in vitro with mycHSP65, humHSP60, p1, p2 or OVA 323-339. Stimulation indexes (SI) were computed as the mean counts per minute (cpm) of cells cultured with antigen, divided by the mean cpm of cells cultured with medium alone. Data represent results of two separate experiments. Similar results were obtained in mandibular lymph nodes. Shown are individual stimulation indices per treatment group. Dotted line indicates SI value of 1,8. ** p<0.001 as measured by analysis of variance (ANOVA) with post-hoc Bonferroni correction for multiple comparisons.



Supplement III Adoptive transfer of CD4 lymphocytes. Spleen cells were harvested from p1-treated or untreated rats (n=6 per treatment group), two days after the last treatment. Cells were pooled per treatment group and stimulated overnight with concanavalin A. Magnetic-activated cell sorting separated CD4 T cells were transferred into recipient rats (n=6 per treatment group) that were injected with complete Freund's adjuvant two days later. Arthritis scores were assessed daily, in a blinded manner, from day 8 after AA induction. Shown are mean area under the curves per treatment group ±SEM (error bars) of recipient rats.



TLR9 agonist CpG enhances protective nasal HSP60 peptide vaccine efficacy in experimental autoimmune arthritis

Evelien Zonneveld-Huijssoon, Femke van Wijk, Sarah TA Roord, Eveline Delemarre, Jenny Meerding, Wilco de Jager, Mark Klein, Eyal Raz, Salvatore Albani, Wietse Kuis, Marianne Boes, Berent J Prakken

Annals of the Rheumatic Diseases, accepted for publication

ABSTRACT

Peptide-based immune tolerance induction is considered an attractive treatment option for autoimmune diseases. Here, we set out to develop a novel method that can facilitate and enhance the induction of protective peptide-specific T cell responses, using a rat arthritis model. We focused on the TLR9 ligand CpG, which was shown to stimulate regulatory T cell proliferation when added to plasmacytoid DC using *in vitro* cultures. The peptide we used is a heat shock protein 60 epitope (p1) that elicits tolerogenic peptide specific immune responses in human arthritis patients and was recently shown to have protective capacity as a bystander antigen in the rat adjuvant arthritis model. Rats were treated with three nasal doses of PBS, p1, CpG or a combination of p1 and CpG. We studied the phenotype of antigen presenting cells in nose-draining mandibular lymph node cells (MLN) after nasal treatment, and analyzed for elicitation of peptide-specific T cell responses and regulatory T cell function in inguinal and popliteal lymph nodes from arthritic joints after arthritis induction. Nasal co-administration of CpG significantly augmented the arthritis-protective effect of p1, while CpG treatment alone could not prevent arthritis. Co-treatment of p1/CpG increased both the number and activation status of plasmacytoid DC in draining MLN, which was accompanied by amplified p1-specific T cell proliferation and IL-10 production compared to control animals receiving p1 alone. During early arthritis, peptide specific IL-10 production was identified at the site of inflammation. P1- and p1/CpG-treated rats showed a higher amount of CD4+FoxP3+ regulatory T cells in the joint draining lymph nodes, which correlated with lower arthritis scores. These clinical and immunological data suggest the use of CpG as a potent adjuvant for mucosal peptide-specific immune therapy in arthritis.

INTRODUCTION

Mucosal immune tolerance induction for autoimmune diseases is a conceptually attractive treatment strategy, as it targets the immune system in an antigen-specific way rather than by generalized immune suppression, which is currently used in clinical practice. Peptide immune therapy has been shown to shift immune responses towards a regulatory phenotype using *in vivo* animal models of autoimmune disease and *in vitro* culture systems of patient cells.[1-8] Extension of these results into human trials has so far been promising but limited in scope.[9-14] One of the major issues that hampered peptide mucosal tolerance clinical trials from happening is that the amount of experimental support that mucosal vaccination can elicit detectable peptide specific immune responses is still limited. Although peptide specific cytokine production was detected in some studies indicating deviation of the immune response, a clear increase in peptide specific T cells is most often lacking.[2,5,8] As a consequence, clear assessment of the proposed immune deviation and monitoring the efficacy of the immune intervention in patients had been challenging.

The weak induction of antigen specific immunity can be considered the consequence of the limited ability of a mucosally delivered peptide to stimulate the immune system,[15-18] due to its ability to trigger the adaptive immune system alone. In a healthy immune system, innate immunity precedes and enhances peptide-triggered adaptive immune responses. Combination therapy of an adjuvant triggering innate immunity and a T-cell epitope triggering adaptive immunity could in this way be a novel concept worth exploring. The success of adjuvants used in mucosal vaccination strategies for infectious diseases including polio and influenza underscores this notion.[19-24] Although adjuvants have been used in parenteral vaccination strategies in auto-immunity[25], the idea of enhancing mucosal vaccination with an adjuvant for preventive peptide therapy in auto-immune disease is new. Prerequisites for such an innate triggering mucosal adjuvant would be applicability at mucosal sites, activation of antigen presenting cells (APCs) and preferably induction of a shift toward regulatory T cell responses to help the peptide in restoring the lost immune balance in autoimmunity. Addition of such an adjuvant would enable the activated APCs to present the peptide more efficiently and enlarge the beneficial effect of nasal peptide treatment.

Unmethylated CpG dinucleotides are effective mucosal adjuvants that, when administered systemically, can trigger Th1 responses by activation of antigen presenting cells via TLR9.[26,27] This might conceptually lead to exacerbation of Th1 mediated auto-immune diseases. CpG was indeed initially considered as an enhancer of CTL responses in cancer therapy,[28-32] and CpG as an adjuvant has been shown to augment peptide specific responses in vaccination against infectious diseases like pneumococcal disease, influenza and malaria.[33-35]

However, both experimental and epidemiological data suggest that in autoimmune diseases, bacterial TLR9 stimulation can also exert a regulatory function. The absence of bacterial stimuli can have adverse effects as animals raised under germ-free conditions acquire poorly efficient immune-regulatory mechanisms and are at greater risk of disease associated with immune dysfunction.[36-39] As a consequence, the presence of bacterial DNA could suppress experimental asthma or multiple sclerosis (EAE), and it has been suggested that this is also the case for human auto-immune disease.[40-44] More specifically, a study in TLR9-/- mice showed that TLR9 down-regulates disease activity in a EAE mouse model.[45] In line with these results, vaccination with CpG sequences alone attenuated disease in two models of autoimmune diabetes.[46,47] Finally, also in human autoimmune disease, TLR9 stimulation by CpG had an anti-inflammatory effect.[48]

Here, we set out to investigate the possible benefits of mucosal CpG-peptide combination therapy against autoimmunity. We chose the rat model of Adjuvant Arthritis (AA), in which the disease reducing effect of heat shock protein (HSP) peptides has been described earlier.[4,49-51] We recently showed that nasal administration of peptide p1, a novel HLA binding HSP60 epitope that elicits tolerogenic peptide specific immune responses in arthritis patients,[1] could suppress AA and skew T cell responses towards a regulatory phenotype.[8] In the current study we found that CpG can be a potent adjuvant for peptide immune therapy in experimental arthritis and that this therapeutic effect is accompanied by increased activation of plasmacytoid DC (pDC), augmentation of peptide-specific T cell responses including IL-10 production and an increase in FoxP3+ regulatory T cells. The fact that the HSP-peptide p1 has also been described to evoke a favourable immune profile in arthritis patients could facilitate translation of the results of this study into peptide specific immunotherapy for human arthritis.

MATERIALS AND METHODS

Animals

Male inbred Lewis rats (RT1.Bl) were obtained from Harlan (Horst, The Netherlands). Adjuvant Arthritis was induced in 8-9 week old rats by a single intradermal injection at the base of the tail of 200µg heat-killed *Mycobacterium tuberculosis* (strain H37Ra) suspended in 100µl Incomplete Freund's Adjuvant (IFA) (Difco, Lawrence, KS, USA). Rats were examined daily, in a blinded manner, for clinical signs of arthritis. Disease severity was determined by assessing weight loss and by scoring each paw (0-4 scale) based on the degree of swelling, redness and deformation of the joints (maximum arthritis score 16).[52] For animal welfare concerns, rats were sacrificed as soon as arthritis scores exceeded 12. We determined the area under the arthritis score curve of the arthritis scores from day 8 to 23 as some rats

had to be sacrificed due to arthritis scores above 12 which occurred first on day 23 (indicated by † in Figure 3A). All experiments were performed according to the guidelines of the Dutch Animal Welfare Committee and were approved by the responsible authorities.

Peptides and adjuvant

Peptides were synthesized as 15-mers by automated simultaneous multiple peptide synthesis as described previously and checked by HPLC for a purity of at least 95% (Ansynth, Roosendaal, The Netherlands).[1]

The following peptides were used: p1 peptide *M. tuberculosis* HSP65 254-268 (GEALSTLVVNKIRGT) and OVA peptide 323-339 (ISQAVHAAHAEINEAGR) as a negative control. Phosphorothioate stabilised oligodeoxynucleotides (ODN) was obtained from Metabion (Martinsried, Germany). The sequence of the used B-type CpG-ODN was: 5' TCC AT***G ACG TT***C CTG ATG CT 3' (CpG1668).

Cytofluorometric analysis

The following monoclonal antibodies were used: anti-rat CD3-Alexa647 or -FITC (clone IF-4); anti-rat CD4-PE-Cy7 or –APC-Cy7 (clone W3/25); anti-rat CD86-PE (clone 24F) from Biolegend (San Diego, CA, USA). Anti-rat CD25-APC (clone OX-39); anti-rat CD45R-PE (clone HIS24); anti-rat/mouse Foxp3-PE, -PerCp-Cy5.5 (clone FJK-16s) were acquired from eBioscience (San Diego, CA, USA). Anti-rat IgD-biotin (clone MARD-3) conjugated to Streptavidin-APC-Cy7; anti-rat CD11b-FITC (clone OX-42) anti-rat CD103-PE (clone OX-62) were purchased from AbD Serotec (Düsseldorf, Germany). Anti-rat RT1B-PerCP (clone OX-6) was obtained from Becton Dickinson (Erembodegem, Belgium).

Freshly harvested or stimulated mandibular lymph nodes (MLN), spleen cells or pooled inguinal and popliteal lymph nodes (ILN/PLN) were used for FACS analyses. FACS stainings were performed according to the manufacturer's instructions. Cells were analyzed on a FACS CantoII flow cytometer using FACSDiva software (Becton Dickinson).

DC subsets were defined according to earlier studies by Hubert (Figure 1, see online supplementary Figure S1).[53] After gating on CD3-IgD- cells, cells were separated into CD11b- and RT1B+ subsets. Within the CD11b- subset, plasmacytoid DC (pDC) were defined by the RT1B+CD4high population. These pDC were all CD45R positive, and expressed CD86 (Figure 1B,C). The RT1B+ subset was further gated on CD11b+CD103+ cells, defining conventional DC (cDC).

T cell proliferation assay

Lymph node or spleen cells were cultured in triplicate (200µl/well) in 96-well, round bottom plates (Nalge Nunc, Roskilde, Denmark) at 2 x10^5 cells/well, in the presence or absence of HSP-peptide p1 or OVA peptide at a final concentration of 20 µg/ml. Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100

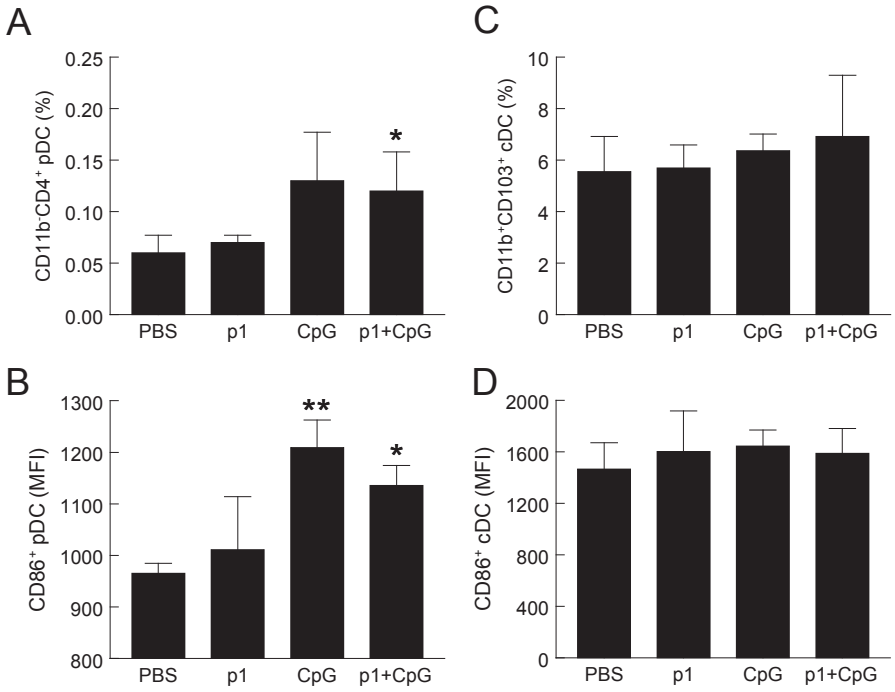


Figure 1 pDC number and activation status are increased in mandibular lymph nodes (MLN) following combination therapy of nasal peptide and CpG, whereas cDC number and activation status remain stable. **Rats were** treated nasally 3 times within 10 days (day 0, 5, 9) with heat shock protein peptide p1 (mycobacterial HSP 254-268), CpG1668 or both. Control rats were treated with PBS in the same schedule. Two days after the last nasal treatment, rats were sacrificed, MLN were harvested and stained for FACS analysis. For gating strategy see supplemental data I. Percentages of pDC (A) and cDC (B) in MLN. Results are expressed as mean percentages of living cells ± SD of four animals per treatment group. Expression of CD86 on gated pDC (C) and cDC (D). To determine CD86 expression, cDC were gated as CD3-IgD-MHCII+CD11b+. Data are depicted as mean fluorescence intensity (MFI) of CD86 of four animals per treatment group ± SD. Results are representative of three independent experiments. *p<0.05 **p<0.01 as measured by Mann Whitney U test (A,B) or two-sided one way ANOVA (C,D).

units/ml penicillin, 100 µg/ml streptomycin (all from Invitrogen, Breda, The Netherlands) and 5 x10^{-5} M 2-mercaptoethanol (Biorad, Hercules, CA, USA) was used as culture medium. Cultures were incubated for 96 hours at 37°C in a humidified atmosphere of 5% CO_2 and pulsed for the final 16-18 hours with ^{3}H thymidine (ICN Radiochemicals, Irvine, USA), 1µCi/well. ^{3}H uptake was measured using a liquid scintillation beta counter.

Cytokine production

Cell culture supernatants were collected after 72 hours of culture in the presence or absence of HSP-peptide p1 or OVA. Rat IL-10 and IFNg were measured with kits for the Bio-Plex system in combination with the Bio-Plex Manager software, version 6.0 (Bio-Rad

Laboratories, Hercules, CA, USA), which employs the Luminex xMAP technology as previously described.[54] Levels of rat IFNa were measured by ELISA (Cusabio, Wuhan, China). The assay was performed according to the manufacturer's instructions. Cytokine levels were expressed as pg/ml based on calibration curves constructed using recombinant cytokines as standards. The lower limits of detection were 0,9 pg/ml for IL-10, 4,0 pg/ml for IFNg and 0,05 ng/ml for IFNa.

Immunotherapy protocol

Rats were lightly anesthetized by inhalation of isoflurane just before nasal administration of 100 µg of peptide or CpG dissolved in PBS, by micropipette in a total volume of 30 µl (15 µl per nostril). For combination therapy, 100µg peptide and 100µg CpG was administered simultaneously in the same total volume of 30µl.

Peptide, adjuvant or combination therapy was administered 14, 9 and 4 days preceding the induction of arthritis. Control rats were treated with 30 µl phosphate-buffered saline (PBS) nasally. All treatment groups had the same weight at the start of experiments. Each experimental and control group consisted of six rats each and all experiments were repeated at least once.

Statistical analysis

To detect differences between treatment groups with normally distributed data (FACS data, proliferation data), two-sided one way ANOVA with posthoc Fisher's LSD was used. If data was not normally distributed (as in cytokine data, arthritis scores, weight loss) differences between multiple treatment groups were evaluated using non-parametric Kruskall Wallis test. When two groups with skewed data were compared Mann Whitney U test for unrelated samples was utilized. To evaluate correlation between two data sets (arthritis scores and percentage Treg) we used Pearson's correlation coefficient. Statistical analysis was carried out with the SPSS version 17.0. (SPSS inc., Chicago, Illinois, USA). Two-tailed p-values of less than 0.05 were considered significant.

RESULTS

Nasally administered CpG increases number and activation status of local plasmacytoid dendritic cells

We first evaluated the effect of combining nasal peptide immune therapy with CpG administration on the amount and activation status of local antigen presenting cells (APC) in nose-draining mandibular lymph nodes. We chose to use CpG1668, which has been described to induce regulatory T cells (Treg) in other models of colitis and allergic disease.[55-57]

CpG can theoretically stimulate all TLR9 expressing APCs, including B cells, conventional dendritic cells (cDC) and plasmacytoid dendritic cells (pDC).
Rats were treated nasally three times within ten days (day 0, 5, 9) with CpG in combination with the earlier described HSP-peptide p1.[1,8] Control animals received PBS, CpG alone or peptide p1 alone. Compared to controls, the percentage of pDC doubled in draining mandibular lymph nodes (MLN) two days after CpG-containing treatment (Figure 1A, for gating strategy see online supplementary Figure S1A-C). These pDC were in an enhanced activation state, as measured by increased CD86 expression per cell (Figure 1B).
The percentage and activation status of cDC did not differ between treatment groups (Figure 1C and D, for gating strategy see online supplementary Figure S1D). Also, no differences were detected in the percentage of B cells in MLN (see online supplementary Figure S2).
As activated pDC are potent producers of type I interferon, we measured levels of IFNα by ELISA in culture supernatants of sorted DC from MLN that were stimulated *in vitro* for 24 hours with CpG. CpG stimulated DC cultures contained elevated levels of IFNα when compared to unstimulated DC (see online supplementary Figure S3A). To test whether *in vivo* CpG treatment stimulated pDC activity as well, we next tested IFNα production in MLN of CpG treated rats four days after the last nasal treatment. Again, IFNα levels in supernatants of cultured MLN of CpG treated rats were raised compared to control animals treated with PBS (max 0,7 pg/ml, see online supplementary Figure S3B). Thus, CpG stimulation *in vitro* and *in vivo* leads to increased IFNα production by local DC.
Summarizing these data, nasal administration of the HSP-peptide p1 combined with CpG is accompanied by an increased number of more activated pDC in nose-draining MLN and raised amounts of the DC cytokine, type I interferon.

Nasal combination treatment of HSP-peptide with CpG results in enhanced local peptide recognition

We next questioned whether the improved activation of pDC by CpG treatment led to enhanced antigen presentation. To test this, we measured peptide-specific T cell proliferation and cytokine production in MLN and spleen two days after the last nasal treatment. Nasal peptide treatment combined with CpG resulted in significantly increased peptide-specific T cell proliferation in the local draining MLN compared to all other treatment groups (Figure 2A, black bars, $p<0.01$). This was specific for the nasally administered HSP-peptide p1 as T cell proliferation upon stimulation by the control peptide ova was not increased (Figure 2A, grey bars). Peptide p1-specific T cell proliferation was confined to MLN as in spleen cells no peptide or ova-specific T cell proliferation could be detected (Figure 2B).

In order to investigate the quality of the early peptide (p1) specific T cell response elicited by nasal p1/CpG combination treatment, we determined cytokine production in culture

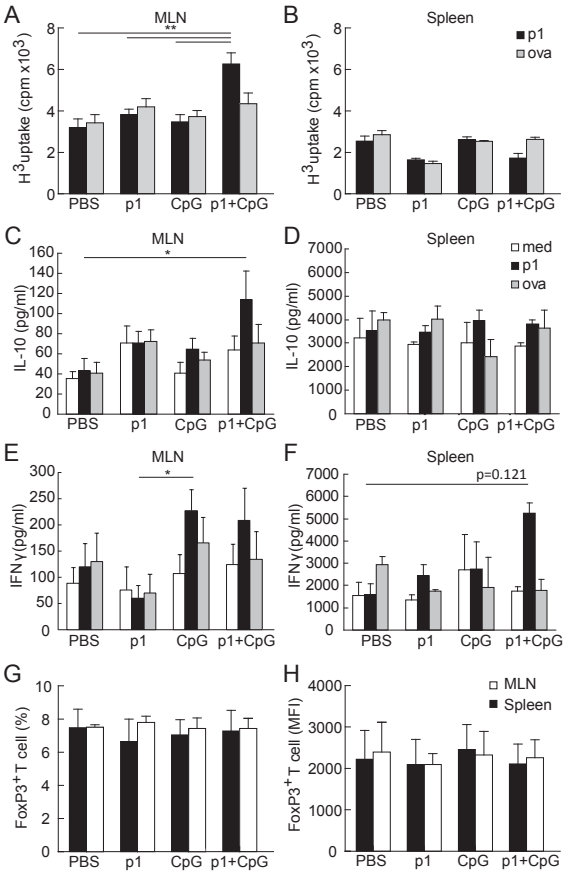


Figure 2 Nasal p1/CpG treatment results in elevated peptide-specific T-cell responses locally. Groups of rats were treated as described in Figure 1.

Mandibular lymph node cells (MLN) (A) or spleen cells (B) were harvested two days after the last nasal treatment and stimulated with peptide p1 (mycobacterial HSP 254-268, black bars) or control peptide (ova 323-339, grey bars) for five days. Proliferation was measured by ^{3}H uptake. Shown are the mean counts per minute (cpm) per treatment group ± SEM. Data represent results of three separate experiments. N=6 rats per treatment group. ** indicates a p-value <0.01 as assessed by one way ANOVA with posthoc LSD.

Peptide-specific cytokine production by MLN cells (C,E) or spleen cells (D,F). Cells were left unstimulated (med, white bars) or stimulated with peptide p1 (mycobacterial HSP 254-268, black bars) or control peptide (ova 323-339, grey bars) for 72 hours. IL-10 (C,D) and IFNg (E,F) were measured in supernatants by Luminex assay. Shown are mean concentrations ± SEM. N=6 rats per treatment group for MLN, n=2 rats per treatment group for spleens. Data represent results of two separate experiments. * indicates a p-value < 0.05 as measured by one way ANOVA with posthoc LSD (for MLN). No significant differences were measured in spleens by Kruskall Wallis test.

MLN (black bars) or spleens (white bars) were harvested four days after the last nasal treatment and stained directly with CD3 Alexa647, CD4 APC-Cy7 and FoxP3-PE for FACS analysis (G,H). Gates were set on living CD3+ lymphocytes. Percentage (G) or mean fluorescence intensity (H) of FoxP3+ cells within the CD3+CD4+ subset (± SD) is depicted. Data represent four separate experiments. N=4 rats per treatment group. No significant differences between treatment groups were measured by one way ANOVA.

supernatants of MLN harvested at the same time point (Figure 2C,E). When compared to medium values, *in vitro* stimulation with p1 showed a trend towards higher p1-specific IL-10 and IFNγ production in rats nasally treated with CpG or p1/CpG (Figure 2C,E). When compared to p1 stimulated cells of PBS treated rats, significantly increased amounts of p1-specific IL-10 were detected only after combination treatment of CpG with p1, with unchanged ova-specific IL-10 production (Figure 2C). Both CpG monotherapy and p1/CpG combination treatment enhanced peptide specific IFNγ in MLN compared to peptide therapy alone (Figure 2E). Thus, nasal treatment with p1/CpG was the only condition that could induce an increase of both p1-specific IL-10 and IFNγ production at the site of peptide treatment.

We next asked whether nasal p1/CpG treatment led to increased peptide specific immunity systemically at this early time point. We therefore also measured p1-specific cytokine production in spleens (Figure 2D,F) but could not yet detect any significant amount of peptide specific IL-10. In p1-specific IFNγ production a non-significant increase was observed (possibly due to low numbers) systemically after p1/CpG combination treatment alone (p=0,121) (Figure 2F).

As pDC have been demonstrated to be important for the CpG-induced peripheral elicitation of Treg,[58] the increased number of pDC in antigen-draining MLN raised the question whether Treg were generated either at the site of peptide administration or at systemic sites. However, four days after the last treatment, the number of CD3+CD4+FoxP3+ Treg was comparable between treatment groups in MLN and spleen (Figure 2G). Also, the amount of FoxP3 per cell (expressed by MFI) did not differ at this time point (Figure 2H).

In summary, mucosal vaccination with p1/CpG combination treatment enhances peptide (p1) recognition locally as shown by increased peptide specific T cell proliferation and cytokine production at the site of treatment.

CpG enhances nasal peptide vaccination against autoimmunity in vivo

As p1/CpG mucosal vaccination lead to increased HSP60 peptide p1-specific T cell proliferation with potential anti-inflammatory capacity, we next tested the clinical efficacy of this approach in the rat model of adjuvant arthritis (AA). Rats were treated three times nasally with HSP-peptide p1, CpG or both at 14, 9 and 4 days before the induction of AA. Control rats were treated with PBS. Pre-treatment with HSP-peptide p1 had a mild disease-reducing effect as measured by arthritis scores (Figure 3A). The combined p1/CpG treatment strongly reduced arthritis scores throughout the disease course (Figure 3A), unlike CpG monotherapy. Figure 3B shows significant amelioration of arthritis by measure of the area under the curve of p1/CpG treated rats compared to PBS treated

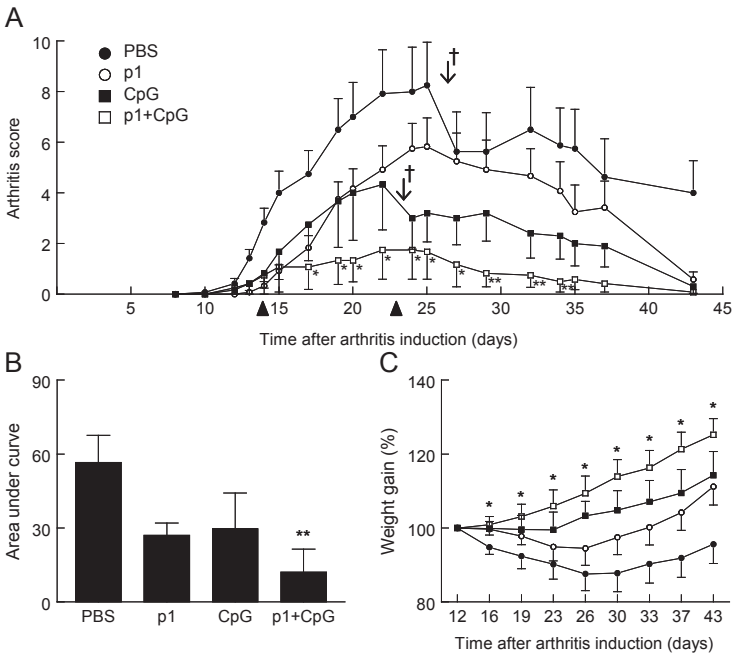


Figure 3 Clinical efficacy of nasal p1/CpG treatment. Reduced adjuvant arthritis scores (A), area under the arthritis score curve (B) and weight gain (C). Groups of rats were treated nasally with PBS (l), heat shock protein peptide p1 (mycobacterial HSP 254-268, i), CpG1668 (n) or both (o) 14, 9 and 5 days before arthritis induction. PBS treated rats show the natural course of adjuvant arthritis. On day 0 rats were immunized with 0.2 mg *Mycobacterium tuberculosis* in 100 µl Freund's incomplete adjuvant to induce AA. Arthritis scores were assessed daily from day 8 after AA induction in a blinded setup. As indicated by the animal welfare committee, rats were sacrificed when arthritis scores were 12 or higher. This was the case for one CpG treated rat (day 23) and two PBS treated rats (day 25) (†). Triangles indicate day 14 and 23, when animals were sacrificed for immunological analysis. Depicted are mean arthritis scores per treatment group per day (A), mean area under the curves from day 8 until day 23 (B) and percentage of rat weight on day 12 (c) ± SEM. N=6 rats per treatment group. Results are representative for three separate experiments. ** indicates a p-value <0.01 and * p<0.05 for p1/CpG treatment in comparison with p1 or PBS treated rats as measured by Kruskall Wallis and Mann Whitney U test.

rats. Weight curves reflected the pattern of arthritis score curves in that combination treatment showed least weight loss during the experiment (Figure 3C). Taken together, we show here experimental support for combined peptide p1/CpG treatment as preventive mucosal peptide immunotherapy in arthritis.

P1 specific IL-10 production in p1/CpG treated rats during early arthritis at inflammatory sites

We established that p1/CpG combination treatment was effective in the Adjuvant Arthritis model, and that this treatment leads to increased p1-specific T cell responses on day 0

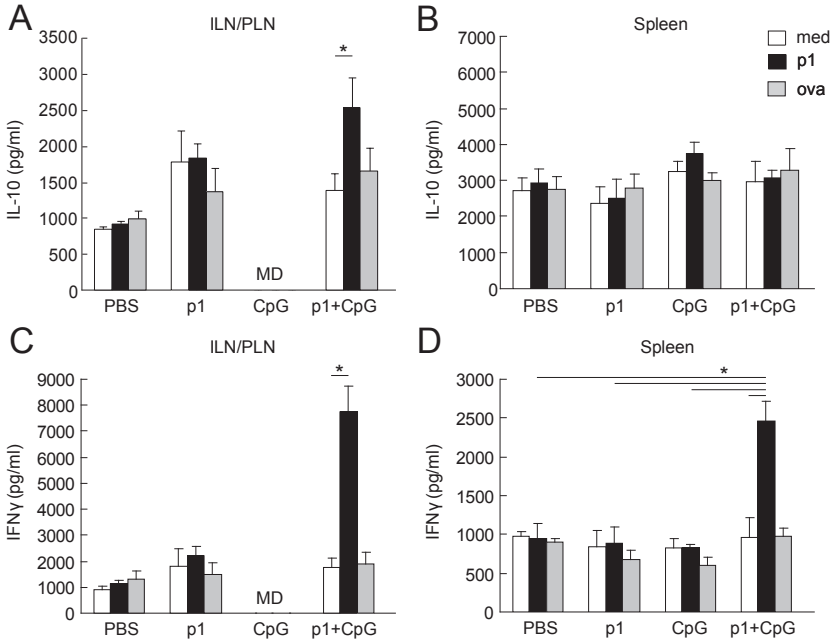


Figure 4 P1 specific cytokine production in p1/CpG treated rats during early arthritis at inflammatory and systemic sites. Groups of rats were treated as in described in Figure 3. Inguinal lymph nodes draining the arthritic joints (A,C) and spleens as a systemic control (B,D) were harvested fourteen days after arthritis induction. Cells were left unstimulated (med, white bars), stimulated with peptide p1 (mycobacterial HSP 254-268) or control peptide (ova 323-339) for 72h. Levels of IL-10 (A,B) and IFN-g (C,D) were measured in culture supernatants by Luminex assay. Shown are mean concentrations above medium values ± SEM. N=2-4 rats per treatment group. Missing data (MD): Data for CpG treated rats is missing due to shortage of lymph node cells. * indicates a p-value < 0.05 as measured by Kruskall Wallis and Mann Whitney U test.

(Figure 2). We next asked whether p1-specific cells could reach the site of inflammation during the early development of arthritis, and determined the quality of the immune response generated. To this end, we harvested the arthritic joint-draining inguinal and popliteal lymph nodes at day 14 after arthritis induction, and measured p1-specific IL-10 and IFNγ production in supernatants of these cells by Luminex multiplex cytokine array analysis. Ova-specific cytokine production was again used as a control. The increase in p1-specific IL-10 and IFNγ production after p1/CpG combination treatment that was already observed on day 0 (before arthritis induction, Figure 2C-F) was more pronounced at day 14 post-arthritis induction in draining lymph nodes (Figure 4A,C) and spleens (Figure 4B,D), now also reaching significance for IFNγ production in spleen. In summary, these data during early onset arthritis indicate preferred homing of anti-inflammatory p1-specific IL-10-producing T cells toward arthritic sites after p1/CpG combination treatment.

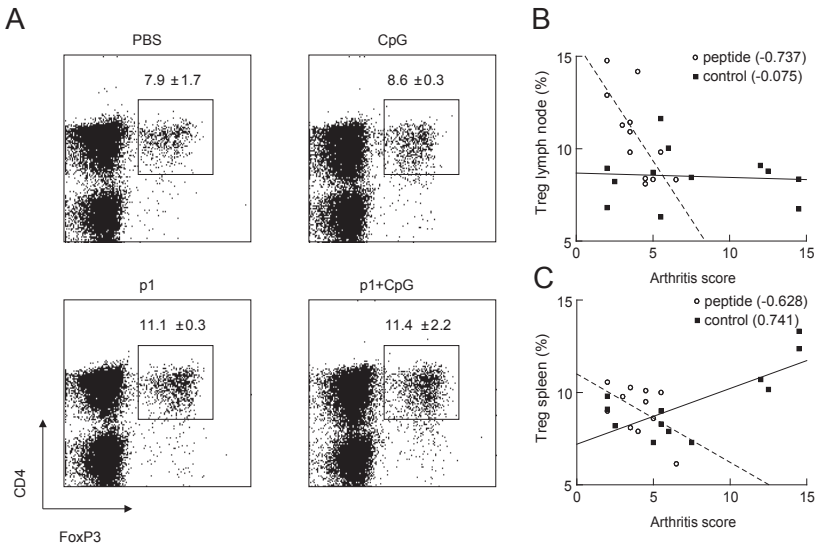


Figure 5 Number of regulatory T cells in joint draining lymph nodes at maximum of disease, that correlate with lower arthritis scores in p1/CpG treated rats. Groups of rats treated as described in Figure 3 were sacrificed 23 days after AA induction. Inguinal and popliteal lymph nodes draining arthritic joints and spleens were harvested.

Representative FACS dot plots for CD4-APC-Cy7 and FoxP3-PE expression in permeabilized pooled inguinal and popliteal lymph node cells (A). Gates were set on living lymphocytes. Numbers represent the mean percentage of FoxP3+ cells of the CD4+ population per treatment group ± SD.

Correlation between the detected percentage of CD4+FoxP3+ Treg cells in pooled inguinal and popliteal lymph nodes (B) or spleens (C) and arthritis scores on day 14 and 23 after AA induction. Peptide treatment (¡, dotted line) represents p1 or p1/CpG treated rats, control treatment (n, solid line) PBS or CpG treated rats. Numbers between brackets represent Pearson's correlation coefficient per group. The higher Pearsons correlation coefficient (0.741, p=0.006) is unreliable because of the uneven distribution of data.

Peptide-containing treatment is associated with suppressive FoxP3+ Treg cells that are retrieved at the site of inflammation and correlate with clinical improvement

To establish whether p1/CpG combination treatment may exert its immune suppressive effect through modulation of the balance in effector T cells versus Tregs, we harvested inguinal and popliteal lymph nodes draining the inflamed joints at day 14 (early onset arthritis) and day 23 (maximal disease activity) post-arthritis induction. We first determined the number of CD4+FoxP3+ regulatory T cells by flow cytometry. On day 14, the percentage Treg and FoxP3 MFI were comparable between treatment groups in the joint draining LNs and spleens (see online supplementary Figure S4A,B). On day 23, however, p1 and p1/CpG treated animals appeared to contain higher percentages of CD4+FoxP3+ Treg within arthritic joint-draining LNs (Figure 5A, see online supplementary Figure S4C) and express more FoxP3 per Treg: MFI FoxP3 PBS: 156.2 +/- 15.3; CpG: 187.4 +/- 40.0: p1: 243.2

+/- 38.5; p1/CpG: 221.3 +/- 54.6, though not reaching statistical significance (see online supplementary Figure S4D). In spleens, we observed no difference in the Treg percentage or their FoxP3 MFI between treatment groups (see online supplementary Figure S4C,D).
We asked whether Treg presence correlated with arthritis scores. We observed that in peptide-containing treatment groups (p1 and p1/CpG groups), lower arthritis scores were accompanied by increased percentages Tregs in the arthritic joint-draining LNs and a diagonal linear regression (Figure 5B; Treg are defined as CD4+FoxP3+, Pearsons correlation coefficient: -0.737, p=0,006). In control treatment groups (PBS and CpG) however, Treg numbers did not correlate with arthritis severity as shown by a horizontal relationship (Pearsons correlation coefficient: -0.075, p>0,05). In spleens we confirmed that increased Treg numbers correlate with lower arthritis scores for peptide-containing treatment groups (Figure 5C, Pearsons correlation coefficient: -0.628, p=0.039). Control groups (PBS and CpG) showed no such association Increased levels of CD4+FoxP3+ cells in samples from control groups might represent the more activated T cell population found in PBS rats.
Taking all data together, p1/CpG treatment is overall the most effective treatment, showing lowest arthritis scores, increased CD4+FoxP3+ Treg cells numbers combined with local IL-10 production in arthritic joint-draining lymph nodes and most weight gain during the course of disease progression.

DISCUSSION

Although mucosal peptide immune therapy has been proven effective in multiple animal models of autoimmune diseases (reviewed by [59]) and translation into humans has provided encouraging results, there is room for improvement.[10,60] Although we and others have shown that disease reduction is accompanied by peptide specific T cell responses ([8], reviewed by [6]) one of the major problems to solve seems to be the limited immunogenicity of the mucosally administered peptide.
In this study we tested the novel concept in autoimmune disease of combining antigen specific therapy triggering adaptive immunity with an adjuvant triggering innate immunity. We showed that the addition of CpG as a mucosal adjuvant to nasal peptide immunotherapy increases peptide immunogenicity and protective capacity, thereby overcoming the problem of limited peptide recognition. To our knowledge this is the first study in which CpG is used as an adjuvant for nasal peptide vaccination in autoimmune disease.

CpG treatment was associated with a selective increase in the number and activation status of pDC at the site of treatment. This was in line with the fact that in rats, pDC almost exclusively express TLR9 whereas B-cells and cDC have a more diverse pattern of TLRs.[53,58]

While it remains possible that (a subset of) cDC after TLR9 administration are involved, we did not detect differences in the number or activation status of cDC using our experimental setup. The increased activation state of pDC in mandibular lymph nodes (MLN) in the presence of p1 may have caused the amplified peptide-specific T cell response in p1/CpG combination-treated rats, with increased peptide specific T cell proliferation, IL-10 and IFNγ. By combining CpG with p1 treatment we potentiated p1-specific responses in such a way that they became detectable in MLN before arthritis induction and at the site of inflammation during arthritis. This is in line with previous results showing p1-specific cytokine production in joint draining lymph nodes 50 days after arthritis induction after p1 treatment.[8]

Since the HSP-peptide p1 used in this study constitutes an MHCII epitope, we consider it most probable that p1-induced cytokines are produced by CD4+ T cells. The antigen specificity of the cytokine responses (which were clearly not elicited by ova) supports this hypothesis.

Both HSP-peptides and CpG-oligodeoxynucleotides have earlier been shown to induce IL-10 responses in animal models of autoimmunity and human arthritis.[10,47,61] The presence of this cytokine in response to HSP-peptide p1 at the site of treatment and later on in the joint-draining lymph nodes could explain the beneficial effect of p1/CpG combination therapy we found in this study. P1-specific IL-10 producing cells were not detected systemically, thus exerting their anti-inflammatory effects locally. The absence of peptide-specific IL-10 production in spleens at these time points could be due to the small size of a peptide signal in general, but might also be an effect of preferred homing of HSP-specific T cells to the site of inflammation, where HSP are expressed ubiquitously. P1-specific IFNγ production was confirmed in spleens, supporting that p1-specific cells induced at the nasal mucosa have the potential to circulate into systemic compartments.
The increased amount of peptide-specific IFNγ however, we initially considered counter-intuitive, because of its known pro-inflammatory effects.[55] Several explanations are possible. First, IFNγ can have a dual role in inflammation, also having anti-inflammatory capacities.[62-64] In this regard, the reduced disease activity associated with increased amounts of IFNγ and mucosal pDC after TLR9 stimulation could be linked by the indoleamine 2,3 dioxygenase (IDO) pathway. IFNγ has been shown (like IFNα) to upregulate IDO.[65,66] Up-regulation of IDO in mucosal pDC has been shown to lead to several immune regulatory mechanisms including the differentiation of Treg, thereby suppressing disease activity.[67,68] Second, IFNγ and IL-10 co-producers are known as Tr1 cells, capable of down regulating inflammation.[69] CpG induced pDC have indeed induced Tr1 cells in mice.[70] However, directly after peptide treatment, the increase in IL-10 production in MLN was not accompanied by an increase in IFNγ, rendering Tr1 cells as a source for this cytokine unlikely at this time

point. Third, the increase in IL-10 could exceed the increase in IFNγ in p1/CpG combination treatment rats, possibly creating an anti-inflammatory environment. This cytokine pattern has earlier been observed in HSP epitope-specific T cell responses of arthritis patients.[1,71] Antigen-specific Treg seem to be more potent than naturally occurring Treg at suppressing pathogenic immune responses that cause autoimmunity and inflammation.[72-75] This underscores the importance of a peptide-containing treatment in autoimmune diseases. Indeed, our study shows a correlation between clinical improvement and the presence of FoxP3+ Treg that seems antigen specific as this occurred only after peptide-containing treatment. This is in support of our previous work[8] and many studies describing the relation between heat shock proteins and regulatory T cells.[76-80] The preferred homing of HSP-specific T cells to the site of inflammation mentioned above could therefore also account for the (non-significant) increase of Treg cells in joint-draining lymph nodes. HSP-specific Treg will find their cognate antigen at the site of inflammation. In support, (HSP-specific) Tregs similarly tend to be more increased in the joint-draining lymph nodes than in mandibular lymph nodes or spleens.

CpG-oligodeoxynucleotides are now also recognized for their ability to induce Tregs in human and mice models of autoimmune disease.[46,55,56,70,81] CpG could for example induce CD4+CD25+FoxP3hi Treg cells in human PBMCs that were potent immune suppressors.[46,81] For the induction of Treg cells, TLR9 expressing pDC were the key antigen presenting cells involved, which was confirmed in later rat-studies by Ouabed et al.[58,81] In this study, we combine these findings in an established arthritis model, showing a synergistic effect of the HSP epitope p1 and CpG.

We think the results of this study can have direct clinical value as the HLA-binding HSP-peptide p1 used in this study is immunogenic in patients with rheumatoid arthritis or Juvenile Idiopathic Arthritis by generating anti-inflammatory T cell responses.[1,71] We now found a way to enhance mucosal peptide therapy with this HSP epitope contributing to eventually safe and effective antigen specific immunotherapy in human arthritis.

ACKNOWLEDGEMENTS

The authors wish to thank Prof. Antony Horner for his useful advice, Maud Plantinga for her technical support and Erica Roks for her excellent secretarial assistance.

REFERENCE LIST

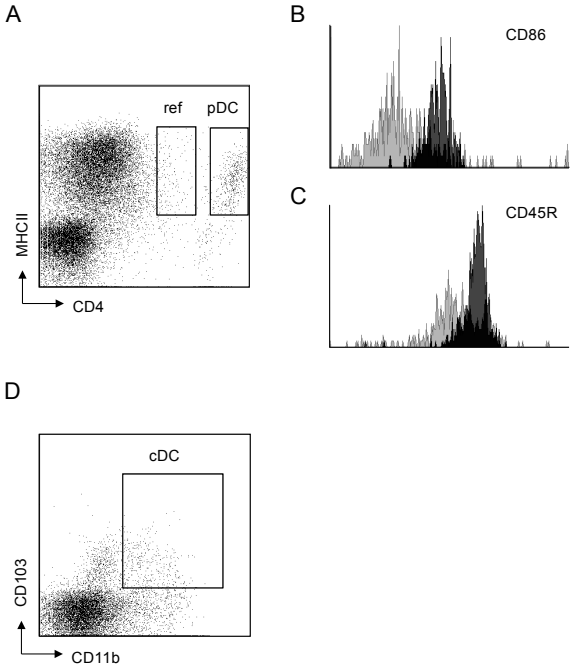
1. Kamphuis S, Kuis W, de Jager W, Teklenburg G, Massa M, Gordon G, et al. Tolerogenic immune responses to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis. *Lancet.* 2005;366:50-56.
2. Hafler DA, Kent SC, Pietrusewicz MJ, Khoury SJ, Weiner HL, Fukaura H. Oral administration of myelin induces antigen-specific TGF-beta 1 secreting T cells in patients with multiple sclerosis. *Ann N Y Acad Sci.* 1997;835:120-131.
3. Prakken BJ, Wendling U, van der Zee R, Rutten VP, Kuis W, van Eden W. Induction of IL-10 and inhibition of experimental arthritis are specific features of microbial heat shock proteins that are absent for other evolutionarily conserved immunodominant proteins. *J Immunol.* 2001;167:4147-4153.
4. Anderton SM, van der Zee R, Prakken B, Noordzij A, van Eden W. Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *J Exp Med.* 1995;181:943-952.
5. Chu CQ, Londei M. Differential activities of immunogenic collagen type II peptides in the induction of nasal tolerance to collagen-induced arthritis. *J Autoimmun.* 1999;12:35-42.
6. Larche M, Wraith DC. Peptide-based therapeutic vaccines for allergic and autoimmune diseases. *Nat Med.* 2005;11:S69-S76.
7. Brownlie RJ, Myers LK, Wooley PH, Corrigall VM, Bodman-Smith MD, Panayi GS, et al. Treatment of murine collagen-induced arthritis by the stress protein BiP via interleukin-4-producing regulatory T cells: a novel function for an ancient protein. *Arthritis Rheum.* 2006;54:854-863.
8. Zonneveld-Huijssoon E, Roord ST, de Jager W, Klein M, Albani S, Anderton SM, et al. Bystander suppression of experimental arthritis by nasal administration of a heat shock protein peptide. *Ann Rheum Dis.* 2011.
9. Huurman VA, Decochez K, Mathieu C, Cohen IR, Roep BO. Therapy with the hsp60 peptide DiaPep277 in C-peptide positive type 1 diabetes patients. *Diabetes Metab Res Rev.* 2007;23:269-275.
10. Koffeman EC, Genovese M, Amox D, Keogh E, Santana E, Matteson EL, et al. Epitope-specific immunotherapy of rheumatoid arthritis: clinical responsiveness occurs with immune deviation and relies on the expression of a cluster of molecules associated with T cell tolerance in a double-blind, placebo-controlled, pilot phase II trial. *Arthritis Rheum.* 2009;60:3207-3216.
11. Corrigall VM, Bodman-Smith MD, Brunst M, Cornell H, Panayi GS. Inhibition of antigen-presenting cell function and stimulation of human peripheral blood mononuclear cells to express an antiinflammatory cytokine profile by the stress protein BiP: relevance to the treatment of inflammatory arthritis. *Arthritis Rheum.* 2004;50:1164-1171.
12. Huurman VA, van der Meide PE, Duinkerken G, Willemen S, Cohen IR, Elias D, et al. Immunological efficacy of heat shock protein 60 peptide DiaPep277 therapy in clinical type I diabetes. *Clin Exp Immunol.* 2008;152:488-497.
13. Thurau SR, Fricke H, Burchardi C, Diedrichs-Möhring M, Wildner G. Long-term follow-up of oral tolerance induction with HLA-peptide B27PD in patients with uveitis. *Ann N Y Acad Sci.* 2004;1029:408-412.
14. Muller S, Monneaux F, Schall N, Rashkov RK, Oparanov BA, Wiesel P, et al. Spliceosomal peptide P140 for immunotherapy of systemic lupus erythematosus: results of an early phase II clinical trial. *Arthritis Rheum.* 2008;58:3873-3883.
15. Holmgren J, Czerkinsky C, Eriksson K, Mharandi A. Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. *Vaccine.* 2003;21 Suppl 2:S89-S95.
16. Moschos SA, Bramwell VW, Somavarapu S, Alpar HO. Adjuvant synergy: the effects of nasal coadministration of adjuvants. *Immunol Cell Biol.* 2004;82:628-637.

17. Decrausaz L, Domingos-Pereira S, Duc M, Bobst M, Romero P, Schiller JT, et al. Parenteral is more efficient than mucosal immunization to induce regression of human papillomavirus-associated genital tumors. *Int J Cancer.* 2011;129:762-772.
18. Bouvet JP, Decroix N, Pamonsinlapatham P. Stimulation of local antibody production: parenteral or mucosal vaccination? *Trends Immunol.* 2002;23:209-213.
19. Dietrich G, Griot-Wenk M, Metcalfe IC, Lang AB, Viret JF. Experience with registered mucosal vaccines. *Vaccine.* 2003;21:678-683.
20. Ivanov AP, Dragunsky EM, Chumakov KM. 1,25-dihydroxyvitamin d3 enhances systemic and mucosal immune responses to inactivated poliovirus vaccine in mice. *J Infect Dis.* 2006;193:598-600.
21. Ichinohe T, Ainai A, Tashiro M, Sata T, Hasegawa H. PolyI:polyC12U adjuvant-combined intranasal vaccine protects mice against highly pathogenic H5N1 influenza virus variants. *Vaccine.* 2009;27:6276-6279.
22. Ivory CP, Chadee K. Intranasal immunization with Gal-inhibitable lectin plus an adjuvant of CpG oligodeoxynucleotides protects against Entamoeba histolytica challenge. *Infect Immun.* 2007;75:4917-4922.
23. Mariotti S, Teloni R, von Hunolstein C, Romagnoli G, Orefici G, Nisini R. Immunogenicity of anti-Haemophilus influenzae type b CRM197 conjugate following mucosal vaccination with oligodeoxynucleotide containing immunostimulatory sequences as adjuvant. *Vaccine.* 2002;20:2229-2239.
24. Holmgren J, Adamsson J, Anjuere F, Clemens J, Czerkinsky C, Eriksson K, et al. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. *Immunol Lett.* 2005;97:181-188.
25. Orban T, Farkas K, Jalahej H, Kis J, Treszl A, Falk B, et al. Autoantigen-specific regulatory T cells induced in patients with type 1 diabetes mellitus by insulin B-chain immunotherapy. *J Autoimmun.* 2009.
26. Krieg AM. CpG motifs: the active ingredient in bacterial extracts? *Nat Med.* 2003;9:831-835.
27. Lenert P. Inhibitory oligodeoxynucleotides - therapeutic promise for systemic autoimmune diseases? *Clin Exp Immunol.* 2005;140:1-10.
28. Mangsbo SM, Sandin LC, Anger K, Korman AJ, Loskog A, Totterman TH. Enhanced tumor eradication by combining CTLA-4 or PD-1 blockade with CpG therapy. *J Immunother.* 2010;33:225-235.
29. Liang X, Moseman EA, Farrar MA, Bachanova V, Weisdorf DJ, Blazar BR, et al. Toll-like receptor 9 signaling by CpG-B oligodeoxynucleotides induces an apoptotic pathway in human chronic lymphocytic leukemia B cells. *Blood.* 2010.
30. Nierkens S, den Brok MH, Roelofsen T, Wagenaars JA, Figdor CG, Ruers TJ, et al. Route of administration of the TLR9 agonist CpG critically determines the efficacy of cancer immunotherapy in mice. *PLoS ONE.* 2009;4:e8368.
31. Westwood JA, Haynes NM, Sharkey J, McLaughlin N, Pegram HJ, Schwendener RA, et al. Toll-Like Receptor Triggering and T-Cell Costimulation Induce Potent Antitumor Immunity in Mice. *Clin Cancer Res.* 2009;15:7624-7633.
32. De Cesare M, Sfondrini L, Campiglio M, Sommariva M, Bianchi F, Perego P, et al. Ascites regression and survival increase in mice bearing advanced-stage human ovarian carcinomas and repeatedly treated intraperitoneally with CpG-ODN. *J Immunother.* 2010;33:8-15.
33. Cooper CL, Davis HL, Morris ML, Efler SM, Krieg AM, Li Y, et al. Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine. *Vaccine.* 2004;22:3136-3143.
34. Bhat AA, Seth RK, Kumar S, Ali R, Mohan T, Biswas S, et al. Induction of cell-mediated immune responses to peptide antigens of P. vivax in microparticles using intranasal immunization. *Immunol Invest.* 2010;39:483-499.

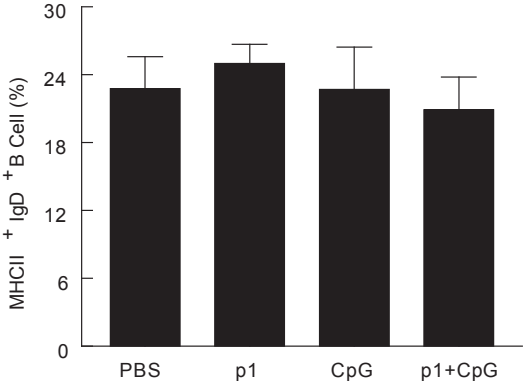
35. Sogaard OS, Lohse N, Harboe ZB, Offersen R, Bukh AR, Davis HL, et al. Improving the immunogenicity of pneumococcal conjugate vaccine in HIV-infected adults with a toll-like receptor 9 agonist adjuvant: a randomized, controlled trial. *Clin Infect Dis.* 2010;51:42-50.
36. Birnbaum G, Kotilinek L, Miller SD, Raine CS, Gao YL, Lehmann PV, et al. Heat shock proteins and experimental autoimmune encephalomyelitis. II: environmental infection and extra-neuraxial inflammation alter the course of chronic relapsing encephalomyelitis. *J Neuroimmunol.* 1998;90:149-161.
37. Feleszko W, Jaworska J, Hamelmann E. Toll-like receptors--novel targets in allergic airway disease (probiotics, friends and relatives). *Eur J Pharmacol.* 2006;533:308-318.
38. Okada H, Kuhn C, Feillet H, Bach JF. The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. *Clin Exp Immunol.* 2010;160:1-9.
39. Bach JF. The effect of infections on susceptibility to autoimmune and allergic diseases. *N Engl J Med.* 2002;347:911-920.
40. Boccaccio GL, Mor F, Steinman L. Non-coding plasmid DNA induces IFNgamma in vivo and suppresses autoimmune encephalomyelitis. *Int Immunol.* 1999;11:289-296.
41. Gilkeson GS, Ruiz P, Pippen AM, Alexander AL, Lefkowith JB, Pisetsky DS. Modulation of renal disease in autoimmune NZB/NZW mice by immunization with bacterial DNA. *J Exp Med.* 1996;183:1389-1397.
42. Chervonsky AV. Influence of microbial environment on autoimmunity. *Nat Immunol.* 2010;11:28-35.
43. Arnold IC, Dehzad N, Reuter S, Martin H, Becher B, Taube C, et al. Helicobacter pylori infection prevents allergic asthma in mouse models through the induction of regulatory T cells. *J Clin Invest.* 2011.
44. Lee YK, Mazmanian SK. Has the microbiota played a critical role in the evolution of the adaptive immune system? *Science.* 2010;330:1768-1773.
45. Marta M, Andersson A, Isaksson M, Kampe O, Lobell A. Unexpected regulatory roles of TLR4 and TLR9 in experimental autoimmune encephalomyelitis. *Eur J Immunol.* 2008;38:565-575.
46. Fallarino F, Volpi C, Zelante T, Vacca C, Calvitti M, Fioretti MC, et al. IDO mediates TLR9-driven protection from experimental autoimmune diabetes. *J Immunol.* 2009;183:6303-6312.
47. Quintana FJ, Rotem A, Carmi P, Cohen IR. Vaccination with empty plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity. *J Immunol.* 2000;165:6148-6155.
48. Meyers JA, Mangini AJ, Nagai T, Roff CF, Sehy D, van Seventer GA, et al. Blockade of TLR9 agonist-induced type I interferons promotes inflammatory cytokine IFN-gamma and IL-17 secretion by activated human PBMC. *Cytokine.* 2006;35:235-246.
49. van Eden W, van der Zee R, Prakken B. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol.* 2005;5:318-330.
50. Prakken BJ, van der Zee R, Anderton SM, van Kooten PJ, Kuis W, van Eden W. Peptide-induced nasal tolerance for a mycobacterial heat shock protein 60 T cell epitope in rats suppresses both adjuvant arthritis and nonmicrobially induced experimental arthritis. *Proc Natl Acad Sci U S A.* 1997;94:3284-3289.
51. Prakken BJ, van der Zee R, Anderton SM, van Kooten P, Kuis W, van Eden W. Tolerance to an arthritogenic T-cell epitope of HSP65 and the regulation of experimental arthritis. *Ann N Y Acad Sci.* 1996;778:425-426.
52. Trentham DE, Townes AS, Kang AH. Autoimmunity to type II collagen an experimental model of arthritis. *J Exp Med.* 1977;146:857-868.

53. Hubert FX, Voisine C, Louvet C, Heslan JM, Ouabed A, Heslan M, et al. Differential pattern recognition receptor expression but stereotyped responsiveness in rat spleen dendritic cell subsets. *J Immunol.* 2006;177:1007-1016.
54. de Jager W, Prakken B, Rijkers GT. Cytokine multiplex immunoassay: methodology and (clinical) applications. *Methods Mol Biol.* 2009;514:119-133.
55. Obermeier F, Strauch UG, Dunger N, Grunwald N, Rath HC, Herfarth H, et al. In vivo CpG DNA/toll-like receptor 9 interaction induces regulatory properties in CD4+CD62L+ T cells which prevent intestinal inflammation in the SCID transfer model of colitis. *Gut.* 2005;54:1428-1436.
56. Bleich A, Janus LM, Smoczek A, Westendorf AM, Strauch U, Mahler M, et al. CpG motifs of bacterial DNA exert protective effects in mouse models of IBD by antigen-independent tolerance induction. *Gastroenterology.* 2009;136:278-287.
57. Jain VV, Kitagaki K, Businga T, Hussain I, George C, O'shaughnessy P, et al. CpG-oligodeoxynucleotides inhibit airway remodeling in a murine model of chronic asthma. *J Allergy Clin Immunol.* 2002;110:867-872.
58. Ouabed A, Hubert FX, Chabannes D, Gautreau L, Heslan M, Josien R. Differential control of T regulatory cell proliferation and suppressive activity by mature plasmacytoid versus conventional spleen dendritic cells. *J Immunol.* 2008;180:5862-5870.
59. Faria AM, Weiner HL. Oral tolerance. *Immunol Rev.* 2005;206:232-259.
60. Thurau SR, Diedrichs-Möhring M, Fricke H, Burchardi C, Wildner G. Oral tolerance with an HLA-peptide mimicking retinal autoantigen as a treatment of autoimmune uveitis. *Immunol Lett.* 1999;68:205-212.
61. Chiaramonte MG, Hesse M, Cheever AW, Wynn TA. CpG oligonucleotides can prophylactically immunize against Th2-mediated schistosome egg-induced pathology by an IL-12-independent mechanism. *J Immunol.* 2000;164:973-985.
62. Muhl H, Pfeilschifter J. Anti-inflammatory properties of pro-inflammatory interferon-gamma. *Int Immunopharmacol.* 2003;3:1247-1255.
63. Kelchtermans H, Billiau A, Matthys P. How interferon-gamma keeps autoimmune diseases in check. *Trends Immunol.* 2008;29:479-486.
64. Kim EY, Moudgil KD. Regulation of autoimmune inflammation by pro-inflammatory cytokines. *Immunol Lett.* 2008;120:1-5.
65. Hayashi T, Beck L, Rossetto C, Gong X, Takikawa O, Takabayashi K, et al. Inhibition of experimental asthma by indoleamine 2,3-dioxygenase. *J Clin Invest.* 2004;114:270-279.
66. Sarkar SA, Wong R, Hackl SI, Moua O, Gill RG, Wiseman A, et al. Induction of indoleamine 2,3-dioxygenase by interferon-gamma in human islets. *Diabetes.* 2007;56:72-79.
67. Hill M, Tanguy-Royer S, Royer P, Chauveau C, Asghar K, Tesson L, et al. IDO expands human CD4+CD25high regulatory T cells by promoting maturation of LPS-treated dendritic cells. *Eur J Immunol.* 2007;37:3054-3062.
68. Fallarino F, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R, et al. Modulation of tryptophan catabolism by regulatory T cells. *Nat Immunol.* 2003;4:1206-1212.
69. Roncarolo MG, 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;212:28-50.
70. Bilsborough J, George TC, Norment A, Viney JL. Mucosal CD8alpha+ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties. *Immunology.* 2003;108:481-492.

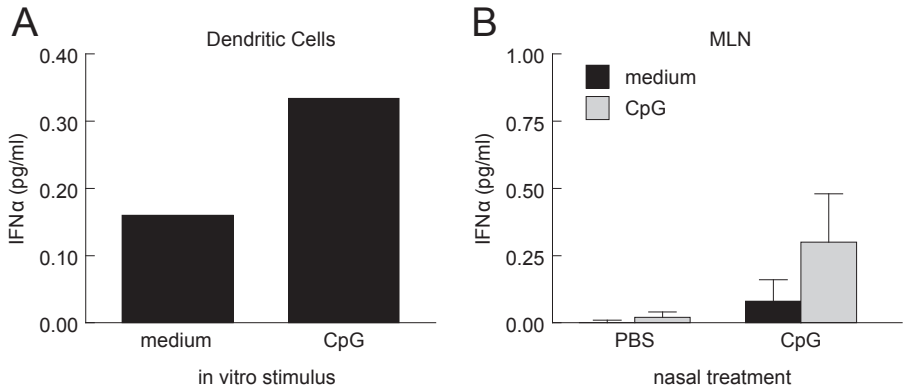
71. de Jong H, Lafeber FF, de Jager W, Haverkamp MH, Kuis W, Bijlsma JW, et al. PAN-DR-Binding Hsp60 self epitopes induce an interleukin-10-mediated immune response in rheumatoid arthritis. *Arthritis Rheum.* 2009;60:1966-1976.
72. Hori S, Haury M, Coutinho A, Demengeot J. Specificity requirements for selection and effector functions of CD25+4+ regulatory T cells in anti-myelin basic protein T cell receptor transgenic mice. *Proc Natl Acad Sci U S A.* 2002;99:8213-8218.
73. Nishimura E, Sakihama T, Setoguchi R, Tanaka K, Sakaguchi S. Induction of antigen-specific immunologic tolerance by in vivo and in vitro antigen-specific expansion of naturally arising Foxp3+CD25+CD4+ regulatory T cells. *Int Immunol.* 2004;16:1189-1201.
74. Tarbell KV, Yamazaki S, Olson K, Toy P, Steinman RM. CD25+ CD4+ T cells, expanded with dendritic cells presenting a single autoantigenic peptide, suppress autoimmune diabetes. *J Exp Med.* 2004;199:1467-1477.
75. Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, et al. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med.* 2004;199:1455-1465.
76. Elst EF, Klein M, de Jager W, Kamphuis S, Wedderburn LR, van der Zee R, et al. Hsp60 in inflamed muscle tissue is the target of regulatory autoreactive T cells in patients with juvenile dermatomyositis. *Arthritis Rheum.* 2008;58:547-555.
77. Quintana FJ, Mimran A, Carmi P, Mor F, Cohen IR. HSP60 as a target of anti-ergotypic regulatory T cells. *PLoS ONE.* 2008;3:e4026.
78. Wang X, Zhou S, Chi Y, Wen X, Hoellwarth J, He L, et al. CD4+CD25+ Treg induction by an HSP60-derived peptide SJMHE1 from Schistosoma japonicum is TLR2 dependent. *Eur J Immunol.* 2009;39:3052-3065.
79. Zanin-Zhorov A, Cahalon L, Tal G, Margalit R, Lider O, Cohen IR. Heat shock protein 60 enhances CD4 CD25 regulatory T cell function via innate TLR2 signaling. *J Clin Invest.* 2006;116:2022-2032.
80. Massa M, Passalia M, Manzoni SM, Campanelli R, Ciardelli L, Yung GP, et al. Differential recognition of heat-shock protein dnaJ-derived epitopes by effector and Treg cells leads to modulation of inflammation in juvenile idiopathic arthritis. *Arthritis Rheum.* 2007;56:1648-1657.
81. Moseman EA, Liang X, Dawson AJ, Panoskaltsis-Mortari A, Krieg AM, Liu YJ, et al. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol.* 2004;173:4433-4442.



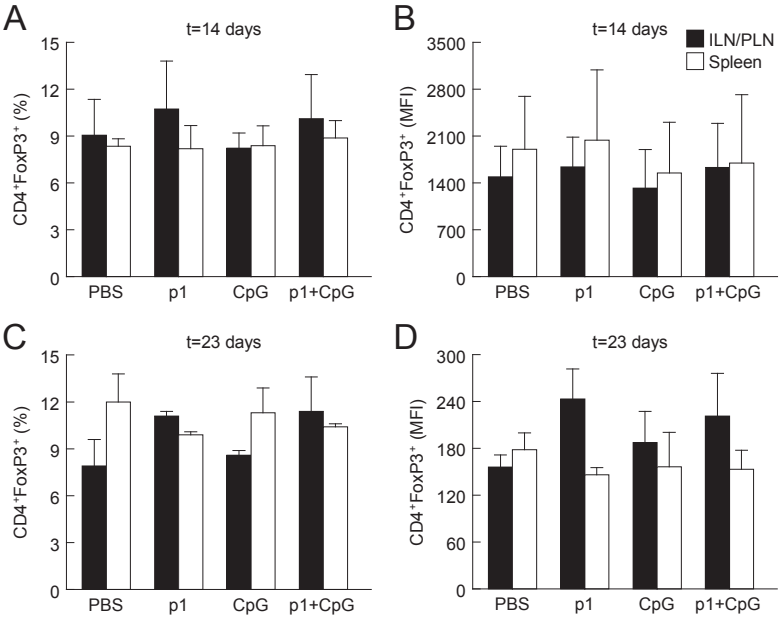
Supplement I Gating strategy for plasmacytoid dendritic cells (pDC) and conventional DC (cDC) in mandibular lymph nodes (MLN). Cells were stained with anti-IgD-APC-Cy7, CD3-APC, CD11b-FITC, CD4-PE-Cy7, and RT1B-PerCP combined with CD86-PE, CD103-PE or CD45R-PE mAbs. For pDC, living CD3-IgD- cells and CD11b- cells were selected. pDC were defined as the MHCII+CD4high population (A). The MHCII+CD4intermediate population was used as a reference population for histograms B and C. Both populations (pDC black, reference shaded) were analysed for their expression of CD86 (B) and CD45R (C). For cDC, CD3-IgD- cells were gated as described for pDC, however within the MHCII positive population, cDC were defined as CD11b+CD103+ (D).



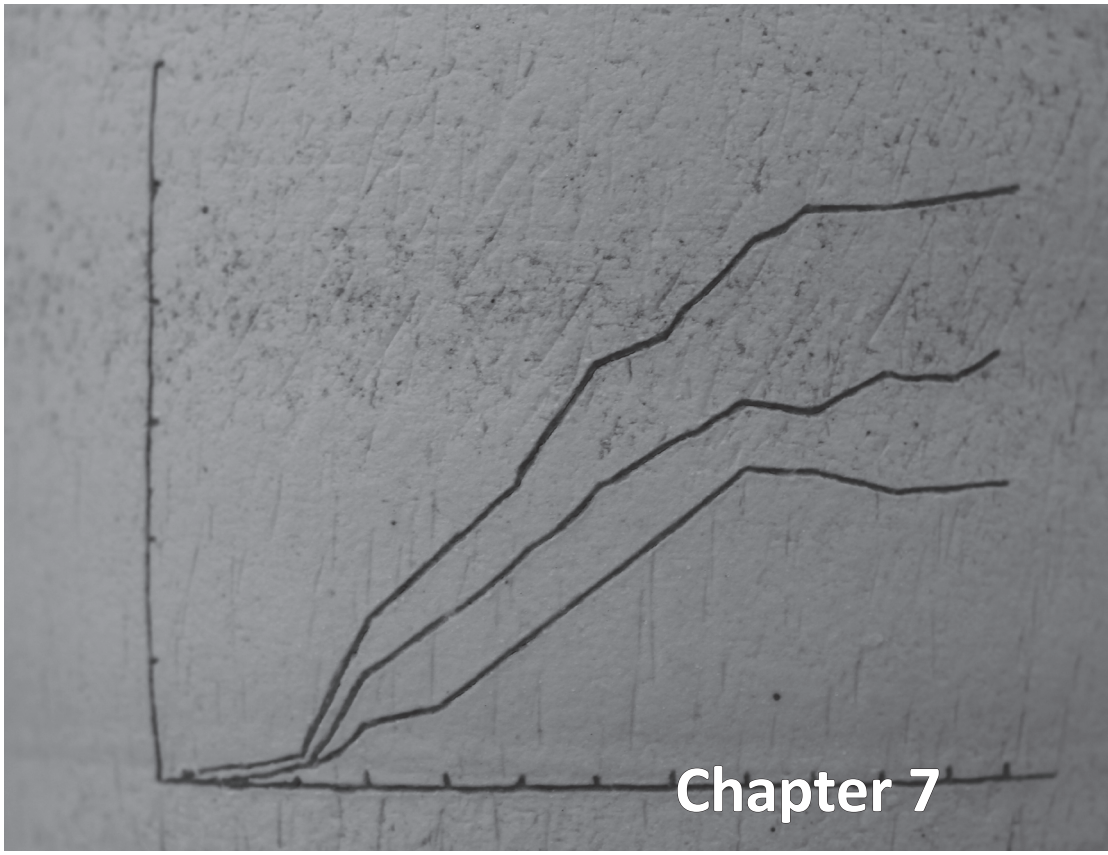
Supplement II Percentage of B cells in MLN after nasal peptide treatment as determined by FACS. Rats were treated and MLN were stained as described in Figure 1. B cells were defined as the CD3-RT1B+IgD+ population. Shown are mean percentages of B cells ± SD in the live gate of four rats per treatment group. No significant differences were measured by one way ANOVA.



Supplement III IFNα production by mandibular DC is elevated after CpG stimulation in vitro (A) and in vivo (B). DC (CD3-IgD-MHCII+) were sorted by FACS from naïve MLN cells and stimulated for 24 hours in vitro with medium or CpG (A). Rats were treated nasally with PBS or CpG as described in Figure 1 (B). Four days after the last nasal treatment, MLN were harvested and cultured for 24 hours with medium or CpG. IFNα was measured in culture supernatants by ELISA. Shown are mean concentrations ± SEM. N=4 rats per treatment group. No significant differences were measured by Mann Whitney U test.



Supplement IV Increased number and MFI FoxP3 of regulatory T cells in joint draining lymph nodes (ILN/PLN) of p1 and p1/CpG treated rats at maximum of disease (day 23). Groups of rats treated as in Figure 3 were sacrificed 14 (A,B) or 23 days (C,D) after AA induction. Inguinal and popliteal lymph nodes draining arthritic joints (black bars) or spleens (white bars) were harvested and stained directly for FACS. Gates for analysis were set on live cells and CD3+CD4+FoxP3+ (A,B) or living CD4+FoxP3+ (C,D) cells. Data represent four (A,B) or two (C,D) rats per treatment group and were repeated in two separate experiments. Shown are means ± SD. No significant differences between treatment groups were measured by one way ANOVA.



Chapter 7

Modulation of T cell function by combination of epitope specific and low dose anticytokine therapy controls autoimmune arthritis

Sarah TA Roord, Evelien Zonneveld-Huijssoon, Tho Le, Gisella Puga Yung, Eva Koffeman, Arash Ronaghy, Negar Ghahramani, Paola Lanza, Rosario Billetta, Berent J Prakken, Salvatore Albani

PLoS One 2006 Dec 20;1:e87

ABSTRACT

Innate and adaptive immunity contribute to the pathogenesis of autoimmune arthritis by generating and maintaining inflammation, which leads to tissue damage. Current biological therapies target innate immunity, eminently by interfering with single pro-inflammatory cytokine pathways. This approach has shown excellent efficacy in a good proportion of patients with Rheumatoid Arthritis (RA), but is limited by cost and side effects. Adaptive immunity, particularly T cells with a regulatory function, plays a fundamental role in controlling inflammation in physiologic conditions. A growing body of evidence suggests that modulation of T cell function is impaired in autoimmunity. Restoration of such function could be of significant therapeutic value. We have recently demonstrated that epitope-specific therapy can restore modulation of T cell function in RA patients. Here, we tested the hypothesis that a combination of anti-cytokine and epitope specific immunotherapy may facilitate the control of autoimmune inflammation by generating active T cell regulation. This novel combination of mucosal tolerization to a pathogenic T cell epitope and single low dose anti-TNFa was as therapeutically effective as full dose anti-TNFa treatment. Analysis of the underlying immunological mechanisms showed induction of T cell immune deviation.

INTRODUCTION

Much progress has recently been achieved on our knowledge of the immunological and molecular mechanisms, which lead to amplification, and perpetuation of autoimmune inflammation. This progress has been translated into a generation of biologic therapeutic agents that target pro-inflammatory cytokines, with the aim of interfering with their mechanism of action. This approach is destined to progressively complement and, in some cases, replace currently used immunosuppressive and anti-inflammatory therapies. Despite their success,[1,2] current anti-cytokine approaches remain hampered with limitations associated eminently with generalized immunosuppression and subsequent increased occurrence of malignancies and infectious diseases, in particular tuberculosis.[3-6]

Conceptually, therapeutic intervention focused on modulation of T cell function leads to the promise of higher specificity and lower toxicity.[7-16] This objective has for long remained a challenge in humans, particularly due to the difficulty of identifying means of intervention that could affect T cell function in a specific fashion.

In a Phase I/IIa trial, we have recently described immunological effects of epitope specific immunotherapy in a group of patients with rheumatoid arthritis. The epitope employed was derived from the heat shock protein (HSP) dnaJ. We have proposed a central role for HSP-specific T cell responses in the physiologic mechanisms of modulation of inflammation.[17-20] We have also suggested impairment of such modulation as one of the mechanisms of amplification of autoimmune inflammation.[21-24] Our treatment sought to restore such control by inducing mucosal tolerization to a peptide with a potential pathogenic, not necessarily etiologic role.[25] Immunological effects of the treatment consisted of immunodeviation from pro-inflammatory to tolerogenic type T cell responses to the peptide employed in the treatment. Restoration of regulatory T cell activity was also observed.

Effects of anti cytokine therapy on T cell function, both effector and regulatory, have been suggested.[13,26-28] These interactions are relevant for many different reasons, including ultimately the design of an optimal biologic therapy based on the combination of anti-cytokine and T cell epitope specific approaches.

The work presented here lays the foundation for this strategy by exploring clinical and immunological effects of the combination of epitope-specific T cell and anti-cytokine therapy. We employed for this purpose Adjuvant Arthritis (AA). This is an experimental form of arthritis that is T cell dependent and can be passively transferred by a T cell clone that is specific for the 180-188 amino acid sequence of mycobacterial HSP60.[29,30] In previous studies we showed that nasal administration of a 15-mer peptide (176-190) encompass-

ing this arthritogenic epitope leads to T cell tolerance[31] and can prevent AA. Treatment with nasal administration of peptide 180-188 after the induction of AA is mildly effective. Here, we compared immunological and clinical effects of different dose regimens, namely full dose anti-TNFa, which is known to be effective[32], mucosal tolerization to the peptide alone, anti-TNFa at one third of the effective dose, and the combination of low dose anti-TNFa and epitope specific therapy.

We found that the combination of low dose anti-TNFa associated with mucosal tolerization to the arthritogenic T cell epitope led to a significant reduction of arthritis clinically as well as histologically, to a degree entirely comparable with what was achieved with full dose anti-TNFa. Interestingly, treatment regimens differed for their influence on immune responses. Indeed, combination therapy induced T cells with a regulatory phenotype, consisting of CD4+CD25+ cells producing IL-10 and expressing FOXP3. Combination treatment also induced immune deviation in CD4+CD25- cells, which were found producing IL-10, as well as IL-4. Such changes were not present in the full dose anti-TNFa therapy group.

Our data provide a compelling rationale for testing the combination of anti-cytokine and epitope specific immunotherapy in human autoimmune disease.

METHODS

Animals

Male inbred Lewis rats (RT1B) were obtained from Harlan (Indianapolis). Rats were 6-9 weeks old at the start of each experiment.

Antigens and adjuvants

Heat killed *Mycobacterium tuberculosis* (Mt, strain H37Ra) was obtained from Difco (Detroit, MI). Incomplete Freund's Adjuvant (IFA; Difco, Detroit, MI) was used as adjuvant. The peptide used in this study was prepared in large quantities by standard solid phase Fmoc chemistry. It was obtained as COOH terminal amide and was analyzed and purified by reverse-phase HPLC. The following peptide was used: *Mycobacterium tuberculosis* HSP60 180-188, containing *Mycobacterium tuberculosis* HSP60 sequence 180-188 (TFGLQLELT). 180-188 is recognized by the arthritogenic T cell clone A2b and is a dominant T cell epitope found after Adjuvant Arthritis (AA) and after immunization with mycobacterial HSP60.

Induction and Clinical Assessment of Experimental Arthritis

Rats were lightly anesthetized using isoflurane and AA was induced by a single intradermal (i.d.) injection in the base of the tail with 0.1 mg *Mycobacterium tuberculosis* (Mt) sus-

pended in 100 ml of IFA (Complete Freund's Adjuvant; CFA). Rats were examined daily for clinical signs of arthritis in a blinded set-up. Severity of arthritis was assessed by scoring each paw from zero to four based on degree of swelling, erythema and deformation of the joints. Thus the maximum score was 16. On day 23 after the induction of arthritis the rats were sacrificed by CO2 inhalation, after which mandibular lymphnodes (MLN), Inguinal Lymphnodes (ILN), spleen and hind limb joints were collected.

Immunotherapy Protocols

Rats were lightly anesthetized using metofane for all nasal treatments or isoflurane for all subcutaneous treatments. etanercept (Enbrel®, Wyeth) was administered subcutaneously (s.c.) at a concentration of 0.3 mg/kg per rat dissolved in 100 ml PBS using a 25-gauge needle. This was done on day 9 after the induction of arthritis with Mt. Some rats in control groups received additional etanercept on day 11 and 13. 100 mg of peptide dissolved in PBS was administered nasally in a total volume of 10 ml (5 ml per nostril, peptide concentration 10 mg/ml) using a micropipette. This was done on day 10, 13, 16, 19 after arthritis induction with Mt.

Adoptive transfer

MLN, ILN and spleen of 2-3 rats per group after combination treatment with etanercept and 180-188 were harvested on day 23 after the induction of arthritis. Cells were cultured in vitro with 2.5µg/ml conA for 48 hours. Subsequently, $13x10^6$ MLN, $11x10^6$ ILN and $11x10^6$ spleen cells were injected i.v. into the tail vein of rats one week after induction of arthritis with Mt. Rats were subsequently examined daily for clinical signs of arthritis in a blinded set-up as described previously.

Histological assessment of hind limb joints

Hind limb joints were collected on day 23 after the induction of arthritis, after the rats were sacrificed by CO2 inhalation. Formalin-fixed tissues were decalcified, and glass slides stained with H&E and Safranin O (for cartilage) were prepared and evaluated by standard methodology (Comparative Biosciences, Inc.). The pathologist examined all of the submitted tissue sections in a blinded fashion by light microscopy and scored for inflammation of the synovium, pannus formation, cartilage damage, inflammation of the bone marrow and periostal proliferation. Each of these parameters was scored for 10 days, severity from 0 (normal) to 4 (severe). A cumulative score was given based upon the sum of all of the parameters measured.

Intracellular Cytokine Staining

MLN cells were cultured for 72 hours with medium alone or antigen. During the last 4 hours of culture 1 M monensin (GolgiStop®, Pharmingen, San Diego, CA) was added. Vi-

able cells were harvested and washed with FACS blocking buffer (PBS with 10% FBS) and 0.03% 1M sodium azide) and subsequently stained for 30 minutes on ice in 100 ml of blocking buffer with the following conjugated monoclonal antibodies for extracellular antigens: PE, FITC or CY-conjugated anti-rat CD4 (clone OX-35, mouse IgG2a), FITC-conjugated anti-rat CD25 (clone OX-39, mouse IgG1) (BD Pharmingen, San Diego CA). The cells were washed twice in staining buffer (PBS containing 3% FBS and 0.03% 1M sodium azide) and resuspended in 100 ml fixation buffer (Cytofix/Cytoperm®, BD Pharmingen, San Diego, CA) for 20 minutes on ice. The cells were washed twice in permeabilization buffer (Perm/Wash®, Pharmingen, San Diego CA) and resuspended in 100 ml permeabilization buffer and stained with the following conjugated monoclonal antibodies: PE-conjugated anti-rat IL-4 (clone OX-81, mouse IgG1), PE-conjugated anti-rat IL-10 (clone A5-4, mouse IgG2b), PE-conjugated anti-rat TNFa (clone TN3.-19.12, hamster IgG) and PE-conjugated anti-mouse CTLA-4 (anti CD152) (clone UC10-4F10-11, armenian hamster IgG, group 1k) (all antibodies from Pharmingen, San Diego, CA). The appropriate isotype controls were used. Finally, the cells were washed twice, resuspended in staining buffer, and transferred to FACS tubes for analysis. Stained cells were analyzed on a FACStar Plus cytometer (Becton and Dickinson). At least 5.000 events were acquired from each sample and subsequently analyzed with Lysis II software.

Sorting of CD4+CD25+ and CD4+CD25- after magnetic bead separation

MLN were incubated for 15 hours with medium or antigen. Viable cells were harvested and first the cell suspensions were depleted of monocytes, phagocytes, NK cells and B cells by magnetic bead separation using the CELLection®Biotin Binder kit (Dynal A.S. Oslo, Norway). In brief, cells were incubated with the following monoclonal antibodies: biotin mouse anti rat mononuclear phagocyte, (C17, Pharmingen), biotin mouse anti rat CD161a (10/78, Pharmingen) and biotin mouse anti rat CD45RA (OX-33, Caltag Laboratories). Positive selection was performed using streptavidin coated magnetic Dynabeads using the Dynal Magnetic Particle Concentrator.

The thus obtained cells were washed in FACS blocking buffer and stained extracellularly with anti rat CD4 and anti rat CD25. Subsequently, cells were sorted by FACS (FACS Vantage, Beckton Dickinson San Jose, CA) into CD4+CD25+ and CD4+CD25- cells.

Real Time Quantitative PCR (Taqman)

MLN were incubated for 15 hours with medium or antigen. Cells were sorted into CD4+CD25+ cells and CD4+CD25- cells as described above, resuspended in Lysis buffer (Qiagen,Valencia, CA) and frozen at –80 °C until analysis. mRNA was extracted from sorted cells by using RNeasy Mini Kit (Qiagen). mRNA was then reverse-transcribed into cDNA with an oligo dT primer (Oligo(dT)12-18, Invitrogen). Subsequently, single stranded cDNA was amplified with the cytokine specific forward and reverse primer sets for GAPDH

(housekeeping gene), IL-10, TNFa and FOXP3. mRNA levels were determined by Real Time Quantitative PCR on an ABI PRISM® 7000 thermal cycler (Perkin Elmer). The following combinations of primers and probes were used: IL-10 Forward 5'GCC TGG CTC AGC ACT GCT AT 3', IL-10 Reverse 5'CGG ATG GAA TGG CCT TTG 3', IL-10 Probe-FAM 5' TTG CCT GCT CTT ACT GGC TGG AGT GAA 3'. TNFα Forward 5'ACA AGG CTG CCC CGA CTA C 3', TNFα Reverse 5'TCC TGG TAT GAA ATG GCA AAC C 3', TNFα Probe-JOE 5'TGC TCC TCA CCC ACA CCG TCA GC 3'. FOXP3 Forward 5'CCA TTG GTT CAC ACG CAT GT 3', FOXP3 Reverse 5'TGG CGG ATG GCA TTC TTC 3', FOXP3 Probe-JOE 5'CGC CTA CTT CAG AAA CCA CCC 3'. GAPDH Forward 5'TGA CTC TAC CCA CGG CAA GTT 3', GAPDH Reverse 5'TTC CCG TTG ATG ACC AGC TT 3', GAPDH Probe-FAM 5'ACG GCA CAG TCA AGG CTG AGA ATG G 3'.

To quantify the amount of mRNA for the different target genes the standard curve method was used.[33] The relative amounts of target gene and GAPDH were quantified by a linear extrapolation of the Ct values using the equation to the line obtained from the standard curve of the respective target genes. Data were normalized for target gene expression, which was obtained by dividing the relative quantity of target gene for each sample divided by the relative quantity of GAPDH for the same sample. The final read outs are expressed as induction index (arbitrary units) defined as stimulated subtracted by reference condition, *i.e.* only media culture.

Statistical analysis

A two tailed, paired t-test was performed to compare clinical scores on day 23 and to compare Area under the arthritis score curve. Kolmogorov-Smirnov Statistics were applied for statistical analysis of FACS histograms.

RESULTS

Combination of epitope specific therapy and a single low dose of etanercept (Enbrel®) has clinical efficacy comparable to full dose etanercept in controlling Adjuvant Arthritis

Lewis rats were immunized with 100 mg Mt to induce AA and randomly divided into 5 treatment groups: i) no treatment; ii) three doses of etanercept s.c., equivalent in this model to a full course of etanercept treatment; iii) one dose of etanercept s.c.; iv) four nasal administrations of HSP60 peptide 180-188 v) combination of one dose of etanercept s.c. followed by four nasal treatments with HSP60 peptide 180-188. Three independent experiments were performed, with 6 rats per treatment group. The lowest effective dose of etanercept was determined in preliminary experiments (not shown). Two different parameters were employed to measure clinical outcomes, in order to ascertain full evaluation of the effects of the various treatment regimens: i) mean arthritis scores on day 23

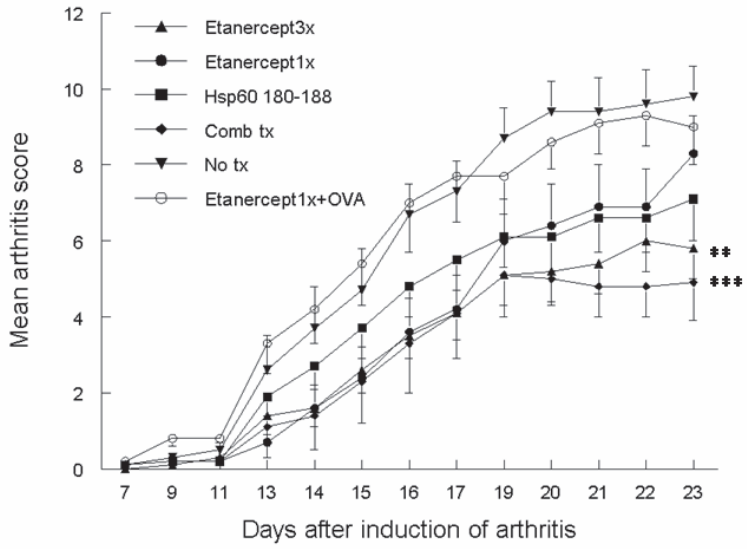


Figure 1 Combination therapy of etanercept with mycobacterial heat shock protein 60 (HSP60) 180-188 led to significant reduction of Adjuvant Arthritis (AA).
Arthritis was induced on day 0 with Complete Freund's Adjuvant (CFA). On day 9, rats were randomly divided into five treatment groups: three doses of etanercept s.c. on day 9, 11, 13 (equivalent to a full course of etanercept treatment); one dose of etanercept on day 9; four doses of mycobacterial HSP60 peptide 180-188 on day 10, 13, 16, 19; combination treatment of one dose of etanercept s.c. on day 9 followed by 180-188 nasally on day 10, 13, 16, 19; or no treatment (PBS). Arthritis scores were assessed every other day from day 8 onward. N=15-18 rats per treatment group. Shown are mean arthritis scores.

(the day of maximum arthritis severity); ii) area under the curve of the corresponding arthritis score curves, thus taking into consideration the whole time course of the treatment.

A significant reduction of AA mean arthritis scores on day 23 (p=0.0004) was achieved with epitope specific and low single dose etanercept combination treatment as well as with a full course of etanercept therapy (p=0.004) compared to no treatment. Similarly, when assessing the areas under the curve (AUC) of the corresponding arthritis score curves, a significant decrease of AA was seen after epitope specific and low single dose etanercept combination treatment (p=0.02 vs. no treatment). Comparable disease control was achieved in the full dose etanercept treatment group (p=0.03 vs. no treatment).

One dose of etanercept alone on day 9 was able to suppress arthritis only temporarily; however, as expected, after day 17 the arthritis revived (Day 23 p=0.3, AUC p=0.1 vs. no treatment). Treatment with HSP60 peptide 180-188 alone showed a trend towards reduction of arthritis, without achievement of statistically significant differences (Day 23 p=0.07,

Table 1 Combination treatment as well as a full course etanercept treatment led to significant reduction of arthritis on day 23, when maximum score of disease is reached, as well as a significant reduction of the area under the arthritis score curve (AUC), representative of the whole treatment period.

Treatment groups	Mean arthritis score on day 23	Arthritis score on day 23 vs. no treatment (p-value)	Area under the curve (AUC)	AUC vs. no treatment (p-value)
Etanercept 3x	5.8	0.004	42.639	0.03
Etanercept 1x	8.3	n.s.	48.858	n.s.
180-188 4x	7.1	n.s.	54.726	n.s.
Etanercept 1x + 180-188 4x	4.9	0.0004	40.396	0.02
Etanercept 1x + OVA 4x	9.0	n.s.	79.597	n.s.
No treatment	9.8		77.748	

Statistical analysis was performed by the paired t-test. Different treatment groups were set out against no treatment.

AUC p=0.26 vs. no treatment). Combination of treatment with an irrelevant peptide, derived from Ovalbumin, and low dose etanercept lacked efficacy in suppressing arthritis, thus confirming the epitope specificity of the treatment (figure 1 and table 1).

Hence, regardless of the outcome parameter employed, combination of epitope specific and low single dose etanercept therapy enabled complete clinical control of the arthritic process to a degree statistically comparable with full dose etanercept, a therapeutic regimen known to fully control AA.

Epitope specific and low single dose etanercept combination therapy leads to a decrease of damage in the hind limb joints

Next we investigated if clinical control of AA with combination therapy was matched in the same treatment groups by a corresponding decrease in joint destruction by the arthritic process. Hind limb joints were collected on day 23 after the induction of arthritis and scored for severity of inflammation in the synovium, pannus formation, cartilage damage, inflammation of the bone marrow and periostal proliferation, with a maximum total score of 20.

Epitope specific and single low dose etanercept combination therapy led to a significant improvement of the histological score in the joints (p=0.014 vs. untreated). Similarly, full course of anti-TNFa therapy led to a significant decrease of histological damage (p=0.001 vs. no treatment). Single dose of etanercept did not lead to significant improvement (p=0.214) (figure 2).

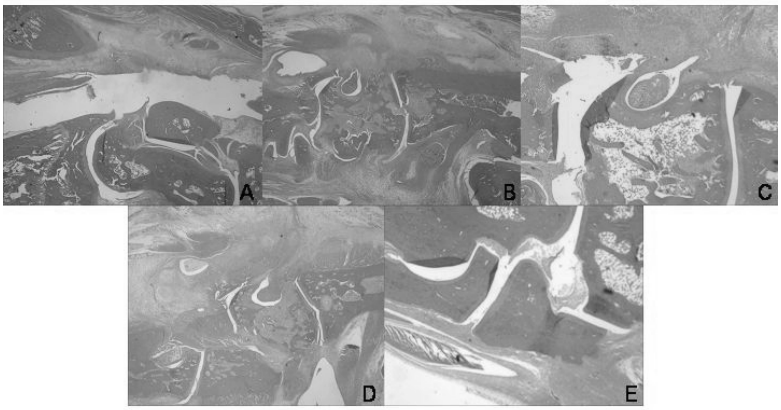


Figure 2 Combination therapy as well as a full course of etanercept treatment led to reduction of histological damage in the ankle joints.
Joints were harvested on day 23 after the induction of arthritis. Formalin-fixed tissues were decalcified, and glass slides stained with H&E were prepared. Submitted tissue sections were examined by light microscopy and scored for severity of inflammation of the synovium, pannus formation, cartilage damage, bone marrow inflammation and periostal proliferation, with a maximum score of 4 per parameter. N=3-4 per treatment group. H&E staining is shown of one rat per treatment, representative for the whole treatment group.
A: combination therapy; B: etanercept 1x; C: peptide Mt. 180-188 4x monotherapy; D: no treatment; E: etanercept 3x.

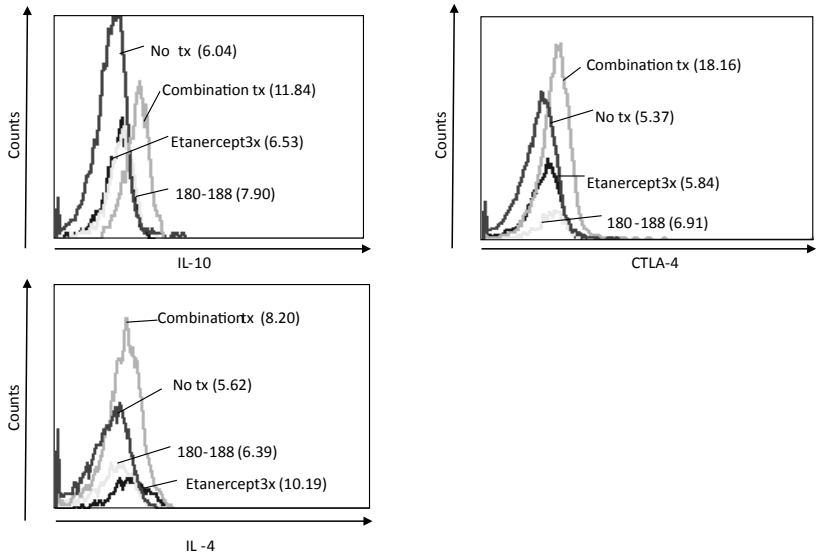


Figure 3 The combination therapy of etanercept and HSP60 180-188 led to an antigen specific increase of IL-10 and IL-4 production and up regulation of CTLA-4 expression in CD4+ T cells in draining Mandibular Lymphnodes (MLN).
MLN were harvested on day 23 after the induction of arthritis. Cells were cultured for 72 hours with medium or antigen. Intracellular production of IL-4, IL-10, and expression of CTLA-4 were measured by FACS. Depicted are Mean Fluorescence Indexes (MFI) of MLN cells cultured with mycobacterial HSP60 peptide 180-188, of cells gated on CD4. Results are representative of one experiment.

Epitope specific and single low dose etanercept combination therapy induces immune deviation of CD4+ T cells

We then analyzed the immune mechanism responsible for the clinical effects of the treatments tested. We focused in this part of our analysis on defining qualitatively CD4+ mediated T cell responses to the inciting antigen. The rationale behind this strategy was to identify qualitative changes in cytokine responses induced by the treatment. To this end, we measured cytokine production and surface marker expression of CD4+ T cells present in the Mandibular Lymphnodes (MLN), the draining site of the nasal mucosa where T cell immune deviation may be induced. MLN were isolated on day 23 after arthritis induction and cultured with HSP60 peptide 180-188 or media. It has to be noted that HSP60 peptide 180-188 is the major immune dominant epitope following induction of AA (due to the presence of mycobacterial HSP65 in CFA) and thus can act as an important surrogate parameter for (immune) therapy in AA.[34,35] After 72 hours viable cells were harvested and stained for surface markers and intracellular cytokines and analyzed by FACS. The results showed differences between treatment groups in the immune mechanisms underlying sometimes comparable clinical efficacy. In fact, only the epitope specific/low dose etanercept combination treatment group showed clear indication of T cell immune deviation, as indicated by the significant difference compared to the untreated group as well as the etanercept groups in the increased production of IL-10 regulatory cytokine (MFI 11.84, isotype control 7.69, $p<0.001$). Expression of CTLA-4, a marker of T cells with regulatory function, also significantly increased when compared both to untreated and etanercept groups (MFI 18.16, isotype control 7.10, $p<0.001$). An increase in IL-4 production was seen after the combination treatment as well as after the full course of etanercept therapy (MFI 8.20 and 10.19, isotype control 6.96, $p<0.001$) (figure 3).

These data are, in our opinion, intriguing as they show differences in underlying immunological mechanism between two equally clinically effective treatments. Indeed, the marked increases in IL-10 production and CTLA-4 expression following combination therapy were both strongly suggestive of restored modulation of T cell function.

Enhancement of CD4+CD25+ regulatory T cell (Treg) function by epitope specific/low dose anticytokine combination therapy

In this part of the project, we addressed the questions on: i) whether certain aspects of regulatory T cell (Treg) function were affected by the combination therapy; ii) whether such induction would affect immune deviation in effector T cells; iii) whether differences in these parameters between full dose etanercept and epitope specific and low single dose etanercept combination therapy could be found.

To this end, we chose to measure by real time PCR (TaqMan) expression of two functional markers of Treg function: IL-10 and FOXP3. FOXP3 is a forkhead transcription factor whose

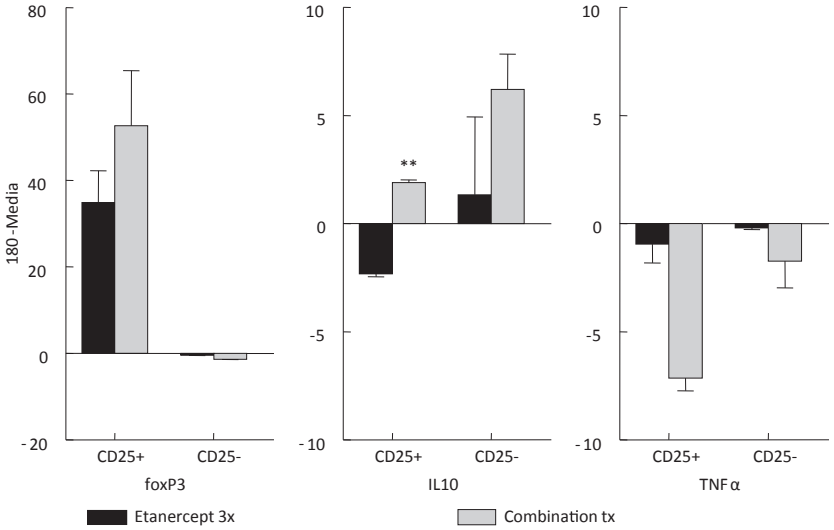


Figure 4 Combination Therapy led to an increase in FOXP3 and IL-10 gene transcription in CD4+CD25+ cells, whereas it also led to an increase in IL-10 transcription in CD4+CD25- cells. TNFa transcription was abolished by the combination therapy as well as by a full course of etanercept treatment.
Results are expressed as the induction index (marker/housekeeping gene GAPDH) of HSP60 peptide 180-188 stimulation subtracted by media alone as measured by Real Time Quantitative PCR. IL-10 production Combination therapy vs. etanercept treatment p=0.002. N= 2-4 per treatment.

expression is deemed crucial for Treg function. IL-10 is considered among the most important soluble mediators for regulatory T cell function. We also measured expression of TNFa, to evaluate if the different therapeutic regimens had a direct effect on the inflammatory response of effector CD4+CD25- cells.

CD4+CD25+ cells were studied, a category of Treg that appears functionally impaired in RA and whose efficiency might not be entirely restored by full dose anti-TNFa treatment.[26] CD4+CD25+ and CD4+CD25- MLN cells were isolated on day 23 after arthritis induction and cultured with HSP60 peptide 180-188 or media. After 15 hours viable cells were harvested, stained for CD4 and CD25 and sorted by FACS. Subsequently mRNA was extracted from sorted CD4+CD25+ and CD4+CD25- cells and levels of FOXP3, IL-10 and TNFa measured by Real Time Quantitative PCR. Figure 4 shows the ratio of the induction index (cytokine/transcription factor divided by housekeeping gene GAPDH) of stimulation with HSP60 peptide 180-188 less the background value.

When FOXP3 expression was measured, a significant increase was found both in the combination and full dose anti-TNFa groups, underscoring likely effects of both therapeutic regimens on some Treg functions, in accordance with recent findings in Rheumatoid

Arthritis patients.[26] Cytokine mediated Treg function however might reportedly not be affected by anti-TNFa therapy, and indeed, when IL-10 expression by CD4+CD25+ cells was measured there was a significant increase (p=0.002) only in the combination therapy group. Interestingly, combination therapy and, to a lesser degree, full dose etanercept, also induced immune deviation of CD4+CD25- effector cells, with higher production of IL-10 consistent with what shown in the FACS analysis. As expected, FOXP3 expression was not induced in CD4+CD25- cells and TNFa expression was abolished by the combination treatment as well as by full dose etanercept.

These data provide evidence for enhancement of Treg function by epitope specific and low dose etanercept combination therapy. Enhanced or restored function of Treg led to immune deviation in effector CD4 cells, with production of IL-10. These immunological changes correlated with the changes in the clinical picture induced by the treatment.

Adoptive Transfer of MLN T cells obtained from animals treated with epitope specific/low dose etanercept combination therapy was able to treat full blown autoimmune arthritis

The purpose of this experiment was to evaluate whether the effects of the combination therapy on T cells could induce clinical amelioration upon adoptive transfer into sick animals. We employed T cells from spleen, Inguinal Lymph nodes (ILN) and MLN after combination treatment with etanercept and 180-188 on day 23 after the induction of arthritis, cultured them for 48 hours with conA and subsequently injected them i.v. into the tail vein of rats one week after arthritis induction with Mt.

Interestingly, only T cells derived from MLN of animals treated with the combination therapy were able to significantly reduce (p=0.0305) clinical symptoms measured as mean arthritis score, when transferred into animals in which disease had been induced (figure 5A). Spleen cells from animals treated with combination therapy transferred to diseased animals failed to exert an efficient control of the disease process (figure 5B) as measured using percent of clinical amelioration. ILN were able to induce a good level of clinical amelioration (figure 5B), however differences with no treatment control group did not reach statistical significance (figure 5A).

Hence, epitope specific mucosal tolerization acts presumably on a population of T cells that resides in the lymph nodes draining the mucosa where the tolerization occurs (MLN) and to a lesser degree ILN. The effect of the treatment on T cells is lasting enough to allow efficient control of the disease process by adoptive transfer in animals in which AA was induced.

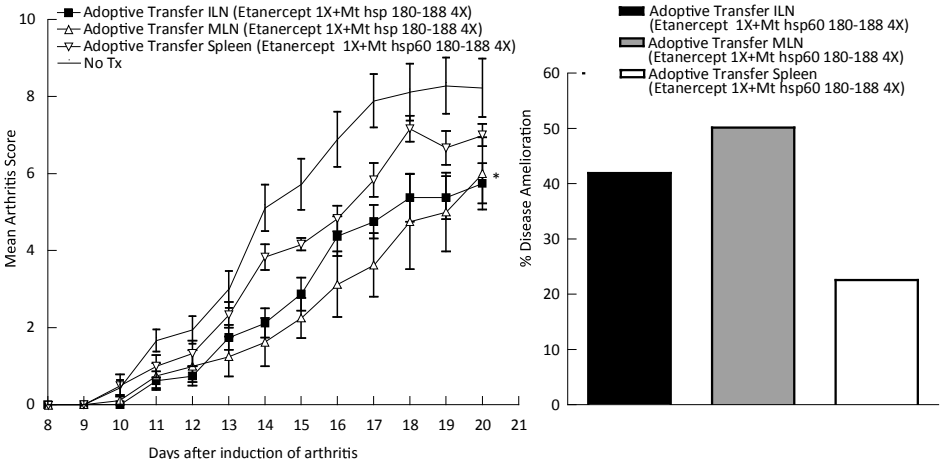


Figure 5 Adoptive Transfer of Mandibular Lymphnode (MLN) T cells from Combination Therapy treated animals led to significant reduction of Adjuvant Arthritis (AA) in diseased animals, measured as Mean Arthritis Score as well as percentage of Disease Amelioration.
A. Adoptive Transfer of T cells from Combination Therapy groups. Adoptive Transfer Groups received $11x10^6$ Inguinal Lymphnode (ILN) cells, $13x10^6$ MLN cells, or $11x10^6$ spleen cells. Data represent Mean $\pm$ SD. Disease induction and scoring was performed as described in the legend to Figure 1.
B. Percentage of Clinical Amelioration for each treatment group in AA rats. The Area Under the Curve (AUC) of each individual treatment group was used to score the Clinical Amelioration (CA) of the distinct treatment groups. AUC was calculated using the curves originated by scoring the disease for the different treatment groups and plotted as percentage of CA with respect to the non-treated group. The non-treated group was considered as having an average percentage of disease = 100%. Formula is as follows: CA = 100 - %AUC.

DISCUSSION

Recent years have witnessed a dramatic progress in our ability to understand mechanisms of autoimmune inflammation and to translate such understanding into novel therapeutic approaches. Particularly remarkable is the success of therapies aimed at interfering with the pro-inflammatory role played by certain cytokines, in particular TNFa. The broadening of clinical applications employing anti-TNFa therapy has led, however, to two interesting developments in clinical immunology, including: i) the recognition of significant generalized immune suppression in treated patients, with a sizable increase in onset or relapse of certain infectious diseases and neoplasias; ii) the need to understand in depth the effects of the treatment on the immune system.

In fact, the effects of anti-TNFα treatment on cytokine production and immunoregulation are still largely unknown and sometimes contradicting. Schotte et al. described the reduction of the number of PBMC producing the pro-inflammatory cytokines TNFa, IFNg and IL-1 after etanercept therapy, whereas the number of IL-10 producing PBMC remained the

same, possibly indicating an immune suppression rather than active immunomodulation due to etanercept.[27] Sieper and colleagues on the other hand investigated the effects of the treatment on the T cell population and postulated that neutralization of peripheral TNFa by etanercept does not lead to a down regulation of the ability to produce TNFa or IFNg by T cells, but rather to an up regulation, possibly due to a counter regulatory mechanism.[28] Ehrenstein et al.[26] found that Treg function in RA is impaired, and that treatment with Infliximab, a monoclonal antibody directed against TNFa, restored it only partially. Namely, Treg mechanisms based on cell-to-cell contact were restored by Infliximab treatment, while Treg mechanisms relying on soluble mediators such as IL-10 remained ineffective despite the treatment. A recent elegant study by Valencia et al. added important insight into the role of TNFa on T regulatory cells. They showed that CD4CD25^{bright} T regulatory cells constitutively express the TNF receptor II. An environment with high levels of circulating TNF led to up regulation of the TNF receptor II, which down regulated both the quantity as well as the quality of FOXP3+ T regulatory cells. Additionally they showed that CD4CD25 ^{bright} cells of patients with active RA expressed high levels of TNF receptor II, reduced levels of FOXP3 and were poor suppressors, which could be reversed by anti- TNFa treatment.[13] These studies, at times contradicting with respect to some mechanisms, underscored that short-term treatment with anti-TNFa may partially restore a more tolerogenic microenvironment, which could be instrumental for the induction of immune tolerance with epitope specific immunotherapy.

Intervention on T cell mediated adaptive immunity would be, in theory, ideal, given the possibility of focusing the approach on one or more possible antigens involved in the disease process, thus sparing the patient generalized immune suppression. Progress is therefore needed in the area of modulation, rather than suppression, of T cells. The most important conceptual development may, however, be the fact that the search for the one inciting and still unidentified antigen should be replaced by approaches targeting mechanisms of control of self-reverberating T cell mediated inflammation. This would realistically shift the focus from etiology to pathogenesis based immune modulation.

A considerable body of evidence, to which we contributed,[21,36,37] supports the concept that peptides derived from heat shock proteins (HSP) may play a role in amplification of autoimmune inflammation. As ubiquitous and bacterial derived products, HSP-derived peptides are in fact perceived as a “danger” signal and elicit a default pro-inflammatory physiologic response. Such response contributes in clearing a possible pathogen invasion but also induces, through cellular stress, increased availability of self-HSP derived peptides. These peptides are recognized by T cells with regulatory function. Such function is impaired in autoimmune arthritis.[17,18]

We have recently reported the results from a Phase I/IIa clinical trial in Rheumatoid Arthritis.[25] The objective of our clinical intervention was to restore natural mechanisms of immune modulation by exploiting the ability of the mucosal route in inducing tolerization to a HSP-derived peptide, which we previously described as part of the pro-inflammatory mechanisms of RA pathogenesis.[21] Interestingly, we were able to induce in treated patients immune deviation from pro-inflammatory to modulatory T cell responses, leading to significant reduction in TNFa and IFNg production and increase in IL-10 and IL-4. These effects were mediated via restoration of function of CD4CD25 ^{bright} Treg, producing IL-10 and expressing FOXP3.[25]

The study reported here addresses the questions on whether epitope specific and anti-cytokine therapy can be complementary, and if such synergy may be advantageous in order to exploit modulation of adaptive immunity while reducing generalized immune suppression, costs and side effects. In order to explore the concept, we chose AA, a T cell, HSP-dependent model of RA, which can be treated with full dose etanercept. We have previously shown in AA that mucosal tolerization to the inciting peptide leads to immune deviation.[31,38]

Combination of epitope specific and anti-cytokine therapy induced full clinical control of AA, to a degree comparable to full dose etanercept and significantly better than the other treatment regimens, including low dose etanercept or epitope specific therapy alone. The comparable clinical efficacy achieved by combination treatment as well as full course etanercept was obtained through distinctly different immune mechanisms in both effector T cells as well as regulatory T cells.

In effector CD4 cells, the combination therapy induced immune deviation while full dose etanercept appeared to be eminently suppressive. Combination therapy led to an increased production of IL-10, which was not found in the other treatment regimens, including full dose etanercept. Both treatments induced suppression of TNFa production and an increase of IL-4 production, which may indicate the presence of a T Helper 2 type tolerogenic mechanism complementing the main effects of the therapy.

Increasing evidence is, however, shifting the focus of modulation of adaptive immunity from effector to regulatory T cells.[39-43] Recent progress in Molecular Immunology has enabled the identification of phenotypical and functional characteristics for these T cells, such as co-expression of CD4, CD25 and CTLA-4, as well as production of certain regulatory cytokines. Several mechanisms of actions have been proposed for Treg, based either on release of cytokines with a tolerogenic function (eminently IL-10), or based on direct cell-to-cell contact by the use of receptors and pathways not yet fully elucidated.[7,39,44-46]

When we sought to analyze the effects of combination therapy and full dose etanercept therapy, it appeared evident that different functions of regulatory T cells were affected by the two treatments. Both treatments significantly increased the expression of the forkhead transcription factor FOXP3, a functional marker of Treg, which act eminently by cell-to-cell contact. A similar observation was described in human RA by Ehrenstein et al.[26] However, CTLA-4 expression and IL-10 production were induced only by combination therapy regimens, and not by anti-TNFa treatment alone, again in agreement with what was found by Ehrenstein. Here we provide evidence that restoration of such function can be achieved by adding epitope specific immunotherapy to low dose etanercept.

Undoubtedly, anti-TNFa therapy creates an environment in which epitope specific immunomodulation can be induced more efficiently. Further evidence for this concept was recently obtained by Bresson et al., who showed that combination of peptide therapy with anti CD3 enhanced the clinical improvement in experimental diabetes compared to anti-inflammatory therapy alone, also through the induction of CD25+FOXP3+ Tregs, as well as insulin specific Tregs producing IL-10 and TGFb.[47]

Recently, it was elegantly shown by Zanin-Zhorov et al, that HSP60 peptides enhance CD4+CD25+ regulatory T cell function via TLR2 signaling,[48] thereby providing an additional explanation for the regulatory effects observed after combination therapy.

Further underscoring the profound immunological differences in mechanisms of action between full dose etanercept and combination therapy, only cells derived from MLN of animals treated with combination therapy were able to control disease when transferred in sick animals. Cells with regulatory properties have been recently described as residing in the MLN.[49]

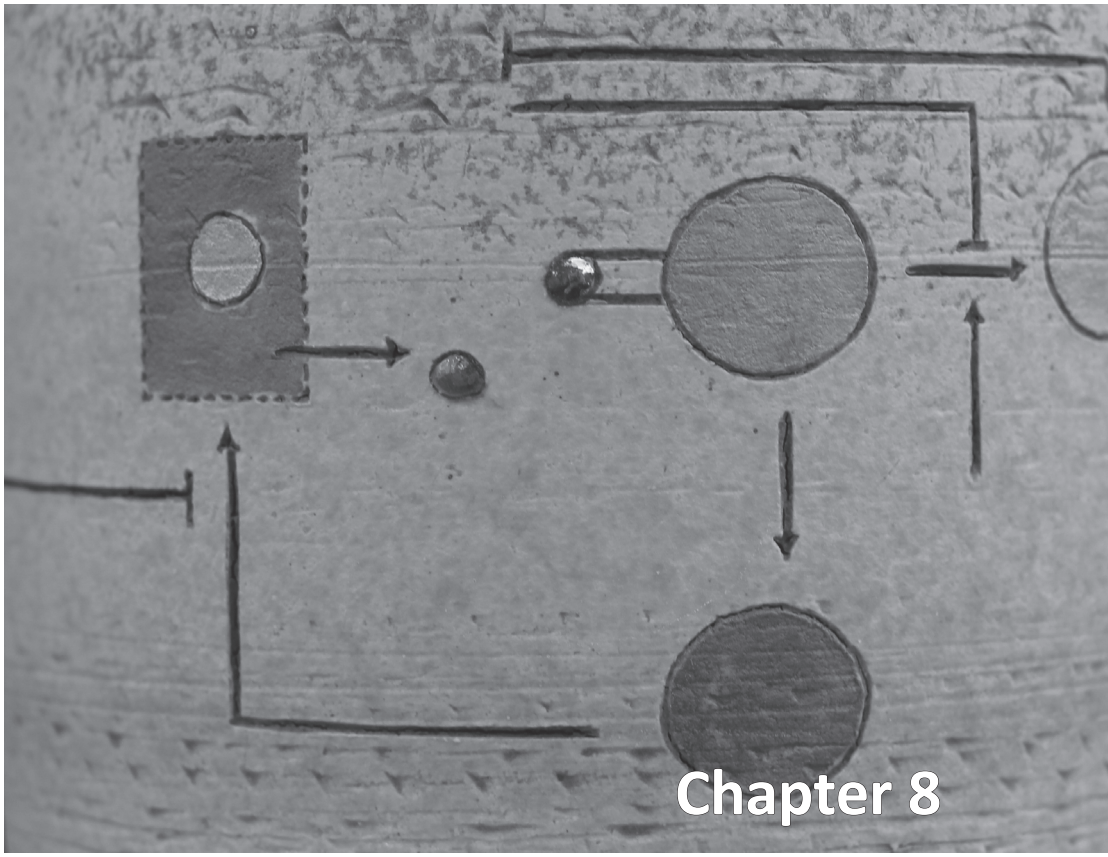
This work lays the foundation for a swift translation of this novel immunotherapeutic concept in human Rheumatoid Arthritis. The implications, should this approach succeed, range from increasing the range of success and utilization of epitope specific immunotherapy, to reducing significantly the costs and undesirable effects of current first generation biologics.

REFERENCE LIST

1. Olsen NJ, Stein CM. New drugs for rheumatoid arthritis. *N Engl J Med.* 2004;350:2167-2179.
2. Taylor PC. Anti-tumor necrosis factor therapies. *Curr Opin Rheumatol.* 2001;13:164-169.
3. Banchereau J, Pascual V, Palucka AK. Autoimmunity through cytokine-induced dendritic cell activation. *Immunity.* 2004;20:539-550.
4. Bloom BJ. Development of diabetes mellitus during etanercept therapy in a child with systemic-onset juvenile rheumatoid arthritis. *Arthritis Rheum.* 2000;43:2606-2608.
5. Dekker L, Armbrust W, Rademaker CM, Prakken B, Kuis W, Wulffraat NM. Safety of anti-TNFalpha therapy in children with juvenile idiopathic arthritis. *Clin Exp Rheumatol.* 2004;22:252-258.
6. Keane J, Gershon S, Wise RP, Mirabile-Levens E, Kasznica J, Schwieterman WD, et al. Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent. *N Engl J Med.* 2001;345:1098-1104.
7. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol.* 2003;3:253-257.
8. Bluestone JA, Boehmer H. Regulatory T cells. *Semin Immunol.* 2006;18:77.
9. Cope AP, Feldmann M. Emerging approaches for the therapy of autoimmune and chronic inflammatory disease. *Curr Opin Immunol.* 2004;16:780-786.
10. Larche M. Peptide immunotherapy. *Immunol Allergy Clin North Am.* 2006;26:321-332.
11. Masteller EL, Tang Q, Bluestone JA. Antigen-specific regulatory T cells -- ex vivo expansion and therapeutic potential. *Semin Immunol.* 2006;18:103-110.
12. Tarzi M, Klunker S, Texier C, Verhoef A, Stapel SO, Akdis CA, et al. Induction of interleukin-10 and suppressor of cytokine signalling-3 gene expression following peptide immunotherapy. *Clin Exp Allergy.* 2006;36:465-474.
13. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood.* 2006;108:253-261.
14. Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J Exp Med.* 2004;199:971-979.
15. Anderson PO, Manzo BA, Sundstedt A, Minaee S, Symonds A, Khalid S, et al. Persistent antigenic stimulation alters the transcription program in T cells, resulting in antigen-specific tolerance. *Eur J Immunol.* 2006;36:1374-1385.
16. Garrood T, Pitzalis C. Targeting the inflamed synovium: the quest for specificity. *Arthritis Rheum.* 2006;54:1055-1060.
17. de Kleer IM, Kamphuis SM, Rijkers GT, Scholtens L, Gordon G, de Jager W, et al. The spontaneous remission of juvenile idiopathic arthritis is characterized by CD30+ T cells directed to human heat-shock protein 60 capable of producing the regulatory cytokine interleukin-10. *Arthritis Rheum.* 2003;48:2001-2010.
18. Prakken AB, van Eden W, Rijkers GT, Kuis W, Toebes EA, Graeff-Meeder ER, et al. Autoreactivity to human heat-shock protein 60 predicts disease remission in oligoarticular juvenile rheumatoid arthritis. *Arthritis Rheum.* 1996;39:1826-1832.
19. van Eden W, van der Zee R, Paul AG, Prakken BJ, Wendling U, Anderton SM, et al. Do heat shock proteins control the balance of T-cell regulation in inflammatory diseases? *Immunol Today.* 1998;19:303-307.
20. Kamphuis S, Kuis W, de Jager W, Teklenburg G, Massa M, Gordon G, et al. Tolerogenic immune responses to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis. *Lancet.* 2005;366:50-56.

21. Albani S, Keystone EC, Nelson JL, Ollier WE, La Cava A, Montemayor AC, et al. Positive selection in autoimmunity: abnormal immune responses to a bacterial dnaJ antigenic determinant in patients with early rheumatoid arthritis. *Nat Med.* 1995;1:448-452.
22. Albani S, Carson DA. A multistep molecular mimicry hypothesis for the pathogenesis of rheumatoid arthritis. *Immunol Today.* 1996;17:466-470.
23. La Cava A, Nelson JL, Ollier WE, MacGregor A, Keystone EC, Thorne JC, et al. Genetic bias in immune responses to a cassette shared by different microorganisms in patients with rheumatoid arthritis. *J Clin Invest.* 1997;100:658-663.
24. Prakken BJ, Carson DA, Albani S. T cell repertoire formation and molecular mimicry in rheumatoid arthritis. *Curr Dir Autoimmun.* 2001;3:51-63.
25. Prakken BJ, Samodal R, Le TD, Giannoni F, Yung GP, Scavulli J, et al. Epitope-specific immunotherapy induces immune deviation of proinflammatory T cells in rheumatoid arthritis. *Proc Natl Acad Sci U S A.* 2004;101:4228-4233.
26. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med.* 2004;200:277-285.
27. Schotte H, Schluter B, Willeke P, Mickholz E, Schorat MA, Domschke W, et al. Long-term treatment with etanercept significantly reduces the number of proinflammatory cytokine-secreting peripheral blood mononuclear cells in patients with rheumatoid arthritis. *Rheumatology (Oxford).* 2004;43:960-964.
28. Zou J, Rudwaleit M, Brandt J, Thiel A, Braun J, Sieper J. Up regulation of the production of tumour necrosis factor alpha and interferon gamma by T cells in ankylosing spondylitis during treatment with etanercept. *Ann Rheum Dis.* 2003;62:561-564.
29. van Eden W, Holoshitz J, Nevo Z, Frenkel A, Klajman A, Cohen IR. Arthritis induced by a T-lymphocyte clone that responds to Mycobacterium tuberculosis and to cartilage proteoglycans. *Proc Natl Acad Sci U S A.* 1985;82:5117-5120.
30. van Eden W, Thole JE, van der Zee R, Noordzij A, van Embden JD, Hensen EJ, et al. Cloning of the mycobacterial epitope recognized by T lymphocytes in adjuvant arthritis. *Nature.* 1988;331:171-173.
31. Prakken BJ, van der Zee R, Anderton SM, van Kooten PJ, Kuis W, van Eden W. Peptide-induced nasal tolerance for a mycobacterial heat shock protein 60 T cell epitope in rats suppresses both adjuvant arthritis and nonmicrobially induced experimental arthritis. *Proc Natl Acad Sci U S A.* 1997;94:3284-3289.
32. Bendele AM, McComb J, Gould T, Frazier J, Chlipala E, Seely J, et al. Effects of PEGylated soluble tumor necrosis factor receptor type I (PEG sTNF-RI) alone and in combination with methotrexate in adjuvant arthritic rats. *Clin Exp Rheumatol.* 1999;17:553-560.
33. Johnson MR, Wang K, Smith JB, Heslin MJ, Diasio RB. Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. *Anal Biochem.* 2000;278:175-184.
34. Prakken BJ, Roord S, Ronaghy A, Wauben M, Albani S, van Eden W. Heat shock protein 60 and adjuvant arthritis: a model for T cell regulation in human arthritis. *Springer Semin Immunopathol.* 2003;25:47-63.
35. Anderton SM, van der Zee R, Prakken B, Noordzij A, van Eden W. Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *J Exp Med.* 1995;181:943-952.
36. Albani S. Infection and molecular mimicry in autoimmune diseases of childhood. *Clin Exp Rheumatol.* 1994;12 Suppl 10:S35-S41.

37. Albani S, Ravelli A, Massa M, De Benedetti F, Andree G, Roudier J, et al. Immune responses to the Escherichia coli dnaJ heat shock protein in juvenile rheumatoid arthritis and their correlation with disease activity. *J Pediatr.* 1994;124:561-565.
38. Prakken BJ, Roord S, van Kooten PJ, Wagenaar JP, van Eden W, Albani S, et al. Inhibition of adjuvant-induced arthritis by interleukin-10-driven regulatory cells induced via nasal administration of a peptide analog of an arthritis-related heat-shock protein 60 T cell epitope. *Arthritis Rheum.* 2002;46:1937-1946.
39. Baecher-Allan C, Viglietta V, Hafler DA. Human CD4+CD25+ regulatory T cells. *Semin Immunol.* 2004;16:89-98.
40. Dieckmann D, Plottner H, Berchtold S, Berger T, Schuler G. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *J Exp Med.* 2001;193:1303-1310.
41. Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *J Exp Med.* 2001;193:1285-1294.
42. Sakaguchi S. The origin of FOXP3-expressing CD4+ regulatory T cells: thymus or periphery. *J Clin Invest.* 2003;112:1310-1312.
43. Walker MR, Kasprowicz DJ, Gersuk VH, Benard A, Van Landeghen M, Buckner JH, et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human CD4+. *J Clin Invest.* 2003;112:1437-1443.
44. Bach JF, Francois BJ. Regulatory T cells under scrutiny. *Nat Rev Immunol.* 2003;3:189-198.
45. Maloy KJ, Powrie F. Regulatory T cells in the control of immune pathology. *Nat Immunol.* 2001;2:816-822.
46. de Kleer I, Vastert B, Klein M, Teklenburg G, Arkesteijn G, Yung GP, et al. Autologous stem cell transplantation for autoimmunity induces immunologic self-tolerance by reprogramming autoreactive T cells and restoring the CD4+CD25+ immune regulatory network. *Blood.* 2006;107:1696-1702.
47. Bresson D, Togher L, Rodrigo E, Chen Y, Bluestone JA, Herold KC, et al. Anti-CD3 and nasal proinsulin combination therapy enhances remission from recent-onset autoimmune diabetes by inducing Tregs. *J Clin Invest.* 2006;116:1371-1381.
48. Zanin-Zhorov A, Cahalon L, Tal G, Margalit R, Lider O, Cohen IR. Heat shock protein 60 enhances CD4 CD25 regulatory T cell function via innate TLR2 signaling. *J Clin Invest.* 2006;116:2022-2032.
49. Nolte-'t Hoen EN, Wagenaar-Hilbers JP, Boot EP, Lin CH, Arkesteijn GJ, van Eden W, et al. Identification of a CD4(+)CD25(+) T cell subset committed in vivo to suppress antigen-specific T cell responses without additional stimulation. *Eur J Immunol.* 2004;34:3016-3027.



Chapter 8

Summary and Discussion

SUMMARY

Vaccination in autoimmunity can have beneficial, but also detrimental effects. Beneficial effects consist of protection against infection or autoimmune disease, whereas detrimental effects could be aggravation of autoimmunity or infection. What determines the outcome of vaccination in autoimmunity depends on multiple factors. In this thesis, we tried to identify factors that contribute to a favourable or an unfavourable outcome of vaccination in Juvenile Idiopathic Arthritis (JIA) and experimental arthritis.

In the first part, we focused on the potential unwanted effects of vaccination against Meningitis type C, such as disease aggravation and the possible lack of immunogenicity in two studies in JIA patients receiving the Meningococcal C vaccine.

In **chapter 2** we describe that no clinical disease aggravation in terms of flare incidence or core set criteria was detected after MenC vaccination in a large cohort of JIA patients. Although geometric mean titers of anti-MenC antibodies were lower in treatment groups receiving immune suppressive medication, functional MenC bactericidal capacity of these antibodies was adequate in all patients, indicating that the protection induced by MenC vaccination was not hampered by immune suppressive medication. These were encouraging results, supporting the positive advice of administration of vaccines to children with JIA. However, the results of **chapter 3** raise a concern for JIA patients with non-remitting disease. The increased pro-inflammatory cytokine profile raised by vaccine specific T cells after vaccination in these patients in combination with an aberrant FOXP3+ regulatory T cell (Treg) response theoretically could hold a risk for aggravating autoimmunity, although this potential clinical effect was not observed in our study.

For the second part of this thesis, we studied the potential protective effects of nasal peptide vaccination against rat adjuvant induced arthritis.

First, we hypothesised that mucosal vaccination with a bystander antigen like heat shock protein (HSP) (peptide immunotherapy) may be able to protect against autoimmune arthritis (reviewed in **chapter 4**). In **chapter 5** we show that mucosal administration of such a bystander peptide derived from bacterial HSP60 protected against experimental arthritis, which was transferable by CD4+ T cells. Skewing of the peptide-specific immune response towards a regulatory phenotype might have played a role, as peptide treatment was associated with a reduction of peptide specific tumour necrosis factor α (TNFα) production by CD4+Tcells and the presence of suppressive CD4+FoxP3+ Treg cells.
Addition of a mucosal adjuvant (CpG) to peptide specific immunotherapy clearly enhanced clinical efficacy in experimental arthritis (**chapter 6**). Co-treatment of p1 with CpG

increased both the number and activation status of plasmacytoid DC in draining MLN, which was accompanied by augmented p1-specific T cell proliferation and IL-10 production. After arthritis induction, p1- and p1/CpG-treated rats showed increased amounts of CD4+FoxP3+ Tregs in the joint draining lymph nodes, which correlated with lower arthritis scores.

In **chapter 7** we moved from a preventive to a therapeutic peptide immunotherapy strategy. The data in this chapter show an improvement in the clinical and immunological effect of nasal HSP peptide immunotherapy in a therapeutic setting. Peptide immunotherapy with a bacterial HSP60 peptide after initiation of adjuvant arthritis was most effective when combined with low dose TNFα receptor blockade (etanercept), reaching the same level of protection as full dose etanercept treatment. Combination treatment led to an increase in peptide specific IL-10 production by CD4+ T cells and an upregulation of FoxP3 gene expression in CD4+CD25+ Treg cells.

These new approaches for more effective peptide immunotherapy could pave the way for a promising future of peptide specific immunotherapy in autoimmune diseases.

DISCUSSION

Part I Vaccination against infectious diseases in chronic arthritis

The two issues addressed in the first part of this thesis were safety and efficacy (immunogenicity) of vaccination in autoimmune disease. As a tool we used vaccination against *N. Meningitides type C* in patients with Juvenile Idiopathic Arthritis (JIA).

Safety of vaccination in autoimmune diseases

Although we did not detect any worsening in flare rate or disease activity of JIA during six months after MenC vaccination in **chapter 2**, the data in **chapter 3** did suggest a potential risk of aggravating inflammation in patients with non-remitting polyarticular JIA (PA-JIA). The failure to raise antigen-induced CD4+FOXP3+ suppressive Tregs after vaccination in combination with the more pro-inflammatory vaccine- and HSP specific T cell responses detected in this patient group could be the result of the pro-inflammatory environment negatively affecting Treg function.[1,2] Whether this pro-inflammatory immune deviation may lead to a potential increased risk of disease aggravation remains unknown as it may not have been detected in our study described in **chapter 2** due to lack of numbers of PA-JIA patients included, but also because presumably multiple hits are necessary for an overt disease flare to occur.

Risk for infection in arthritis patients

Vaccination in patients with autoimmune arthritis to prevent infectious diseases is of importance as this patient group is prone to infections. In patients with JIA, controlled data on the incidence of infectious diseases is largely unavailable, but in rheumatoid arthritis the increased risk of infectious diseases has been estimated as high as approximately double that of the general population, also after adjusting for glucocorticoid use.[3] Although the contributions to this increased susceptibility to infections either by the disease itself or by its treatment are hard to distinguish, there have been observations pre-dating the routine use of glucocorticoid therapy in adult arthritis that indicate a role for the disease itself.[4]
The largest risk factor for infections in JIA seems to be the use of immune suppressive medication. Obviously, for the clearance of invading micro-organisms an adequately functioning immune system is required. This defence system may be hampered in patients with a secondary immunodeficiency due to the use of immune suppressive medication, leading to increased risk of infections. In JIA, some reports mention infections as adverse events of immunosuppressive therapy[5-7], but more detailed evidence comes from research in adults with arthritis, showing a significant increase in the risk of infection during the use of corticosteroids[3] or TNFα receptor inhibitors.[8,9] With the increasing use of biologicals already early in the disease course[10], it can be expected that the risk of infections in this patient group will further raise.

If an infection occurs in an arthritis patient, the infection itself may have more serious sequelae than in others. Children with rheumatic diseases are more likely to experience infections requiring hospitalization, including septicaemia and pneumonia.[11] Moreover, several vaccine preventable diseases are known to negatively affect the course of established autoimmune diseases.[12-14]

Risk factors for autoimmunity after vaccination

As autoimmune diseases are rare and the latency time between the initial disease triggering event and overt autoimmune disease could be long, immunological markers of autoimmunity could be early signs and thereby help to identify patients at risk for the further development of autoimmune diseases. In this regard, the induction or increase of auto-antibodies by vaccines, such as the hepatitis A vaccine, has been studied quite extensively in healthy controls[15,16] and in SLE patients.[17,18] It has long been thought that an increase in auto-antibodies could be a marker for the onset of autoimmune disease. Results are however hard to interpret as it is widely known that auto-antibodies may be present in a certain percentage of apparently healthy subjects.[19] It has even been reasoned that auto-antibodies could be present in every healthy person.[20] Interpretation of transient elevated auto-antibodies is moreover complicated as this could also be related to a preceding infection[21] without clinical consequences.

Another risk factor for aggravation of autoimmunity is administration of the vaccine during active disease. After BCG vaccination, half of the patients with active Kawasaki disease showed local inflammation at the injection site, a reason to withhold BCG vaccination during active Kawasaki disease.[22]

Chapter 3 ads new information on the less well-studied T cell mediated immune responses after vaccination. Of course, the above mentioned limitations could also be applicable to the results described in **chapter 3**. Therefore we argue that vaccinations should not be withheld in PA-JIA patients until the clinical significance of these findings have been elucidated.

Based on current literature, it is possible to identify vaccines with properties that may form an increased risk of inducing or aggravating autoimmune disease.

First, live-attenuated vaccines have the advantage of good protection rates as they reproduce natural infection with the exposure of a large number of immunogenic epitopes. However, this wide array of antigens presented does increase chances for autoimmunity to occur via molecular mimicry and the larger amount of tissue disruption can generate innate stimuli that may further enhance this risk.[12] Moreover, live-attenuated vaccines entail the danger of transmission or persistence of the virus, or the back mutation of the virus into a more virulent strain, that can all result in infection. For these reasons, live-attenuated vaccines are currently contraindicated in patients on high immunosuppressive

medication, explaining why studies of live-attenuated vaccines in these patients are scarce and underpowered. Nevertheless, limited data so far indicate that live-attenuated booster vaccines are safe in patients on regular MTX dosages, low dose glucocorticosteroids and even anti-TNFα therapy.[23,24]

Inactivated vaccines are safer as they do not contain infectious agents, but are less immunogenic and therefore often need adjuvants or booster vaccinations to induce protective immunity entailing other risks.[25] Adjuvants by themselves have been suggested as possible inducers of autoimmunity. Certain adjuvants (e.g. alum) might induce an autoimmune condition named 'the adjuvant diseases' of which macrophage myofasciitis (MMF) is perhaps the best defined.[26] Another group of adjuvants (i.e. pristine, squalene) also seems to be associated with adverse effects such as the induction of SLE and arthritis in genetically susceptible animal models.[27]

Third, if the infectious disease against which is vaccinated has been associated with the induction of autoimmune disease (like arthritis after measles[28], *C. jejuni*, *B. burgdorferi*[29], VZV[30], Yellow fever), the occurrence of self-reactive immune responses through molecular mimicry or bystander activation could also be more likely to occur after the vaccine containing microbial or viral antigens.

In conclusion, administration of live-attenuated vaccines containing adjuvant against infectious diseases that have been associated with autoimmunity theoretically holds risks for inducing or aggravating autoimmunity. However, as high level evidence is lacking, these observations should not lead to the discontinuation of these vaccines in children with autoimmune diseases.[31]

The chance that vaccination induces aggravation of autoimmunity does not outweigh the chance that an infection with more serious effects occurs in children with rheumatic diseases. Especially when taken into account that non-vaccinated children in the USA have a 35 times increased risk of contracting measles in comparison with vaccinated children, despite presumed herd immunity.[32,33] Therefore these findings stress the important task for clinicians to advocate vaccination, especially in patients with increased risk of infectious complications.

Efficacy of vaccination in autoimmune disease

Influence of autoimmune disease on vaccine efficacy

The dysbalanced immune system in autoimmune diseases like arthritis could not only be the cause of increased susceptibility to infections, but also have a negative effect on the generation of protective immunity after vaccination.

Antibody titres in a population can be expressed as geometric mean titre (GMT), as seroconversion rate defined as the proportion of persons with a fourfold or greater increase

in titre, or as seroprotection rate that defines the proportion of persons with an antibody level above a threshold for protection. In **chapter 2**, 2.5% of JIA patients failed to raise adequate vaccine specific antibody titres, as opposed to none of the healthy controls. A large study concerning 1668 patients with autoimmune rheumatic diseases also showed reduced seroprotection rates, seroconversion rates and a lower increase in GMTs in patients with rheumatic diseases as opposed to healthy controls in response to the 2009 non-adjuvanted influenza A/H1N1 vaccine. Interestingly, the reduced B cell response reported in this study could not be explained by the small number of patients (0,8%) using Rituximab, indicating that the disease itself may also account for lower immunogenicity of the vaccine.[34] Active disease during vaccination may also play a role by masking vaccine antigens, impairing the generation of protective immunity.[17,22] Although numbers in **chapter 2** were low, fifty percent of the low responders indeed had active disease during vaccination.

Influence of treatment on vaccine efficacy

In **chapter 2**, we observed significantly lower vaccine specific antibody titres in patient groups on biological and non-biological disease modifying anti rheumatic drugs (DMARDS) like MTX, sulfasalazine and a small proportion of patients receiving anti-TNFα blockade. All low responders detected in this study belonged to these patient groups. The functional bactericidal capacity of these antibodies was however intact. This implies that immune suppressive medication can impair vaccine specific B cell responses after vaccination, but does not affect protection rates.

The conclusions from a recent extensive literature survey of vaccination in juvenile patients with rheumatic disease were that the immunogenicity of vaccines appeared to be good in this patient group, with a few exceptions.[35] High dose glucocorticoids or azathioprine reduced responses to influenza and varicella zoster vaccination, whereas low dose glucocorticosteroids had no detrimental effect on immunogenicity of vaccines or established antibody concentration. Responses to T cell independent pneumococcal polysaccharide vaccine or the varicella zoster vaccine were impaired in patients on high dose methotrexate. Although lower antibody concentrations were often noted by others in adults with rheumatic diseases after vaccination during TNFα blockade,[36-38] also in these studies protection rates were similar, except for a lower rate of protection after vaccination against Hepatitis A. Life attenuated vaccines were generally not administered to patients using TNFα blockers due to lack of safety data.[35] Rituximab, a chimeric monoclonal antibody against CD20, which is primarily found on B cells, had the strongest impact, reducing antibody responses to T cell independent and T cell dependent vaccines (reviewed by [35]).

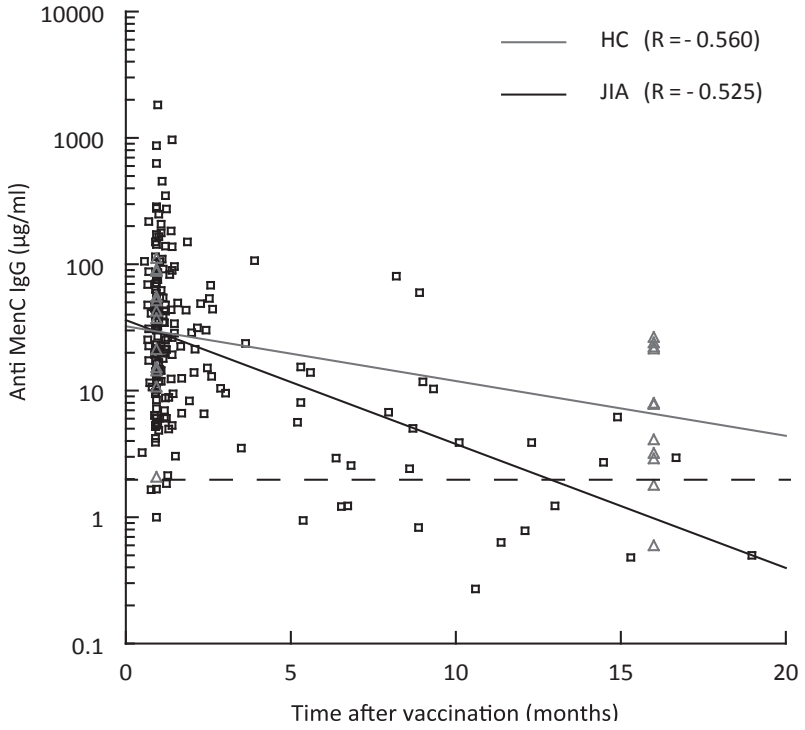


Figure 1 Anti-MenC antibody titres after MenC vaccination in JIA patients (JIA, open squares, o) and healthy controls (HC, open triangles, Δ). Dotted line indicates the generally accepted protective level of anti-MenC antibody, 2 µg/ml).[126]

Although vaccination responses may be adequate shortly after vaccination, persistence of protective antibody titres may be reduced in patients with rheumatic diseases (figure 1). In SLE patients a more rapid decline of measles-specific antibody titres and seroprotection rates was observed compared to healthy controls. This decline may be pathogen specific as in the same patient group, antibody titres against tetanus toxoid did not differ in time from healthy controls.[39] To our knowledge, no data are available regarding this subject in JIA patients.

Recommendations for vaccination of patients with JIA

Recently, recommendations for vaccinations in paediatric patients with rheumatic diseases like JIA have been developed based on the available evidence in literature.[40] The results of our study in **chapter 3** contributed to these recommendations and appeared to be the only data available on safety and efficacy of Meningococcal vaccination in JIA patients.

Because of the presumed increased risk of infections in JIA patients with a more complicated course deducted from studies in adult arthritis, these patients should be offered vaccination according to the national vaccination guidelines, to which annual influenza vaccinations should be added and, in the case of high dose immunosuppressive medication use, vaccinations against encapsulated bacteria and varicella. However, based on current literature, certain precautions should be taken. Inactivated vaccines can be administered safely in JIA patients,[25,40] but live-attenuated vaccines are recommended to withhold in patients on high dose DMARD, high dose glucocorticosteroids or biological agents. Safe opportunities to administer live-attenuated vaccines seem to be either 3-4 weeks before the initiation of high dose immune suppressive therapy or 3 months after discontinuation.[25,41] However, it may not be an option to wait several weeks before initiation of adequate therapy or to discontinue effective medication. In these cases, the risks of infection should be weighed against the hypothetical risk of inducing infection by vaccination.[40]

Pneumococcal or influenza vaccination during Rituximab treatment however results in inadequate immune responses, so that these vaccinations are advised to be offered before initiation of this therapy. When vaccinating during the use of high dose glucocorticosteroids, Rituximab or TNFα blocking agents, vaccine specific antibody concentrations should be measured to evaluate protective immune responses and assess the requirement for booster vaccinations.
Although there is enough evidence to refrain from administration of BCG vaccination during active Kawasaki disease, there are currently no recommendations concerning vaccination in active JIA. In adult arthritis it is advised to postpone vaccinations in the case of severe rheumatic disease or if the vaccine has previously caused a relapse.[42] However for children with chronically active disease in combination with an extensive vaccinations schedule this may not be realistic.

Future perspectives for vaccination against infectious diseases in chronic arthritis

Safety

Ascertaining causality between vaccination and autoimmune disease is difficult due to rarity of autoimmune diseases and their subacute presentation with a latency time varying from days to even years.[43] Therefore the available reports yield unsatisfactory evidence. To be able to answer the question whether vaccinations are truly safe in autoimmune diseases large, controlled international studies are needed on vaccine related onset or aggravation of autoimmunity. The availability of networks within paediatric rheumatology like the Paediatric Rheumatology INternational Trials Organisation (PRINTO), Paediatric Rheumatology Collaborative Study Group (PRCSG) and the Childhood Arthritis and Rheumatology

Alliance (CARRA) can facilitate these multi-centre international clinical trials. Recently, a European collaboration on long-term outcome and pharmacovigilance for biologics used in JIA (PharmaChild) has started. As the monitoring of adverse events like infections due to biologicals is one of the main goals, studies on the efficacy and safety of vaccination in JIA patients using such biologicals will also contribute to the current lack of knowledge on the risk of infections in JIA. Next to these epidemiological data, including internationally validated core set criteria for JIA disease activity,[44] more insight in the mechanism of possible vaccination induced autoimmunity could help identify biomarkers for the identification of patients at risk for aggravation or induction of autoimmune diseases. To facilitate this and other biological studies in childhood arthritis a new international federation was founded: UCAN (Understanding Childhood Arthritis Network). Recently the first UCAN platform for biological studies was set up with support from the Dutch Arthritis Foundation in Utrecht, the Netherlands (www.ucan-u.org).

Monitoring of immunological markers like the quantity and quality of vaccine specific T and B cell responses, but also the generation of cross-reactive immune responses and auto-antibody formation would certainly be of interest. To define relevant cross-reactive auto reactive immune responses is a major challenge. The generation of GBS after influenza vaccination has been contributed to cross-reactive antiGM1 antibodies. In human arthritis however, the disease inducing cross-reactive immune responses are unknown, although heat shock proteins and cartilage proteoglycan seem to play a role.[45,46] Finally, new vaccines, like the vaccine against human papilloma virus, should, after extensive pre registration studies monitoring clinical and serological data, be evaluated by thorough postmarketing surveillance, hopefully revealing more insight in the incidence of autoimmunity or infection after vaccination.

Efficacy

Efficacy of a vaccine is preferably demonstrated by the percentual risk reduction of a clinical significant infection[47], instead of the surrogate markers evaluating B cell generated antibodies like seroconversion rates, titres, binding avidity and bactericidal assays.[48] Although the risk of infections may be increased in JIA patients, the incidence of a particular infection (e.g. meningococcal disease) may however be so low within this relatively small patient group, that the patient number needed for sufficient power of such studies may be unreachable, even with the use of the international collaborations available. Therefore, there is an urgent need for markers that correlate well with disease protection.

Research in JIA patients is needed on the persistence of antibody titres and the possible concomitant risk of infection. Booster vaccinations may be needed if protective immune responses indeed decline more rapidly than in healthy controls.

Part II Vaccination against autoimmunity in chronic arthritis

In the search for safe and effective antigen specific therapy in human arthritis, three important issues needed to be explored: the choice of antigen, the route of administration and peptide immunogenicity. In the second part of this thesis, we used the rat adjuvant induced arthritis model to address these issues.

Antigen

First, we explained why mucosal vaccination with a bystander antigen (peptide immunotherapy) like heat shock protein (HSP) may be able to protect against autoimmune arthritis (**chapter 4**). In **chapter 5** we show that mucosal administration of such a bystander peptide derived from bacterial HSP60 could protect against experimental arthritis, and that this protection was transferable by CD4+ T cells.

An interesting finding from this study was that the self HSP60 peptide p2 (280-294) had no arthritis reducing effect, while the homologous non-self peptide p1 (mycobacterial HSP65 254-268) did. A difference in binding affinity between p1 and p2 does not seem to be the cause as both peptides have the same binding affinity for the rat MHC RT1Bl as predicted by the model designed by Wauben et al.[49] Possibly, the differences in T cell populations induced by the two peptides, account for the different clinical outcome. We showed that cytokine responses of the T cells reactive to p1 differed from the p2 reactive T cell populations. Moreover, self cross-reactivity was induced by p1 but not by p2. Data from Anderton et al. support this explanation. In their study, administration of mycobacterial HSP65 (256-270) (which is a peptide at a two amino acid frame shift from p1), induced protection against experimental arthritis which was not reproduced by the self homologue (rat HSP60 256-270). Like in our study, where p1 treatment induced cross-reactive T cell responses between mammalian and bacterial HSP, protection against arthritis was associated with cross-recognition of the conserved core epitope 256-265 in rat and bacterial HSP.[50]

Route of administration

The dual role of HSPs (pro- and anti-inflammatory) in modulating the immune system is probably regulated by multiple factors (figure 2). The cytokine profile of HSP reactive cells, phase of the immune response or the tissue in which the recognition takes place may determine whether HSP60 autoreactivity is noxious or beneficial.[51-53] T cells induced by mucosal immunisation can thus be directed by the non-inflammatory, tolerogenic tissue towards a tolerogenic response. In **chapter 5**, **6** and **7** we chose the nasal route for peptide administration and showed that it is indeed possible to generate tolerogenic T cell responses via this mucosal route with clinical efficacy. Peptide specific T cell responses were retrieved locally and systemically.

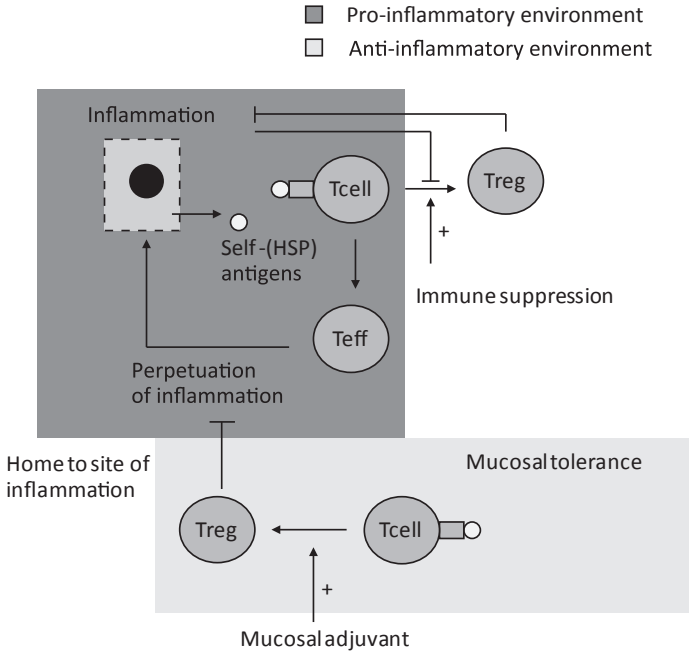


Figure 2 The dual role of HSPs (pro- and anti-inflammatory) in modulating the immune system is influenced by multiple factors. The cytokine profile of HSP reactive cells, phase of the immune response or the tissue in which the recognition takes place may determine whether HSP60 autoreactivity is noxious or beneficial. T cells induced via the mucosal route can thus be directed by the anti-inflammatory environment towards a predominantly tolerogenic response. The mucosally induced antigen specific T cells consist of multiple kinds of regulatory cells and are thought to migrate to the site of inflammation as their cognate antigen (e.g. HSP) is expressed there. At the site of inflammation, these antigen specific Tregs skew the pro-inflammatory T cell response toward an anti-inflammatory phenotype by cytokine release like IL-10 or cell-cell contact.
Mucosal adjuvant could enhance peptide presentation by APCs at the site of tolerance induction, enlarging the pool of Treg formed after mucosal tolerance induction.
The beneficial effect of peptide therapy with heat shock proteins in autoimmune diseases is probably not only due to mucosal tolerance induction to this disease maintaining agent, but also due to the known immunoregulatory properties of self-HSP cross-reactive T cell responses augmented by peptide immune therapy. The inflammatory environment hampers the development of self-HSP specific Tregs. Combination therapy of antigen specific mucosal tolerance induction with immune suppression could therefore reduce systemic inflammation, creating a more favourable environment for the development of these Tregs.

Peptide specific responses in **chapter 5** were however limited to subtle changes in cytokine production, which may be due to the supposed limited peptide immunogenicity when delivered via the mucosal route.[54,55] This brings us to the next issue of peptide immunogenicity.

Peptide immunogenicity

Although HSPs have been associated with the induction of Treg responses,[56] in an inflamed environment, HSP specific T cell responses may be skewed to a more pro inflammatory

profile, especially at the site of inflammation.[53] We therefore had two goals to reach for effective peptide immunotherapy against arthritis by nasal administration of an HSP peptide. First, we aimed to skew the (pro-inflammatory) HSP specific response towards a lesser pro-inflammatory profile, and second, we wanted to boost this regulatory response. For a strong bystander effect induced by the HSP peptide specific T cells, the peptide specific response has to be potent enough for skewing and boosting. Therefore, it is important to generate well-detectable peptide specific responses. Moreover, it would allow monitoring of vaccination efficacy.

Non-self homologue

A solution to the limited recognition of self-epitopes could be the use of microbial homologues of a selected self-antigen. The subtle changes in amino acids in homologous microbial peptides could possibly enhance the immunogenicity of the peptide. Furthermore, as discussed previously, cross-reactivity to self antigens elicited by a non-self epitope may be important in eliciting the required regulating response. Thus, the use of a microbial counterpart of a self epitope could enhance peptide responses and create a possibility for the tolerogenic cross-recognition of self. The increased efficacy of the microbial HSP peptide p1 over the self-peptide p2 in experimental arthritis could therefore, next to the induction of self-cross reactive responses also have been the result of increased peptide recognition (**chapter 5**), underlining the need for adequate peptide immunogenicity.

Adjuvant

The weak induction of antigen specific immune responses observed in **chapter 5** could be the consequence of intrinsic weakness of the mucosal peptide signal alone as it triggers the adaptive immune system only.[54,55,57,58] In a healthy immune system, innate triggering may enhance peptide-triggered adaptive immune responses. Combining an adjuvant triggering innate immunity and a T-cell epitope triggering adaptive immunity was therefore a novel concept worth exploring. The success of adjuvants used in mucosal vaccination strategies for infectious diseases including polio and influenza underscores this notion.[59-64] Although adjuvants have been used in non-mucosal vaccination strategies in autoimmunity such as by intramuscular injection [65], the idea of enhancing mucosal vaccination with an adjuvant for preventive peptide therapy in autoimmune disease was rather new. Prerequisites for such an innate triggering mucosal adjuvant were applicability at mucosal sites, activation of antigen presenting cells (APCs) and preferably shifting toward Treg responses to help the peptide in restoring the lost immune balance in autoimmunity. The activated APCs could in this way present the peptide more efficiently and enlarge the beneficial effect of nasal peptide treatment.

Unmethylated CpG dinucleotides are effective mucosal adjuvants that, when administered systemically, can trigger the innate immune system by activation of antigen presenting cells via TLR9 on dendritic cells and B cells. The resulting T cell response has been shown to be Th1 mediated.[66,67] CpG was indeed initially considered an enhancer of cytotoxic lymphocyte responses in cancer therapy upon intra- or peritumoral injection.[68-72] CpG as an adjuvant has been shown to augment peptide specific responses in humans after subcutaneous vaccination against infectious diseases like pneumococcal disease or influenza[73,74] and is effective in the treatment of a Th2 mediated disease like allergy.[75] In this perspective, administration of CpG could theoretically lead to the exacerbation of Th1 mediated autoimmune diseases.
However, both experimental and human data suggest that in autoimmune diseases, bacterial TLR9 stimulation can skew towards a regulatory response, possibly down regulating autoimmune disease. A study in TLR9-/- mice showed that TLR9 down regulates disease activity in a myelin oligodendrocyte glycoprotein (MOG)-induced EAE mouse model.[76] In line with these results, intraperitoneal or intramuscular vaccination with CpG sequences attenuated disease in two models of autoimmune diabetes.[77,78] CpG has been shown to play a role in the induction of Tregs in human cells and in animal models of colitis.[79-81] For the induction of T cells with regulatory capacity, mucosal plasmacytoid dendritic cells (pDC) have shown to be required.[82] Co-administration of CpG and allergen via the nasal route and not the intradermal route has been shown to be effective in reducing allergic disease in mice.[83] Mucosal administration of CpG in the genital tract could also enhance protection against type 2 herpes simplex virus infection, indicating the adjuvant activity is still intact via this route.[84] Based on these data, we hypothesized that mucosal co-administration of CpG may enlarge the efficacy of peptide immunotherapy in arthritis by enhancement of peptide presentation (leading to skewing and boosting of the HSP peptide specific T cell response) and its potency to skew towards a (non antigen-specific) regulatory immune response.

The study presented in **chapter 6** is to our knowledge the first study described in literature in which CpG is tested as a mucosal adjuvant for peptide specific therapy in a model for an autoimmune disease. In this study we show that addition of CpG to HSP peptide specific immunotherapy indeed clearly enhanced clinical efficacy in the rat adjuvant arthritis model, which seems to be a solution for the limited immunogenicity of mucosally administered peptides. The induction of Tregs seemed to play a role as p1 specific IL-10 production was induced at the site of treatment as well as the site of inflammation. Moreover, p1- and p1/CpG-treated rats showed a higher amount of CD4+FoxP3+ Tregs in the joint draining lymph nodes, which correlated with lower arthritis scores.

In **chapter 6**, we also describe that nasal co-treatment of p1 with CpG increased the number and activation status of plasmacytoid DCs in draining mandibular lymph nodes, with enhancement of p1-specific T cell proliferation and a counter-intuitive induction of p1 specific interferon-γ (IFNγ) production. This combination of reduced disease activity after CpG containing treatment with increased amounts of IFNγ and activated mucosal pDC, may suggest involvement of the indoleamine 2,3 dioxygenase (IDO) pathway. IDO participates in a natural immunoregulatory mechanism that creates potent and dominant T cell suppression at sites of inflammation.[85] The administered TLR9 ligand CpG in the study described in **chapter 6** may have stimulated mucosal pDC (as shown by increased number and activity of mucosal pDC), leading to the detected production of IFNα and IFNγ. The theoretically ensuing upregulation of IDO in pDC could be the missing link that could explain the therapeutic efficacy of CpG. The next step will be to evaluate the role of IDO in our system.
Translation of these results to human autoimmune disease could be hampered by acquired resistance to IDO mediated inhibition by T cells, as shown in RA patients.[86] and T1D[87] Also in SLE, elevated levels of IDO activity have been measured prior to worsening of disease, indicating that naturally induced IDO may be insufficient to overcome autoimmunity.[88] More research is needed to evaluate the role of IDO in TLR9 enhanced peptide immunotherapy and if so, identify the factors that cause the resistance to IDO mediated inhibition in human autoimmune disease. Possibly, immune suppressive therapy prior to and during peptide/CpG administration could be beneficial.

Increased efficacy of antigen specific therapy may be reached by ascertaining delivery of the antigen and CpG to the same APC. Following stimulation with CpG DNA, TLR9 and DNA have been found in the same endocytic vesicles,[89] suggesting that CpG conjugation to the peptide may facilitate antigen uptake in the cell. This would allow activation of the same APC that presents the peptide to T cells thereby enhancing peptide recognition and therapeutic efficacy. In cancer research it has indeed been shown that antigen/CpG co-localization is directly correlated with antigen presentation by DCs and protective antitumor immunity.[90] Also in a mouse model of allergic disease, conjugation of an allergen (Amb-a-1) with CpG (AIC) was significantly more effective than administration of the unlinked compounds.[91] The first results in humans show a possible long-lasting effect of short course subcutaneous immunotherapy with AIC. In rhinitis patients, immune responses of the nasal mucosa to subsequent allergen challenge were modified by AIC, even in the second ragweed season following immunotherapy.[92] Thus, although no results have been described in human autoimmune diseases yet, CpG conjugation to the peptide might enhance peptide immunogenicity and thereby the efficacy of antigen specific immunotherapy in these patients as well.

Safety of (adjuvant enhanced) peptide specific immunotherapy

The omnipresence of HSPs during stress and inflammation might also implicate that manipulating HSP-specific responses with antigen specific immunotherapy could have widespread consequences. In this line of thinking, generating tolerogenic immune responses to HSP might conceptually lead to general immune suppression with for example defective responses to infection and impaired tumour surveillance. The more T cell clones would be deviated by antigen specific therapy, the larger this risk. To limit this theoretical risk, a single HSP peptide was used in our studies. The use of a single epitope probably limits the strength of therapeutic efficacy but increases safety as immune responses against whole HSP protein (as shown in **chapter 5**) and bacterial or viral agents remain intact. Moreover, the bystander suppression generated by peptide specific immunotherapy is presumably transient as it is cytokine mediated (**chapter 5**). The effect will therefore last as long as the peptide is present, but will disappear as soon as peptide therapy is discontinued.

In fact, the great advantage of mucosal tolerance induction is its safety, as illustrated by the many studies performed in experimental and clinical settings without any side effects. The major problem is actually on the other side: the limited efficacy of mucosal peptide immunotherapy. To improve peptide recognition at the mucosal site, adjuvants are needed. The use of adjuvants however, brings about more risks.

First, toxicity to the central nervous system (CNS) is a concern in the development of nasal adjuvants as described in vaccines against infectious diseases.[93] A correlation was found between nasal influenza vaccination containing *E. Coli* enterotoxin as an adjuvant and a higher incidence of facial paresis.[94] Cholera toxin (CT) and *E.coli* enterotoxin are powerful adjuvants that can enter the CNS upon nasal administration. After nasal administration in mice, both toxins have been retrieved in the olfactory neurons and the olfactory bulbs together with signs of inflammation of the meninges.[95,96] These effects have not been described for CpG ODN as an adjuvant. A safer option for nasal antigen administration could be the oral route, but, as it is a less efficient route, this would require large quantities of antigens and adjuvants with greater risk of unwanted side effects. Sublingual immunization can be an interesting option, as it shows significant efficacy and safety.[97] Since dendritic cells seem to be necessary for effective sublingual immunization,[98] CpG could be an interesting adjuvant for peptides delivered via this route.

Second, CpG administration in a non-inflammatory environment, as we did in **chapter 6** using a preventive regimen, was safe in rats. However, administration of CpG in a pro-inflammatory environment, which would be the case in ongoing human autoimmunity, could hold the risk of aggravation of autoimmunity or onset of new inflammation. CpG have been shown to activate autoreactive encephalitogenic T cells in vivo, suggesting that

CpG could potentially induce autoimmune disease in a susceptible individual.[99] Any dose reduction in CpG achieved, for example by conjugation of CpG with a peptide will also reduce the potential risk of induction of autoimmune disease or inflammatory responses.[100] Although CpG as an adjuvant resulted in therapeutic efficiency in **chapter 6**, other innate stimulating agents could also be attractive adjuvants for mucosal peptide specific immunotherapy. In diabetes, alum and the non-toxin B subunit of the cholera enterotoxin from Vibrio Cholerae (CTB) have shown promising results as adjuvants to the disease related peptide GAD65 in human and experimental diabetes respectively (reviewed by [101,102]) CTB seems to be the most promising of the two as it markedly enhances antigen specific therapy in various other experimental autoimmune diseases like EAE, CIA and uveitis, even in already sensitized animals.[103-105] The first support of safety and efficacy of an oral vaccine, comprised of a disease associated selfHSP60 peptide conjugated to CTB, has been established by a small phase 1-2 trial in patients with Behcet disease.[106] Whether alum and CTB are safe upon nasal administration remains to be elucidated.

Combination therapy

Although we now seem to be able to skew autoaggressive T cells towards a less pro-inflammatory profile by antigen specific therapy (**chapter 5**-**7**), and we have indications that the induction of Treg play a role in the efficacy of peptide immunotherapy, the induction and functionality of Treg generated may be hampered by the pro-inflammatory environment that is present during overt autoimmune disease. Widespread inflammation could not only mask the peptide signal, but has also been described to impede the generation and adequate functioning of Tregs or to render activated T cells insusceptible for suppression by Treg.[2] IL-6, IL-7 and IL-15 can indeed interfere with Treg function [107,108] and can convert Treg into IL-17 producing Th17 cells.[2,109,110] Moreover, an environment with high levels of circulating TNFα leads to upregulation of the TNFIIR on Treg, which down regulates both the quality and the quantity of FOXP3+Treg cells, abolishing suppression.[1] This is of importance as human autoimmune diseases are already characterized by an ongoing non-specific inflammatory process even at the time of diagnosis, as epitope spreading generally takes place before symptoms occur.[111]

Like general (innate) immune suppressive therapy cannot be discontinued because the autoaggressive T cell response remains and revives inflammation as soon as aspecific therapy is stopped, skewing of the adaptive immune response alone may also be insufficient unless the inflammation sustained by the innate immune system is dampened. Combining antigen specific therapy targeting the adaptive immune system with general suppression of (innate) inflammation therefore seems preferable. One could compare this strategy with treating autoimmunity at an earlier time point in disease, when wide spread inflammation is not present yet. In type 1 diabetes (T1D) and multiple sclerosis

(MS) efficacy of antigen-specific therapy in phase II trials was indeed most striking when the drug was administered after recent onset.[112] Unfortunately, very little early markers of autoimmune disease in humans are currently available in the clinic, limiting early intervention mostly to time of diagnosis.
By the use of combination therapy, two goals are pursued. The first goal would be to lower the need for general immune suppressive therapy, thereby reducing side effects of this life-long therapy. Second, immune suppressive therapy could artificially recreate the immunological environment that was present before initiation of autoimmunity, thereby creating an environment in which peptide specific therapy can sustain disease remission.

Candidate immune modulators/suppressors that could create the favourable environment for antigen specific immunotherapy are anti-CD3 and TNFα blocking agents. Anti-CD3 monotherapy has been shown effective in various models of autoimmune disease (reviewed in [113]) by selective anergy and apoptosis of T effector cells and induction of Tregs.[114] Combination therapies of anti-CD3 with proinsulin peptide or GAD65 have been shown to be successful in experimental models of new onset type I diabetes, with increased efficacy compared to peptide or anti-CD3 monotherapy.
TNFα blocking agents are currently the most widely used cytokine blocking drugs in JIA, because of their high efficacy. Previous work has shown that TNFα may be a particularly interesting target for combination therapy. Several studies have indicated that next to immune suppression, anti TNFα therapy improves Treg function and numbers in RA patients,[1,115,116] and shifts T cells toward a more anti-inflammatory profile.[117] Anti-TNFα therapy reduces the number of autoreactive T cells in experimental diabetes[118] and increases the frequency of FOXP3+ T cells in children with Crohns Disease.[119] In **chapter 7** we show that nasal peptide therapy combined with low dose TNFα blockade (etanercept) was equally effective as a full dose etanercept treatment. Combination treatment led to an increase in peptide specific IL-10 production by CD4+ T cells and an upregulation of FoxP3 gene expression in CD4+CD25+ Treg cells. The combination of anti-cytokine and peptide specific therapy may therefore act complementarily and synergistically, thereby enhancing efficacy.

Future perspectives for vaccination against autoimmunity in arthritis

An experimental model for arthritis for future research
Although the adjuvant induced arthritis model has many advantages as discussed in **chapter 1**, it also has shortcomings. The disease inducing antigen *M. tuberculosis* contains mHSP65, which could hamper the interpretation of efficacy of HSP peptide immunotherapy in this model. However, as described in **chapter 5**, CFA administration alone did not result in p1 recognition, indicating that this peptide is not presented during disease induction,

which agrees with earlier observations that most prominent T cell responses after CFA are directed towards mHSP65 180–188, the disease-inducing epitope.[120] Another shortcoming could be that the monophasic disease course fails to represent the relapsing remitting nature of human arthritis and the disease is primary T cell mediated, while human arthritis is both B and T cell mediated.

Proteoglycan Induced Arthritis (PGIA) in retired BALB/C mice is induced by human cartilage PG in the adjuvant DDA, has a chronic relapsing remitting disease course and is B and T cell mediated.[121,122] PGIA could therefore be a useful model for future studies needed to unravel remaining questions in HSP peptide specific therapy for autoimmune arthritis.

Bench-bedside-bench

The studies presented in this thesis are the result of a true translational effort that preceded these data: from bench/model (description of the role of HSP60 in adjuvant arthritis [50,123]) to bedside (definition of bystander epitopes of HSP60 in JIA and RA [46,124]). In part II of this thesis, we went back to bench/model again (testing the defined HSP60 epitopes in the adjuvant arthritis model). Especially this step is often difficult to make. It was possible in this case because of the highly conserved nature of HSP and the extensive characterization of the role of HSP60 in both experimental arthritis and humans. The findings in these studies underscore the value of such a translational approach as they provide the basis for the final step: namely back again to patients, this time for therapy.

Translation of peptide specific immunotherapy into the clinic

We showed that nasal administration of HSP peptides reduces experimental arthritis and we explored ways to improve peptide immunogenicity by addition of an adjuvant or shutting down inflammation by combination therapy with cytokine blockade. Before these results can be translated into a clinical trial testing peptide specific immunotherapy in human arthritis, several issues remain to be resolved.

First, for the further development of safe and effective mucosal peptide immunotherapy it is of great importance that further research will be done concerning the safety of mucosal adjuvants.

Second, as dosage of antigen is of importance for oral tolerance to be effective,[125] dose finding studies are needed to define the optimal dose of peptide needed in humans.

Third, patient selection in terms of presence and quality of the peptide response before the start of treatment may enhance the success rate of peptide immunotherapy.

Proposal for future clinical trial

For the design of a future clinical trial it should be realized that although immune suppression is beneficial for peptide recognition by dampening the background noise of inflammation it could also hamper the generation of an adequate immune response to the

peptide if administered simultaneously. Combination therapy with immune suppressive medication could therefore theoretically be optimised in a trial by a sequential regimen. Patients in such a trial would start with a primary phase of induction of disease remission by general immune suppression followed by a secondary phase of peptide administration in which the immune suppressive medication is discontinued. This regimen would aim for sustained disease remission by peptide specific immunotherapy after initial down regulation of pro-inflammatory responses by general immune modulation.

As several concerns remain considering the safety of the use of mucosal adjuvants, there is a need for further development of safe and effective mucosal vaccines before administration to human patients can be considered.

Translation of combination therapy into clinical trials seems to be more within reach, although also here obstacles remain. Collaboration between companies is needed for a successful combination therapy. It is imaginable however, that the pharmaceutical industry that produces cytokine blockers will be reluctant to support research that will lower the need for their product. Still, the effectiveness of combination therapy is worth exploring, while the possibility of lowering the dose of anti-inflammatory treatment will have great impact on patient care, since it reduces the severe side effects that can occur with lifelong drug administration.

CONCLUSION

In this thesis we combined clinical and experimental studies for a better insight in the detrimental and beneficial effects of vaccination in autoimmunity. We were able to assess clinical and immunological safety and efficacy of Meningococcal vaccination in a large group of simultaneously vaccinated JIA patients, identifying a subgroup of patients with immunological risk factors for disease aggravation by vaccination.
Further research is needed on safety and efficacy of vaccination against infectious diseases in JIA. By making advantage of international collaborations, large multicentre studies could be generated with sufficient power to detect rare adverse events. Reliable outcome measures should be used that are directly related to protection against infectious disease.

Using the rat adjuvant arthritis model, we showed that bystander peptides derived from heat shock protein protected against arthritis and explored the immunological mechanisms. It was possible to improve the arthritis protective effect by addition of a mucosal adjuvant and therapeutic efficacy could be improved when combined with low dose cytokine blockade.

Peptide immunotherapy with bystander antigens such as HSP thus shows promising results in the experimental model used for this thesis. New approaches for enhanced peptide recognition such as combination treatment with adjuvants or generalized immune suppression hold promise for a successful future for directed peptide specific immunotherapy for autoimmune diseases (figure 2).

Although some obstacles remain, peptide specific immune therapy with HSP60 epitopes against autoimmunity is ready to be translated to JIA patients, hopefully creating opportunities to lower the need for immune suppressive therapy to sustain disease remission.

REFERENCE LIST

1. Valencia X, Stephens G, Goldbach-Mansky R, Wilson M, Shevach EM, Lipsky PE. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood.* 2006;108:253-261.
2. Wehrens EJ, Mijnheer G, Duurland CL, Klein M, Meerding J, van Loosdregt J, et al. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. *Blood.* 2011.
3. Doran MF, Crowson CS, Pond GR, O'Fallon WM, Gabriel SE. Frequency of infection in patients with rheumatoid arthritis compared with controls: a population-based study. *Arthritis Rheum.* 2002;46:2287-2293.
4. Cobb S, Anderson F, Bauer W. Length of life and cause of death in rheumatoid arthritis. *N Engl J Med.* 1953;249:553-556.
5. Hashkes PJ, Laxer RM. Medical treatment of juvenile idiopathic arthritis. *JAMA.* 2005;294:1671-1684.
6. Murray KJ, Lovell DJ. Advanced therapy for juvenile arthritis. *Best Pract Res Clin Rheumatol.* 2002;16:361-378.
7. Silverman E, Mouy R, Spiegel L, Jung LK, Saurenmann RK, Lahdenne P, et al. Leflunomide or methotrexate for juvenile rheumatoid arthritis. *N Engl J Med.* 2005;352:1655-1666.
8. Salliot C, Gossec L, Ruyssen-Witrand A, Luc M, Duclos M, Guignard S, et al. Infections during tumour necrosis factor-alpha blocker therapy for rheumatic diseases in daily practice: a systematic retrospective study of 709 patients. *Rheumatology (Oxford).* 2007;46:327-334.
9. Furst DE. The risk of infections with biologic therapies for rheumatoid arthritis. *Semin Arthritis Rheum.* 2010;39:327-346.
10. Beukelman T, Patkar NM, Saag KG, Tolleson-Rinehart S, Cron RQ, DeWitt EM, et al. 2011 American College of Rheumatology recommendations for the treatment of juvenile idiopathic arthritis: initiation and safety monitoring of therapeutic agents for the treatment of arthritis and systemic features. *Arthritis Care Res (Hoboken).* 2011;63:465-482.
11. Atzeni F, Bendtzen K, Bobbio-Pallavicini F, Conti F, Cutolo M, Montecucco C, et al. Infections and treatment of patients with rheumatic diseases. *Clin Exp Rheumatol.* 2008;26:S67-S73.
12. Wraith DC, Goldman M, Lambert PH. Vaccination and autoimmune disease: what is the evidence? *Lancet.* 2003;362:1659-1666.
13. Lehmann HC, Hartung HP, Kieseier BC, Hughes RA. Guillain-Barre syndrome after exposure to influenza virus. *Lancet Infect Dis.* 2010;10:643-651.
14. Mantadakis E, Farmaki E, Buchanan GR. Thrombocytopenic purpura after measles-mumps-rubella vaccination: a systematic review of the literature and guidance for management. *J Pediatr.* 2010;156:623-628.
15. Toplak N, Avcin T. Vaccination of healthy subjects and autoantibodies: from mice through dogs to humans. *Lupus.* 2009;18:1186-1191.
16. Karali Z, Basaranoglu ST, Karali Y, Oral B, Kilic SS. Autoimmunity and hepatitis A vaccine in children. *J Investig Allergol Clin Immunol.* 2011;21:389-393.
17. Lu CC, Wang YC, Lai JH, Lee TS, Lin HT, Chang DM. A/H1N1 influenza vaccination in patients with systemic lupus erythematosus: safety and immunity. *Vaccine.* 2011;29:444-450.
18. Wiesik-Szewczyk E, Romanowska M, Mielnik P, Chwalinska-Sadowska H, Brydak LB, Olesinska M, et al. Anti-influenza vaccination in systemic lupus erythematosus patients: an analysis of specific humoral response and vaccination safety. *Clin Rheumatol.* 2010;29:605-613.

19. Hilario MO, Len CA, Roja SC, Terreri MT, Almeida G, Andrade LE. Frequency of antinuclear antibodies in healthy children and adolescents. *Clin Pediatr (Phila).* 2004;43:637-642.
20. Cohen IR. Peptide therapy for Type I diabetes: the immunological homunculus and the rationale for vaccination. *Diabetologia.* 2002;45:1468-1474.
21. Avcin T, Toplak N. Antiphospholipid antibodies in response to infection. *Curr Rheumatol Rep.* 2007;9:212-218.
22. Uehara R, Igarashi H, Yashiro M, Nakamura Y, Yanagawa H. Kawasaki disease patients with redness or crust formation at the Bacille Calmette-Guerin inoculation site. *Pediatr Infect Dis J.* 2010;29:430-433.
23. Heijstek MW, Pileggi GC, Zonneveld-Huijssoon E, Armbrust W, Hoppenreijs EP, Uiterwaal CS, et al. Safety of measles, mumps and rubella vaccination in juvenile idiopathic arthritis. *Ann Rheum Dis.* 2007;66:1384-1387.
24. Borte S, Liebert UG, Borte M, Sack U. Efficacy of measles, mumps and rubella revaccination in children with juvenile idiopathic arthritis treated with methotrexate and etanercept. *Rheumatology (Oxford).* 2009;48:144-148.
25. Rahier JF, Moutschen M, Van Gompel A, Van Ranst M, Louis E, Segaert S, et al. Vaccinations in patients with immune-mediated inflammatory diseases. *Rheumatology (Oxford).* 2010;49:1815-1827.
26. Israeli E, Agmon-Levin N, Blank M, Shoenfeld Y. Adjuvants and autoimmunity. *Lupus.* 2009;18:1217-1225.
27. Reeves WH, Lee PY, Weinstein JS, Satoh M, Lu L. Induction of autoimmunity by pristane and other naturally occurring hydrocarbons. *Trends Immunol.* 2009;30:455-464.
28. Rosenau BJ, Schur PH. Association of measles virus with rheumatoid arthritis. *J Rheumatol.* 2009;36:893-897.
29. Regner M, Lambert PH. Autoimmunity through infection or immunization? *Nat Immunol.* 2001;2:185-188.
30. Pileggi GS, de Souza CB, Ferriani VP. Safety and immunogenicity of varicella vaccine in patients with juvenile rheumatic diseases receiving methotrexate and corticosteroids. *Arthritis Care Res (Hoboken).* 2010;62:1034-1039.
31. Salemi S, D'Amelio R. Could autoimmunity be induced by vaccination? *Int Rev Immunol.* 2010;29:247-269.
32. Omer SB, Salmon DA, Orenstein WA, deHart MP, Halsey N. Vaccine refusal, mandatory immunization, and the risks of vaccine-preventable diseases. *N Engl J Med.* 2009;360:1981-1988.
33. Salmon DA, Haber M, Gangarosa EJ, Phillips L, Smith NJ, Chen RT. Health consequences of religious and philosophical exemptions from immunization laws: individual and societal risk of measles. *JAMA.* 1999;282:47-53.
34. Saad CG, Borba EF, Aikawa NE, Silva CA, Pereira RM, Calich AL, et al. Immunogenicity and safety of the 2009 non-adjuvanted influenza A/H1N1 vaccine in a large cohort of autoimmune rheumatic diseases. *Ann Rheum Dis.* 2011;70:1068-1073.
35. Heijstek MW, Ott de Bruin LM, Borrow R, van der Klis F, Kone-Paut I, Fasth A, et al. Vaccination in paediatric patients with auto-immune rheumatic diseases: A systemic literature review for the European League against Rheumatism evidence-based recommendations. *Autoimmun Rev.* 2011.
36. Farmaki E, Kanakoudi-Tsakalidou F, Spoulou V, Trachana M, Pratsidou-Gertsi P, Tritsoni M, et al. The effect of anti-TNF treatment on the immunogenicity and safety of the 7-valent conjugate pneumococcal vaccine in children with juvenile idiopathic arthritis. *Vaccine.* 2010;28:5109-5113.
37. Erguven M, Kaya B, Hamzah OY, Tufan F. Evaluation of immune response to hepatitis A vaccination and vaccine safety in juvenile idiopathic arthritis. *J Chin Med Assoc.* 2011;74:205-208.

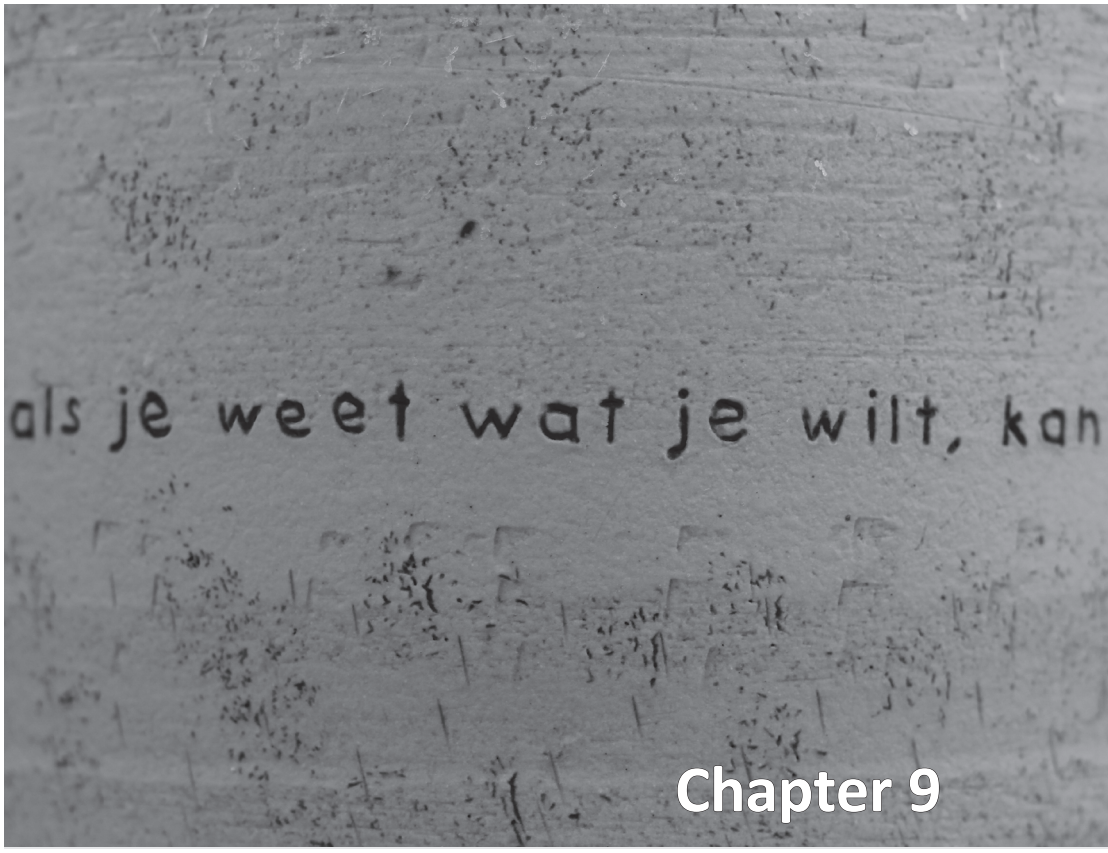
38. Lu Y, Jacobson DL, Ashworth LA, Grand RJ, Meyer AL, McNeal MM, et al. Immune response to influenza vaccine in children with inflammatory bowel disease. *Am J Gastroenterol.* 2009;104:444-453.
39. Miyamoto M, Ono E, Barbosa C, Terreri M, Hilario M, Salomao R, et al. Vaccine antibodies and T- and B-cell interaction in juvenile systemic lupus erythematosus. *Lupus.* 2011;20:736-744.
40. Heijstek MW, Ott de Bruin LM, Bijl M, Borrow R, van der Klis F, Kone-Paut I, et al. EULAR recommendations for vaccination in paediatric patients with rheumatic diseases. *Ann Rheum Dis.* 2011;70:1704-1712.
41. Kroger AT, Atkinson WL, Marcuse EK, Pickering LK. General recommendations on immunization: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep.* 2006;55:1-48.
42. Dell' EL, Esposito S, Corona F, Principi N. Vaccination of children and adolescents with rheumatic diseases. *Rheumatology (Oxford).* 2011;50:1358-1365.
43. Shoenfeld Y, Aron-Maor A. Vaccination and autoimmunity-'vaccinosis': a dangerous liaison? *J Autoimmun.* 2000;14:1-10.
44. Giannini EH, Ruperto N, Ravelli A, Lovell DJ, Felson DT, Martini A. Preliminary definition of improvement in juvenile arthritis. *Arthritis Rheum.* 1997;40:1202-1209.
45. van Eden W, Holoshitz J, Nevo Z, Frenkel A, Klajman A, Cohen IR. Arthritis induced by a T-lymphocyte clone that responds to Mycobacterium tuberculosis and to cartilage proteoglycans. *Proc Natl Acad Sci U S A.* 1985;82:5117-5120.
46. Kamphuis S, Kuis W, de Jager W, Teklenburg G, Massa M, Gordon G, et al. Tolerogenic immune responses to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis. *Lancet.* 2005;366:50-56.
47. Qin L, Gilbert PB, Corey L, McElrath MJ, Self SG. A framework for assessing immunological correlates of protection in vaccine trials. *J Infect Dis.* 2007;196:1304-1312.
48. Plotkin SA. Vaccines: correlates of vaccine-induced immunity. *Clin Infect Dis.* 2008;47:401-409.
49. Wauben MH, van der Kraan M, Grosfeld-Stulemeyer MC, Joosten I. Definition of an extended MHC class II-peptide binding motif for the autoimmune disease-associated Lewis rat RT1.BL molecule. *Int Immunol.* 1997;9:281-290.
50. Anderton SM, van der Zee R, Prakken B, Noordzij A, van Eden W. Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *J Exp Med.* 1995;181:943-952.
51. Quintana FJ, Cohen IR. The HSP60 immune system network. *Trends Immunol.* 2011;32:89-95.
52. Matzinger P, Kamala T. Tissue-based class control: the other side of tolerance. *Nat Rev Immunol.* 2011;11:221-230.
53. van Wijk F, Prakken B. Heat shock proteins: Darwinistic immune modulation on dangerous grounds. *J Leukoc Biol.* 2010;88:431-434.
54. Decrausaz L, Domingos-Pereira S, Duc M, Bobst M, Romero P, Schiller JT, et al. Parenteral is more efficient than mucosal immunization to induce regression of human papillomavirus-associated genital tumors. *Int J Cancer.* 2011;129:762-772.
55. Bouvet JP, Decroix N, Pamonsinlapatham P. Stimulation of local antibody production: parenteral or mucosal vaccination? *Trends Immunol.* 2002;23:209-213.
56. van Eden W, van der Zee R, Prakken B. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nat Rev Immunol.* 2005;5:318-330.
57. Holmgren J, Czerkinsky C, Eriksson K, Mharandi A. Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. *Vaccine.* 2003;21 Suppl 2:S89-S95.

58. Moschos SA, Bramwell VW, Somavarapu S, Alpar HO. Adjuvant synergy: the effects of nasal coadministration of adjuvants. *Immunol Cell Biol.* 2004;82:628-637.
59. Dietrich G, Griot-Wenk M, Metcalfe IC, Lang AB, Viret JF. Experience with registered mucosal vaccines. *Vaccine.* 2003;21:678-683.
60. Ivanov AP, Dragunsky EM, Chumakov KM. 1,25-dihydroxyvitamin d3 enhances systemic and mucosal immune responses to inactivated poliovirus vaccine in mice. *J Infect Dis.* 2006;193:598-600.
61. Ichinohe T, Ainai A, Tashiro M, Sata T, Hasegawa H. PolyI:polyC12U adjuvant-combined intranasal vaccine protects mice against highly pathogenic H5N1 influenza virus variants. *Vaccine.* 2009;27:6276-6279.
62. Ivory CP, Chadee K. Intranasal immunization with Gal-inhibitable lectin plus an adjuvant of CpG oligodeoxynucleotides protects against Entamoeba histolytica challenge. *Infect Immun.* 2007;75:4917-4922.
63. Mariotti S, Teloni R, von Hunolstein C, Romagnoli G, Orefici G, Nisini R. Immunogenicity of anti-Haemophilus influenzae type b CRM197 conjugate following mucosal vaccination with oligodeoxynucleotide containing immunostimulatory sequences as adjuvant. *Vaccine.* 2002;20:2229-2239.
64. Holmgren J, Adamsson J, Anjuere F, Clemens J, Czerkinsky C, Eriksson K, et al. Mucosal adjuvants and anti-infection and anti-immunopathology vaccines based on cholera toxin, cholera toxin B subunit and CpG DNA. *Immunol Lett.* 2005;97:181-188.
65. Orban T, Farkas K, Jalahej H, Kis J, Treszl A, Falk B, et al. Autoantigen-specific regulatory T cells induced in patients with type 1 diabetes mellitus by insulin B-chain immunotherapy. *J Autoimmun.* 2009.
66. Krieg AM. CpG motifs: the active ingredient in bacterial extracts? *Nat Med.* 2003;9:831-835.
67. Lenert P. Inhibitory oligodeoxynucleotides - therapeutic promise for systemic autoimmune diseases? *Clin Exp Immunol.* 2005;140:1-10.
68. Mangsbo SM, Sandin LC, Anger K, Korman AJ, Loskog A, Totterman TH. Enhanced tumor eradication by combining CTLA-4 or PD-1 blockade with CpG therapy. *J Immunother.* 2010;33:225-235.
69. Liang X, Moseman EA, Farrar MA, Bachanova V, Weisdorf DJ, Blazar BR, et al. Toll-like receptor 9 signaling by CpG-B oligodeoxynucleotides induces an apoptotic pathway in human chronic lymphocytic leukemia B cells. *Blood.* 2010.
70. Nierkens S, den Brok MH, Roelofsen T, Wagenaars JA, Figdor CG, Ruers TJ, et al. Route of administration of the TLR9 agonist CpG critically determines the efficacy of cancer immunotherapy in mice. *PLoS ONE.* 2009;4:e8368.
71. Westwood JA, Haynes NM, Sharkey J, McLaughlin N, Pegram HJ, Schwendener RA, et al. Toll-Like Receptor Triggering and T-Cell Costimulation Induce Potent Antitumor Immunity in Mice. *Clin Cancer Res.* 2009;15:7624-7633.
72. De Cesare M, Sfondrini L, Campiglio M, Sommariva M, Bianchi F, Perego P, et al. Ascites regression and survival increase in mice bearing advanced-stage human ovarian carcinomas and repeatedly treated intraperitoneally with CpG-ODN. *J Immunother.* 2010;33:8-15.
73. Cooper CL, Davis HL, Morris ML, Efler SM, Krieg AM, Li Y, et al. Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine. *Vaccine.* 2004;22:3136-3143.
74. Sogaard OS, Lohse N, Harboe ZB, Offersen R, Bukh AR, Davis HL, et al. Improving the immunogenicity of pneumococcal conjugate vaccine in HIV-infected adults with a toll-like receptor 9 agonist adjuvant: a randomized, controlled trial. *Clin Infect Dis.* 2010;51:42-50.
75. Asai K, Foley SC, Sumi Y, Yamauchi Y, Takeda N, Desrosiers M, et al. Amb a 1-immunostimulatory oligodeoxynucleotide conjugate immunotherapy increases CD4+CD25+ T cells in the nasal mucosa of subjects with allergic rhinitis. *Allergol Int.* 2008;57:377-381.

76. Marta M, Andersson A, Isaksson M, Kampe O, Lobell A. Unexpected regulatory roles of TLR4 and TLR9 in experimental autoimmune encephalomyelitis. *Eur J Immunol.* 2008;38:565-575.
77. Fallarino F, Volpi C, Zelante T, Vacca C, Calvitti M, Fioretti MC, et al. IDO mediates TLR9-driven protection from experimental autoimmune diabetes. *J Immunol.* 2009;183:6303-6312.
78. Quintana FJ, Rotem A, Carmi P, Cohen IR. Vaccination with empty plasmid DNA or CpG oligonucleotide inhibits diabetes in nonobese diabetic mice: modulation of spontaneous 60-kDa heat shock protein autoimmunity. *J Immunol.* 2000;165:6148-6155.
79. Obermeier F, Strauch UG, Dunger N, Grunwald N, Rath HC, Herfarth H, et al. In vivo CpG DNA/toll-like receptor 9 interaction induces regulatory properties in CD4+CD62L+ T cells which prevent intestinal inflammation in the SCID transfer model of colitis. *Gut.* 2005;54:1428-1436.
80. Bleich A, Janus LM, Smoczek A, Westendorf AM, Strauch U, Mahler M, et al. CpG motifs of bacterial DNA exert protective effects in mouse models of IBD by antigen-independent tolerance induction. *Gastroenterology.* 2009;136:278-287.
81. Moseman EA, Liang X, Dawson AJ, Panoskaltsis-Mortari A, Krieg AM, Liu YJ, et al. Human plasmacytoid dendritic cells activated by CpG oligodeoxynucleotides induce the generation of CD4+CD25+ regulatory T cells. *J Immunol.* 2004;173:4433-4442.
82. Bilsborough J, George TC, Norment A, Viney JL. Mucosal CD8alpha+ DC, with a plasmacytoid phenotype, induce differentiation and support function of T cells with regulatory properties. *Immunology.* 2003;108:481-492.
83. Suzuki M, Nakamura Y, Matsumoto T, Yokota M, Ohno N, Murakami S. Intranasal therapy with CpG DNA alone for the control of allergic rhinitis. *Adv Otorhinolaryngol.* 2011;72:113-115.
84. Gallichan WS, Woolstencroft RN, Guarasci T, McCluskie MJ, Davis HL, Rosenthal KL. Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract. *J Immunol.* 2001;166:3451-3457.
85. Johnson S, Zhan Y, Sutherland RM, Mount AM, Bedoui S, Brady JL, et al. Selected Toll-like receptor ligands and viruses promote helper-independent cytotoxic T cell priming by upregulating CD40L on dendritic cells. *Immunity.* 2009;30:218-227.
86. Zhu L, Ji F, Wang Y, Zhang Y, Liu Q, Zhang JZ, et al. Synovial autoreactive T cells in rheumatoid arthritis resist IDO-mediated inhibition. *J Immunol.* 2006;177:8226-8233.
87. You S, Belghith M, Cobbold S, Alyanakian MA, Gouarin C, Barriot S, et al. Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells. *Diabetes.* 2005;54:1415-1422.
88. Munn DH, Sharma MD, Lee JR, Jhaver KG, Johnson TS, Keskin DB, et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. *Science.* 2002;297:1867-1870.
89. Takeshita F, Leifer CA, Gursel I, Ishii KJ, Takeshita S, Gursel M, et al. Cutting edge: Role of Toll-like receptor 9 in CpG DNA-induced activation of human cells. *J Immunol.* 2001;167:3555-3558.
90. Nierkens S, den Brok MH, Sutmuller RP, Grauer OM, Bennink E, Morgan ME, et al. In vivo colocalization of antigen and CpG [corrected] within dendritic cells is associated with the efficacy of cancer immunotherapy. *Cancer Res.* 2008;68:5390-5396.
91. Tighe H, Takabayashi K, Schwartz D, Van Nest G, Tuck S, Eiden JJ, et al. Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhances its immunogenicity and reduces its allergenicity. *J Allergy Clin Immunol.* 2000;106:124-134.
92. Tulic MK, Fiset PO, Christodoulopoulos P, Vaillancourt P, Desrosiers M, Lavigne F, et al. Amb a 1-immunostimulatory oligodeoxynucleotide conjugate immunotherapy decreases the nasal inflammatory response. *J Allergy Clin Immunol.* 2004;113:235-241.

93. Kataoka K, Fujihashi K. Dendritic cell-targeting DNA-based mucosal adjuvants for the development of mucosal vaccines. *Expert Rev Vaccines.* 2009;8:1183-1193.
94. Mutsch M, Zhou W, Rhodes P, Bopp M, Chen RT, Linder T, et al. Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland. *N Engl J Med.* 2004;350:896-903.
95. van Ginkel FW, Jackson RJ, Yuki Y, McGhee JR. Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues. *J Immunol.* 2000;165:4778-4782.
96. van Ginkel FW, Jackson RJ, Yoshino N, Hagiwara Y, Metzger DJ, Connell TD, et al. Enterotoxin-based mucosal adjuvants alter antigen trafficking and induce inflammatory responses in the nasal tract. *Infect Immun.* 2005;73:6892-6902.
97. Song JH, Nguyen HH, Cuburu N, Horimoto T, Ko SY, Park SH, et al. Sublingual vaccination with influenza virus protects mice against lethal viral infection. *Proc Natl Acad Sci U S A.* 2008;105:1644-1649.
98. Song JH, Kim JI, Kwon HJ, Shim DH, Parajuli N, Cuburu N, et al. CCR7-CCL19/CCL21-regulated dendritic cells are responsible for effectiveness of sublingual vaccination. *J Immunol.* 2009;182:6851-6860.
99. Segal BM, Chang JT, Shevach EM. CpG oligonucleotides are potent adjuvants for the activation of autoreactive encephalitogenic T cells in vivo. *J Immunol.* 2000;164:5683-5688.
100. Krieg AM. The role of CpG motifs in innate immunity. *Curr Opin Immunol.* 2000;12:35-43.
101. Nicholas D, Odumosu O, Langridge WH. Autoantigen based vaccines for type 1 diabetes. *Discov Med.* 2011;11:293-301.
102. Bergerot I, Ploix C, Petersen J, Moulin V, Rask C, Fabien N, et al. A cholera toxoid-insulin conjugate as an oral vaccine against spontaneous autoimmune diabetes. *Proc Natl Acad Sci U S A.* 1997;94:4610-4614.
103. Sun JB, Rask C, Olsson T, Holmgren J, Czerkinsky C. Treatment of experimental autoimmune encephalomyelitis by feeding myelin basic protein conjugated to cholera toxin B subunit. *Proc Natl Acad Sci U S A.* 1996;93:7196-7201.
104. Tarkowski A, Sun JB, Holmdahl R, Holmgren J, Czerkinsky C. Treatment of experimental autoimmune arthritis by nasal administration of a type II collagen-cholera toxoid conjugate vaccine. *Arthritis Rheum.* 1999;42:1628-1634.
105. Phipps PA, Stanford MR, Sun JB, Xiao BG, Holmgren J, Shinnick T, et al. Prevention of mucosally induced uveitis with a HSP60-derived peptide linked to cholera toxin B subunit. *Eur J Immunol.* 2003;33:224-232.
106. Stanford M, Whittall T, Bergmeier LA, Lindblad M, Lundin S, Shinnick T, et al. Oral tolerization with peptide 336-351 linked to cholera toxin B subunit in preventing relapses of uveitis in Behcet's disease. *Clin Exp Immunol.* 2004;137:201-208.
107. Ruprecht CR, Gattorno M, Ferlito F, Gregorio A, Martini A, Lanzavecchia A, et al. Coexpression of CD25 and CD27 identifies FoxP3+ regulatory T cells in inflamed synovia. *J Exp Med.* 2005;201:1793-1803.
108. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. *Science.* 2003;299:1033-1036.
109. Koenen HJ, Smeets RL, Vink PM, van Rijssen E, Boots AM, Joosten I. Human CD25highFoxp3pos regulatory T cells differentiate into IL-17-producing cells. *Blood.* 2008;112:2340-2352.
110. Radhakrishnan S, Cabrera R, Schenk EL, Nava-Parada P, Bell MP, Van Keulen V, et al. Reprogrammed FoxP3+ T regulatory cells become IL-17+ antigen-specific autoimmune effectors in vitro and in vivo. *J Immunol.* 2008;181:3137-3147.

111. Sabatos-Peyton CA, Verhagen J, Wraith DC. Antigen-specific immunotherapy of autoimmune and allergic diseases. *Curr Opin Immunol.* 2010;22:609-615.
112. Bresson D, von Herrath M. Moving towards efficient therapies in type 1 diabetes: to combine or not to combine? *Autoimmun Rev.* 2007;6:315-322.
113. Ochi H, Abraham M, Ishikawa H, Frenkel D, Yang K, Basso A, et al. New immunosuppressive approaches: oral administration of CD3-specific antibody to treat autoimmunity. *J Neurol Sci.* 2008;274:9-12.
114. Boettler T, von Herrath M. Immunotherapy of type 1 diabetes--how to rationally prioritize combination therapies in T1D. *Int Immunopharmacol.* 2010;10:1491-1495.
115. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, et al. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med.* 2004;200:277-285.
116. Toubi E, Kessel A, Mahmudov Z, Hallas K, Rozenbaum M, Rosner I. Increased spontaneous apoptosis of CD4+CD25+ T cells in patients with active rheumatoid arthritis is reduced by infliximab. *Ann N Y Acad Sci.* 2005;1051:506-14.:506-514.
117. Schuerwegh AJ, Van Offel JF, Stevens WJ, Bridts CH, De Clerck LS. Influence of therapy with chimeric monoclonal tumour necrosis factor-alpha antibodies on intracellular cytokine profiles of T lymphocytes and monocytes in rheumatoid arthritis patients. *Rheumatology (Oxford).* 2003;42:541-548.
118. Ban L, Zhang J, Wang L, Kuhtreiber W, Burger D, Faustman DL. Selective death of autoreactive T cells in human diabetes by TNF or TNF receptor 2 agonism. *Proc Natl Acad Sci U S A.* 2008;105:13644-13649.
119. Ricciardelli I, Lindley KJ, Londei M, Quaratino S. Anti tumour necrosis-alpha therapy increases the number of FOXP3 regulatory T cells in children affected by Crohn's disease. *Immunology.* 2008;125:178-183.
120. Anderton SM, van der Zee R, Noordzij A, van Eden W. Differential mycobacterial 65-kDa heat shock protein T cell epitope recognition after adjuvant arthritis-inducing or protective immunization protocols. *J Immunol.* 1994;152:3656-3664.
121. Glant TT, Finnegan A, Mikecz K. Proteoglycan-induced arthritis: immune regulation, cellular mechanisms, and genetics. *Crit Rev Immunol.* 2003;23:199-250.
122. Glant TT, Mikecz K. Proteoglycan aggrecan-induced arthritis: a murine autoimmune model of rheumatoid arthritis. *Methods Mol Med.* 2004;102:313-338.
123. Prakken BJ, Wendling U, van der Zee R, Rutten VP, Kuis W, van Eden W. Induction of IL-10 and inhibition of experimental arthritis are specific features of microbial heat shock proteins that are absent for other evolutionarily conserved immunodominant proteins. *J Immunol.* 2001;167:4147-4153.
124. de Jong H, Lafeber FF, de Jager W, Haverkamp MH, Kuis W, Bijlsma JW, et al. PAN-DR-Binding Hsp60 self epitopes induce an interleukin-10-mediated immune response in rheumatoid arthritis. *Arthritis Rheum.* 2009;60:1966-1976.
125. Faria AM, Weiner HL. Oral tolerance. *Immunol Rev.* 2005;206:232-259.
126. Maslanka SE, Tappero JW, Plikaytis BD, Brumberg RS, Dykes JK, Gheesling LL, et al. Age-dependent Neisseria meningitidis serogroup C class-specific antibody concentrations and bactericidal titers in sera from young children from Montana immunized with a licensed polysaccharide vaccine. *Infect Immun.* 1998;66:2453-2459.



Chapter 9

Nederlandse samenvatting

Dankwoord

Curriculum Vitae

List of contributors

List of publications

NEDERLANDSE SAMENVATTING

INLEIDING

Juveniele Idiopathische Arthritis

Juveniele Idiopathische Arthritis (JIA, ook wel jeugdreuma genoemd), is de meest voorkomende chronisch reumatische ziekte op de kinderleeftijd. Jaarlijks zijn er per 100.000 kinderen ongeveer 16 tot 150 nieuwe ziektegevallen. De oorzaak van JIA is onbekend, maar de ziekte lijkt zich te ontwikkelen in genetisch vatbare personen na blootstelling aan een trigger uit de omgeving. Het resultaat hiervan is een ongecontroleerde immuunrespons, gericht tegen lichaamseigen eiwitten (een auto-immuunrespons), met gewrichtsschade tot gevolg.

Er zijn verschillende subtypen van JIA. De meest gunstige vorm is persisterende oligoarticulaire arthritis (OA-JIA), waarbij maximaal vier gewrichten zijn aangedaan. Bij deze vorm van arthritis heeft het immuunsysteem klaarblijkelijk een manier gevonden om op een bepaald moment de tolerantie voor lichaamseigen eiwitten te herstellen, waardoor de ziekte zich niet verder uitbreidt. Dit herstel schiet in het geval van polyarticulaire arthritis (PA-JIA) tekort. Bij deze ernstige vorm van arthritis zijn meer dan vier gewrichten aangedaan in het eerste half jaar van de ziekte en blijft de ziekte vaak langdurig bestaan. Een vergelijking van de immunologische processen die bij deze twee ziektebeelden een rol spelen kan ons daarom meer leren over immuunregulatie in chronische arthritis.

De behandeling van JIA is multidisciplinair, maar voornamelijk gericht op algehele immuunsuppressie. Sinds de beschikbaarheid van biologicals (monoclonale antistoffen die specifiek kunnen binden aan een cytokine of diens receptor en daarmee de werking van een dergelijk cytokine blokkeren, bijvoorbeeld TNFα blokkade) is er veel vooruitgang geboekt in de behandeling van ernstige vormen van JIA. Deze biologicals zijn echter wel levenslang nodig voor blijvende remissie. Deze levenslange behandeling wordt beperkt door het ontstaan van resistentie voor het middel, maar ook door bijwerkingen zoals het verhoogde risico op (opportunistische) infecties als tuberculose en daarnaast een verhoogd risico op lymfomen. Om infecties zoveel mogelijk te voorkomen is vaccinatie tegen infectieziekten bij deze patiënten van groot belang. Bovendien is het wenselijk om een meer gerichte immuunmodulerende behandeling te geven, zodat bijwerkingen kunnen worden gereduceerd en mogelijk een meer langdurig effect kan worden behaald.

Deel 1: Vaccinatie tegen infectie bij chronische arthritis

Hoewel vaccinaties belangrijk zijn voor patiënten met chronische arthritis, ontbreken richtlijnen hiervoor. Door een gebrek aan goede klinische studies hierover is het opstellen van een richtlijn ook niet gemakkelijk. Een eerste voorstel is recent gedaan door de EULAR (het Europese verband voor reumatologen en onderzoek naar reuma), maar ook in dit voorstel wordt de behoefte uitgesproken aan goede klinische en immunologische studies naar de effectiviteit en veiligheid van vaccinaties bij patiënten met auto-immuunziekten in het algemeen en reuma in het bijzonder.

Veiligheid van vaccinatie bij auto-immuniteit

Doordat auto-immuunziekten zoals jeugdreuma vaak ontstaan op of rond de leeftijd waarop ook gevaccineerd wordt, rijst regelmatig de vraag of dit berust op toeval of dat er sprake is van een causaal verband. Van een aantal infectieziekten waartegen gevaccineerd wordt (zoals rubella) is bekend dat ze kunnen leiden tot reactieve arthritis. Voor wat betreft de vaccinatie zelf is het minder duidelijk. Hoewel er vele studies gedaan zijn naar een causaal verband tussen vaccinatie en het ontstaan van auto-immuunziekten, hebben slechts een paar studies dit kunnen aantonen.

Het veronderstelde mechanisme berust enerzijds op moleculaire mimicry, waarbij T cellen die specifiek zijn voor bacteriële antigenen, kruisreageren met zelf-antigenen doordat de bacteriële antigenen lijken op de lichaamseigen antigenen. Anderzijds wordt gedacht dat ‘bystander ‘activatie een rol speelt. Door het vrijkomen van lichaamseigen antigenen in een inflammatoir milieu, ontstaat een proinflammatoire (auto-) immuunrespons. Men kan zich hierbij afvragen waarom niet iedereen een auto-immuunziekte krijgt na infectie of vaccinatie. Bij gezonde personen zijn er immunologisch regulatoire mechanismen aanwezig die auto-immuniteit voorkomen. Een daarvan wordt verzorgd door regulatoire T cellen (Treg) die FoxP3 tot expressie brengen. Deze Tregs kunnen de immuunrespons remmen door direct celcontact of het vrijmaken van regulerende cytokinen. In patiënten met een auto-immuunziekte lijken deze regulatoire mechanismen te falen. Het bestuderen van de immunologische responsen na vaccinatie bij patiënten met een auto-immuunziekte zoals jeugdreuma, geeft ons meer inzicht in het al dan niet bestaan van een verhoogde kans op verergering van de ziekte na vaccinatie.

Effectiviteit van vaccinatie bij auto-immuniteit

Aan de effectiviteit van vaccinatie bij het gebruik van immuunsuppressieve medicatie wordt regelmatig getwijfeld. Immuunsuppressiva geven een verworven immuundeficiëntie die afhankelijk is van dosering en medicament. Het is de vraag of de gewenste antistofrespons na vaccinatie hoog genoeg is voor bescherming tegen de infectie bij gebruik van de sterke immuunsuppresiva voor de behandeling van reuma.

Meningococcen C vaccinatie

In 2002 werd de Meningococcen C vaccinatie aan het Rijksvaccinatieprogramma toegevoegd. Omdat er een toename was van het aantal kinderen met Meningococcen C ziekte werd er in Nederland een grote inhaalvaccinatie campagne opgezet, waarin alle kinderen tussen de 1 en 18 jaar (inclusief de kinderen met JIA) werden gevaccineerd. Deze campagne gaf ons de gelegenheid om de studies in hoofdstuk 2 en 3 uit te voeren in een groot aantal patiënten met JIA.

Deel 2: Vaccinatie tegen auto-immuniteit zoals chronische arthritis

Bij patiënten met JIA is er in het gewrichtskapsel een continue ontsteking (inflammatie) gaande die (onder andere) specifiek is voor de eiwitten die op deze plaats tot expressie komen. Specifieke tolerantie inductie van het immuunsysteem voor deze eiwitten zou een meer gerichte behandeling kunnen vormen, die gepaard gaat met minder bijwerkingen en mogelijk een langduriger effect zou kunnen hebben. Het induceren van tolerantie voor een immunologisch actief eiwit (antigeen) dat aanwezig is op de plaats van inflammatie wordt antigeen specifieke tolerantie inductie genoemd. Dit kan worden bewerkstelligd door mucosale toediening van het antigeen. Hierbij is gebleken dat nasale toediening effectiever is dan orale toediening.

Het antigeen dat chronische arthritis veroorzaakt is niet bekend en zal waarschijnlijk ook nooit gevonden worden. Het is de vraag of dit ene ziekte-inducerende antigeen wel bestaat. Wel weten we dat door celschade op de plaats van inflammatie verschillende zelf-antigenen aanwezig zijn. De zelf-antigenen die immunogeen zijn en bovendien bij inflammatie opgereguleerd zijn, zijn goede kandidaten voor antigeen specifieke tolerantie inductie. Een voorbeeld van een dergelijk zelf-antigeen is het heat shock eiwit (HSP). Door mucosale toediening van HSP zal tolerantie ontstaan voor dit eiwit. Deze tolerogene immuunrespons komt van pas op de plek van inflammatie, waar veel HSP tot expressie komt. Effectiviteit van mucosale tolerantie inductie met HSP is gebleken uit meerdere dierexperimentele studies in diverse arthritis modellen.

HSPs zijn grote eiwitten met uiteenlopende functies in het immuunsysteem. Toediening van het hele eiwit zou aanleiding kunnen geven tot ongewenste reacties van het afweersysteem. Omdat een T cel via zijn T cel receptor niet een heel eiwit herkent, maar delen hiervan (peptiden), is het ook mogelijk om antigeen specifieke tolerantie te induceren met peptiden in plaats van met het hele eiwit. Hierdoor wordt de therapie nog gerichter en veiliger.

Peptide-specifieke tolerantie inductie is, in tegenstelling tot resultaten uit dierexperimenteel onderzoek, bij de mens niet zo eenvoudig. De belangrijkste oorzaak hiervoor is

dat de genetische achtergrond bij de proefdieren die gebruikt worden vrijwel identiek is, terwijl dit bij de mens veel complexer is. Welk peptide door een antigeen presenterende cel wordt aangeboden aan de T cel wordt namelijk bepaald door een genetisch sterk variabel eiwit: het HLA eiwit. Om effectief te zijn in een groot deel van de patiënten is het daarom belangrijk dat een peptide kan binden aan meerdere verschillende HLA-eiwitten. Recent zijn er acht HSP-peptiden met behulp van computermodellen geïdentificeerd met een hoge bindingsscore aan verschillende HLA eiwitten die veel voorkomen bij patiënten met arthritis. Deze peptiden worden herkend door T cellen van de meerderheid van de patiënten met reumatoide arthritis en JIA. Deze HSP peptiden zijn vanwege deze immunogeniciteit in combinatie met hun aanwezigheid op de plaats van inflammatie interessante kandidaten voor mucosale antigeen specifieke tolerantie inductie.

Versterken van de immunogeniciteit van nasaal toegediende antigenen

Een groot probleem bij mucosale toediening van een peptide is de beperkte immunogeniciteit in deze tolerogene omgeving. Een peptide stimuleert namelijk alleen het adaptieve immuunsysteem. Omdat ook het induceren van tolerantie een actief proces is, is herkenning van en reactie op peptiden door het immuunsysteem noodzakelijk. Het activeren van het aspecifieke immuunsysteem door toevoeging van een adjuvant zou een manier kunnen zijn om de immunogeniciteit van een nasaal toegediend peptide te verhogen. Bij vaccinatie tegen infectieziekten is dit al effectief gebleken.

Combinatie therapie

Bij de diagnose van reumatoïde arthritis of JIA is de inflammatie al zodanig gaande, dat immuunmodulatie door mucosale tolerantie inductie waarschijnlijk te subtiel is om werkzaam te zijn in dit sterk inflammatoire milieu. Aspecifieke immuunsuppressie met biologicals zoals TNFα blokkade zou de inflammatie zodanig kunnen remmen dat antigeen specifieke tolerantie inductie weer mogelijk is. Combinatie van antigeen specifieke tolerantie inductie met aspecifieke immuunsuppressie (anti-CD3) is inderdaad effectief gebleken in patiënten met diabetes. Het is daarom denkbaar dat combinatietherapie van een HSP-peptide met TNFα blokkade soortgelijke resultaten boekt.

Het adjuvant arthritis model

Voor de studies in het tweede deel van dit proefschrift is gebruikt gemaakt van het adjuvant arthritis model in de Lewis rat. Deze dieren ontwikkelen het klinische en immunologische beeld van arthritis na een injectie met hitte-geïnactiveerd *M. tuberculosis* in Incomplete Freund's Adjuvant (een olieachtige substantie) in de staartbasis. De arthritis die volgt is een monofasische ziekte die zijn maximum bereikt na 23 dagen en die vanzelf verdwijnt.

Doelen van dit proefschrift

In dit proefschrift staan twee onderzoeksvragen centraal:

1. Is de Meningococcen C vaccinatie veilig en effectief in patiënten met verschillende vormen van jeugdreuma, ook als ze immuunsuppressive medicatie gebruiken?

2. Als gebruik gemaakt wordt van nasale toediening van heat shock eiwit (HSP)-peptiden die op de plaats van inflammatie tot expressie komen en bovendien door het immuunsysteem van patiënten met arthritis herkend worden, is het dan mogelijk om tolerantie te induceren voor deze HSPs, en daarmee arthritis te voorkomen? Met andere woorden: werkt antigeen-specifieke tolerantie inductie door nasale toediening van HLA-bindende HSP-peptiden in een diermodel voor arthritis?

Met de volgende vervolgvragen op vraag 2:

2a. Kan toevoeging van een adjuvant dat het aspecifieke immuunsysteem activeert de effectiviteit van antigeen specifieke tolerantie inductie vergroten en hoe is het werkingsmechanisme hiervan?

2b. Kan een therapeutisch effect bewerkstelligd worden van nasale antigeen specifieke tolerantie inductie op arthritis als dit gecombineerd wordt met immuunsuppressieve medicatie zoals TNFα blokkade?

RESULTATEN

Deel 1: Vaccinatie tegen infectie bij chronische arthritis

In **hoofdstuk 2** van dit proefschrift beschrijven we dat de Meningococcen C (MenC) vaccinatie bij 234 JIA patiënten niet leidt tot een verergering van ziekteactiviteit binnen 6 maanden na vaccinatie. Hoewel gemiddelde anti-MenC antistof titers lager waren in patiëntgroepen met zwaardere immuunsuppressieve medicatie, was de functionele bactericide werking van de ontstane antistoffen adequaat. Dit betekent dat de Men C vaccinatie klinisch veilig en serologisch effectief is bij patiënten met JIA. Dit ondersteunt het advies om deze patiënten te vaccineren.

Echter, de resultaten in **hoofdstuk 3** wijzen erop dat vaccinatie van kinderen met de polyarticulaire vorm van JIA toch in theorie gepaard gaat met een risico op verergering van de ziekte. De MenC-specifieke T cellen genereerden namelijk vooral proinflammatoire cytokinen, zonder een adequate regulatoire T cel respons. We hebben dit risico klinisch niet kunnen bevestigen in **hoofdstuk 2**, mogelijk door een te klein aantal PA-JIA patiënten in deze studie. Daarnaast is het denkbaar dat meerdere factoren nodig zijn om klinische ziekteverergering te induceren. De kans dat een vaccinatie een auto-immuunziekte verer-

gert is echter vele malen kleiner dan de kans op een ernstige infectie bij kinderen met JIA. Het blijft daarom belangrijk dat deze kinderen zoveel mogelijk beschermd worden tegen infecties door vaccinatie.

Deel 2: Vaccinatie tegen auto-immuniteit zoals chronische arthritis

Hoofdstuk 4 vormt de theoretische onderbouwing van de studies in het tweede deel van dit proefschrift. Hierin leggen we uit waarom mucosale vaccinatie met een peptide van een eiwit dat aanwezig is op de plaats van inflammatie zoals HSP, zou kunnen beschermen tegen chronische arthritis. In **hoofdstuk 5** laten we vervolgens zien dat dit inderdaad het geval is. Vaccinatie door nasale toediening van een van de acht geselecteerde HLA-bindende HSP-peptiden (peptide p1) beschermde tegen arthritis in het adjuvant arthritis model in de rat. Deze bescherming kon worden overgebracht naar niet-gevaccineerde ratten door CD4 positieve (CD4+) T cellen. De door HSP-vaccinatie geïnduceerde CD4+ T cel populatie had een minder inflammatoir cytokine profiel en bevatte meer regulatoire T cellen dan CD4+ T cellen van niet-gevaccineerde ratten met arthritis. Dit betekent dat nasale antigeen specifieke tolerantie inductie met een HSP peptide dat geschikt is voor toepassing in de mens, leidt tot immuunmodulatie en daarmee een goede kandidaat is voor antigeen specifieke immunotherapie van arthritis.

Combinatie van nasale peptide vaccinatie met een mucosaal adjuvant (CpG) verbeterde sterk de klinische effectiviteit van peptide vaccinatie (**hoofdstuk 6**). Combinatie therapie van een HSP peptide (p1) met CpG leidde tot activatie van plasmacytoide dendritische cellen (pDC) en deed ze in aantal toenemen. Deze pDCs zijn van belang omdat ze een rol lijken te spelen in het opwekken van regulatoire immuunresponsen. Daarbij zijn ze gespecialiseerd in het presenteren van peptiden aan het immuunsysteem (T cellen). De toegenomen p1-specifieke T cel proliferatie en p1-specifieke IL-10 productie (een immuunregulatoir cytokine) in de met p1+CpG behandelde groep, is dus mogelijk verklaard door betere peptide presentatie door deze geactiveerde dendritische cellen. Na arthritis inductie waren er in de gewrichtsdrainerende lymfeklieren bij de ratten die nasale tolerantie inductie ondergingen met p1 (p1 of p1+CpG) grotere hoeveelheden regulatoire T cellen aantoonbaar, die correleerden met lagere arthritisscores. Dit laat opnieuw het immuunmodulerende effect laat zien van nasale toediening van het HSP-peptide p1. De data in **hoofdstuk 6** geven aan dat CpG een goed werkzaam adjuvant is voor nasale antigeen specifieke immunotherapie van arthritis.

In **hoofdstuk 7** hebben we de stap gemaakt van een preventieve strategie naar een therapeutische strategie. Door nasale peptide therapie met een HSP-peptide te combineren met een lage dosering immuunsuppressieve medicatie in de vorm van TNFα-blokkade, konden we een therapeutisch effect bereiken van nasale peptide toediening in het

adjuvant arthritis model. Deze combinatietherapie zorgde voor toegenomen peptide-specifieke IL-10 productie door CD4+T cellen en een opregulatie van het voor regulatoire T cellen kenmerkende gen FoxP3 in Treg cellen. Het therapeutisch effect van deze combinatie therapie met lage dosering TNFα-blokkade was net zo groot als het effect van hoge dosering TNFα-blokkade alleen. Vertaling van deze combinatie therapie naar de mens kan daarom een belangrijke reductie van kosten en bijwerkingen van immuunsuppressieve medicatie opleveren.

CONCLUSIE

In dit proefschrift hebben we klinische en experimentele studies gecombineerd om tot een beter inzicht te komen in de gewenste en ongewenste effecten van vaccinatie bij een auto-immuunziekte zoals chronische arthritis.

Hoewel de klinische en antistofresponsen na Meningococcen C vaccinatie in de totale groep JIA patiënten gunstige resultaten lieten zien, bleek er een subgroep bestaande uit polyarticulaire JIA patiënten te zijn met immunologische risicofactoren voor ziekte verergering na vaccinatie. Daarnaast waren er lagere antistoftiters zichtbaar bij gebruik van sterkere immuunsuppressie en leken titers in de loop van de tijd bij patiënten met auto-immuunziekten sneller af te nemen dan bij gezonde controles. Gezien de lage frequentie van bijwerkingen van vaccinaties en van patiënten die sterke immuunsuppressiva gebruiken zijn daarom grotere studies nodig naar de veiligheid en effectiviteit van vaccinaties tegen infectieziekten in het bijzonder bij polyarticulaire JIA patiënten. Door gebruik te maken van bestaande internationale samenwerkingsverbanden moet dit mogelijk zijn. Het is belangrijk dat er in deze studies voor beoordeling van de effectiviteit van vaccinatie gebruik gemaakt wordt van betrouwbare uitkomstmaten die direct gerelateerd zijn met bescherming tegen de ziekte.

Door gebruik te maken van het adjuvant arthritis model in de rat hebben we in het tweede deel van dit proefschrift kunnen aantonen dat nasale toediening van HSP-peptiden door immuunmodulatie kan beschermen tegen arthritis. Bovendien is het gelukt om de immunogeniciteit van nasale peptide toediening (en daarmee het klinisch effect) te verbeteren door het toevoegen van een mucosaal adjuvant (CpG). Om tot therapeutische effectiviteit te komen van mucosale tolerantie inductie met HSP-peptiden hebben we nasale peptide immunotherapie gecombineerd met lage dosis cytokine blokkade. Nasale behandeling met HSP-peptiden heeft dus in de studies in dit proefschrift veelbelovende resultaten laten zien. Er zijn echter nog verschillende horden te nemen. Zo is het bijvoorbeeld belangrijk om op zoek te gaan naar een mucosaal adjuvant dat veilig toegepast kan worden in de

mens en naar de optimale dosering van peptide. Peptide specifieke immunotherapie met HSP peptiden tegen auto-immuniteit is dan klaar voor vertaling naar JIA patiënten. Om het slagingspercentage vergroten kan selectie van patiënten overwogen worden op basis van immunologische herkenning van het peptide voor de start van immunotherapie. Antigeen specifieke tolerantie inductie met HSP-peptiden zou op deze manier de behoefte aan immuunsuppressieve medicatie om ziekteremissie in stand te houden drastisch kunnen verlagen, en daarmee de kwaliteit van leven voor JIA patiënten vergroten.

DANKWOORD

De balans opmaken

A balancing act: dat was het de afgelopen jaren zeker. Niet alleen zoeken naar de balans tussen activatie en regulatie van het immuunsysteem, maar ook balanceren tussen kliniek en onderzoek, tussen werk en thuis. Ik heb er veel van geleerd en daarvoor ben ik vele mensen dankbaar. Ik wil een aantal van hen hier in het bijzonder noemen.

(Co)-promotoren en begeleiders

Allereerst **Berent**, wat een bijzonder mens ben jij. Ik ben erg blij dat je me na die uitgebreide sollicitatieprocedure in 2002 hebt aangenomen en dat we sindsdien hebben kunnen bouwen aan dit proefschrift. Al mijn schema's waar niets van terecht kwam, al mijn honger naar details, jij begreep als geen ander dat je mij niet moet stimuleren maar afremmen om tot het beste resultaat te komen. Je hebt in de afgelopen tien jaar veel van mijn life-events meegemaakt. Je hield steeds een rotsvast vertrouwen in mij en droeg dat ook uit naar anderen. Dat heeft voor mij heel veel betekend en deuren geopend en ik ben je daar dan ook zeer dankbaar voor. Het enige nadeel van het voltooien van dit proefschrift is dat ik geen excuus meer heb om nog eens bij je binnen te lopen. Ik hoop dat onze samenwerking hier niet ophoudt, ik ben namelijk graag gast in jouw Hotel California.

Wietse, wat ik altijd aan jou heb bewonderd is dat je steeds de juiste vragen weet te stellen om weer een stap verder te komen. Menig promovendilunch eindigde met plannen voor jaren van experimenten, waarvan de meeste ook daadwerkelijk zijn uitgevoerd. Dank voor je betrokkenheid, zelfs nu, na je pensioen. Ik ben blij dat er een regel bestaat die ervoor zorgt dat je mijn promotor kunt blijven en dat we dit proefschrift samen hebben kunnen afronden.

Nico, jij hebt mijn eerste schreden in klinisch onderzoek begeleid en heel wat eigenwijzigheden van mij moeten verduren. Samen naar de statisticus was een belevenis! Dank voor je geduld hierin. Het blijft fantastisch om te zien hoe je telkens weer nieuwe projecten op poten weet te zetten. Je enthousiasme voor vaccinatieonderzoek is en blijft aanstekelijk.

Femke, jouw komst is voor mij een geschenk geweest. Jij wist voor mij overzicht te creëren op het moment dat ik het kwijt was en had (en hebt) slimme ideeën waarmee je het onderzoek naar een hoger niveau getild hebt. Hoofdstuk 4 van dit proefschrift was er zonder jou nooit in zo'n korte tijd gekomen. Dank je wel voor al je hulp!

Marianne, ook jouw komst was een prettige verrassing. Je positieve instelling en frisse blik brachten mij weer op de rit voor hoofdstuk 6. Mede dankzij jouw inbreng en kennis over dendritische cellen staat er nu een prachtig stuk. Dankjewel daarvoor.

Salvo, you gave me the opportunity to participate in research in your lab in San Diego. Together with Sarah, I had an inspiring time which I look back upon with lots of pleasure. Thank you for your hospitality and your useful advice throughout my project.

Het Prakkenlab

Een hele bijzondere groep slimme en enthousiaste mensen, die elkaar versterken en ondersteunen. Ik heb het altijd een voorrecht gevonden om van deze groep deel uit te mogen maken.

Allereerst de fundamenten van het Prakkenlab: Wilco, Mark, Mariska, Jenny. Terwijl promovendi komen en gaan zijn jullie degenen die zorgen voor continuïteit. **Wilco**, wat hebben wij veel uren samen doorgebracht: of het nu ratten scoren was, 's ochtends vroeg ratten offeren of milten prakken in de flowkast (het letterlijke 'prakken'-lab!), je was er, soms na wat morren, maar uiteindelijk altijd bij. Dank je wel voor al die inzet. **Mark**, jij bent uitgegroeid tot een fantastisch sociale labmanager, maar ik kon ook altijd bij je terecht voor een middagje FACS sorten of weer een nieuwe portie 'p1'. Daarnaast dank voor je altijd belangstellende vragen, vooral ook naar het leven buiten het lab. **Mariska**, dank voor je hulp op de drukke offerdagen. **Jenny**, ook jij kon buffelen op offerdagen. Jouw werktempo is onwaarschijnlijk hoog. Ik ben jaloers op je efficiëntie.

Een ander deel van het fundament wordt gevormd door Erica en Angela, onze altijd behulpzame secretaresses. **Erica**, jij wist me altijd op de meest onmogelijke tijden nog in te plannen in de agenda van Berent. Dank voor je flexibiliteit hierin. **Angela**, je was voor mij lang de secretaresse van Wietse, maar nu ben je mede-steunpilaar van het Prakken lab.

En dan de promovendi van het Prakkenlab:
Arash, you taught me being a simple clinical doctor how to work with pipettes, tubes, stoves and FACS. It was a privilege to be educated in lab skills by such an experienced professional. I'm still impressed by the path you took: from lab technician to MD, PhD, wow!
Ismé, je bent voor mij in vele dingen een voorbeeld. Zowel in de manier waarop je onderzoek doet, maar ook in het maken van keuzes daarna. Ontzettend knap hoe jij je in het buitenland hebt gestort op het onderzoek. Ik vond het heel fijn om over die weg in Gent eens met je van gedachten te wisselen. Dank je wel ook voor jouw steun in mijn keuze-traject.

Sylvia, met de identificatie van de HLA-bindende HSP epitopen legde jij de basis voor het tweede deel van dit proefschrift. Dank je wel voor je goede ideeën (uveitis!), je raad bij presentaties en de vele gezellige congressen samen.
Lianne, onze lieve lange dokter. Wij deelden vaak dezelfde frustraties en dat was dan weer een verademing. Ik bewonder het pad wat jij bewandelt: klasse.
Lise en **Joost**, dank jullie wel voor jullie altijd warme belangstelling en een ontzettend tof congres in San Francisco. **Annet**, dank voor de tip van het jaar: Anna Hinze. Zij heeft mij echt 'back on track' gekregen.
Eva, mijn HSP-maatje van het laatste uur en kamergenoot. Als ik uit de kliniek kwam en het hele HSP concept weer vergeten was, was jij er gelukkig om dit weer op te frissen. Dank je wel voor je leestips, je gezelligheid en voor het feit dat je in Hilversum woont. Ik kijk uit naar onze volgende eetdate!
Huib, dat de combinatie opleiding tot kinderarts en promoveren niet ideaal is, weten we inmiddels, maar de combinatie kinderarts zijn en promoveren is helemaal een uitdaging. Knap hoe je nu desondanks toch toewerkt naar jouw eindstreep. Heel veel succes daarbij.
Alvin, in het lab maar ook in de kliniek hebben we veel ervaringen gedeeld. Samen in Eindhoven, later de NICU en de PICU, het was me steeds een waar genoegen. Had ik maar een fractie van de rust die jij uitstraalt. Dank voor je luisterend oor en goede raad.
Joost A., Yvonne, Ellen, Berber, Lieke, Sytze, Annemarie, Selma, Jorg, Henk, Maja, Ruud, Theo, Gijs, allemaal bedankt voor die fantastische teamspirit die de Prakken-groep zo eigen is. En dat dan niet alleen op het gebied van de immunologie, maar ook daarbuiten. Het was lang zo leuk niet geweest zonder jullie.

De mensen die echt 'met de voeten in de klei' van dit onderzoek hebben gestaan zijn natuurlijk de studenten. Zonder hun leergierigheid, werklust en briljante opmerkingen was dit proefschrift er niet geweest.
Eline, ´mijn´ eerste student, dank voor al je hulp met die enorme database voor het vaccinatie stuk. **Marloes**, jij kwam daarna en bent teruggekeerd (of nooit weggeweest) als een fantastisch onderzoeker en bovendien een ontzettend goede en gezellige collega. **Wai Ming**, jij schreef met je scriptie de basis voor hoofdstuk 1. Dank voor je harde werken. **Maud**, jij timmert inmiddels hard aan de weg in Gent. Met jouw moleculair biologische achtergrond voegde jij echt iets toe. Dank voor al die uren samen bij de ratten, prakken in de flowkast, kweken en FACSen, het was me een genoegen. **Eveline**, je bleek een gouden sleutel uit Leiden en combineert nu de opleiding tot kinderarts met je eigen promotie. Petje af daarvoor.

Flex kamer genoten

Je kamergenoten maken misschien nog wel het meeste mee van de toppen en dalen die een promotietraject met zich meebrengt. Daarom wil ik ook hen hier bedanken voor de

vele kopjes thee, koekjes en luisterende oren die ze mij in de afgelopen 10 jaar hebben geboden.

Titia en **Marieke van S., Coralie, Marieke vd Z** en **Judith**, dank voor jullie steun en gezelligheid. **Lieke de V., Sanne H, Eva, Sanne N., Gerwin, Annick, Arjan**, jullie maakten het mogelijk dat ik in de afrondende fase van dit proefschrift toch een min of meer vaste werkplek had in de voor mij zo vertrouwde kamer van zes. Dank jullie wel voor jullie flexibiliteit en betrokkenheid.

Analisten

Carla, jij was degene die ontzettend geholpen heeft met de MenC ELISA's voor hoofdstuk 2. Dank voor al je hulp hierbij. **Ger A.** en **Gerrit S**. Is het toeval dat 'de mannen van het sorten' soortgelijke namen hebben? Dank voor jullie geduld bij het sorten van de suppressie-assays (ook als het eindeloos duurde). Mensen van het 'Routine lab': **Wil, Pirrko, Koos**. Jullie stonden altijd klaar om vragen over FACS isolatie-dienst en aanverwante zaken te beantwoorden. Dank daarvoor.

RIVM

Patricia Vermeer en **Fiona van der Klis**, dank voor jullie enthousiasme voor het MenC-project en voor het organiseren van de broodnodige serum bactericidal assays.

Diergeneeskunde

Suzanne, ik ken maar weinig mensen met zoveel doorzettingsvermogen en kracht als jij. Je draagt ook je eigen steentje aan arthritis onderzoek bij. Ik hoop van harte dat je ooit zelf nog eens mag profiteren van de vooruitgang op dit gebied in de wetenschap. **Femke, Teun, Lotte**, naast de HSP-meeting zagen we elkaar op menig congres en het was altijd even gezellig. Dank voor de leuke tijd.

De **medewerkers van het GDL** wil ik bedanken voor de goede zorgen voor 'mijn' ratjes en voor alle hand- en spandiensten die ze in de loop van het onderzoek hebben verricht.

En dan is er nog een onderzoek wat niet in dit proefschrift staat, maar waar wel vele uren werk aan besteed zijn. Niet alleen door mij, maar zeker ook door **prof. Boevé**, dierenoogarts. De resultaten zijn ernaar en ik heb u en mezelf de belofte gedaan het artikel hierover te schrijven. Helaas heeft het de deadline van het proefschrift niet gehaald. Maar van uitstel komt wat mij betreft geen afstel. Ontzettend veel dank voor uw hulp en betrokkenheid.

De vele **JIA patiënten**, **hun ouders**, en de **gezonde vrijwilligers** die zich lieten vaccineren met het MenC vaccin en twee maal bloed gaven voor de studie wil ik graag bedanken voor hun deelname. Zonder hen waren hoofdstuk 1 en 2 er nooit gekomen.

Kinderartsen van het WKZ

Joost Frenkel, jij als bevlogen opleider Kindergeneeskunde, wil ik graag bedanken voor de tijd en ruimte die je mij gaf om een nieuwe weg te vinden en mijn proefschrift af te ronden. **Marije Hennus**, dank je wel dat je me hebt wakker geschud en voor al je support sindsdien.

Klinische Genetica

Nine Knoers, ik vind het een eer dat je in mijn commissie wil plaatsnemen. Dank voor je vertrouwen in mij en de fantastische kans die je me biedt binnen de Klinische Genetica. Ik voel me na een paar maanden al helemaal thuis. Dit is zeker ook dankzij mijn collega AIOS: **Saskia S, Albertien, Annette, Saskia B, Merel, Marijke, Jeske, Lianne**: bedankt voor de gezellige werksfeer, jullie teamspirit en flexibiliteit.

Paranimfen

Sarah, wij hebben in het lab zoveel samen gedaan: van groepje 1 tot in San Diego, van rat tot muis en van theetje drinken tot liedje schrijven: natuurlijk moest je mijn paranimf zijn. Daarnaast ben je ook nog eens een heel fijn mens. Dank je wel voor je altijd warme belangstelling en daadkracht. Heel fijn dat je straks naast me staat!

Bas, je ging me voor in Eindhoven, zat naast me in de flexkamer en je stond achter me in tijden van moeilijke keuzes. Ik bewonder de manier waarop je kliniek, onderzoek en thuis combineert en ook nog zo kunt meeleven met anderen. Dank je wel voor je steun en luisterend oor in de afgelopen jaren, maar ook voor je slimme ideeën en veel gezelligheid op de vele congressen die we samen bezochten. Dank je wel dat je ook deze mijlpaal mee wilt beleven als paranimf.

Sociale balans

Vrienden en **vriendinnen**, heel veel dank voor al die uren oppas, voor de blijvende belangstelling in dit schijnbaar eeuwig durende project en voor het onderhouden van onze vriendschap als ik daarin tekort schoot. We gaan elkaar de komende tijd vaker zien wat mij betreft!

Lieve **Niek** en **Wilma**, jullie hebben mij de afgelopen jaren zien balanceren en desondanks mij altijd gesteund in de afronding van dit project. Ik wil jullie bedanken voor al voor jullie

oprechte geïnteresseerdheid in de materie van dit proefschrift, maar vooral ook voor het feit dat jullie altijd voor ons klaarstaan.

Lieve schoonfamilie: **Willemijn, Jimmie, Berend Jan, Boukje, Merijn**, de leukste en liefste ooms en tantes die Emma en Julia zich kunnen wensen. Dankjewel voor jullie goede zorgen voor onze meisjes en voor ons.

Lief **zusje**, hoewel ik je nog steeds graag zo noem, ben je inmiddels een zelfbewuste, stijlvolle vrouw geworden die haar mannetje staat. Ik weet zeker dat je een hele lieve mama zal zijn voor jullie kleine mensje, net zoals je een hele lieve tante bent voor Emma en Julia. Dankjewel voor al je kledingadvies, creatieve ideeën en voor het feit dat je altijd mijn zusje blijft.

Lief thuisfront, lieve **ouders**, jullie zorgen tot op de dag van vandaag voor een goede basis en een veilige thuishaven. Jullie hebben me altijd de kans gegeven om mijn eigen weg te gaan en hebben me altijd gesteund in mijn keuzes. Ik prijs me daarmee zeer gelukkig. Heel veel dank voor het feit dat jullie deur altijd openstaat voor ons en onze meisjes en jullie onvoorwaardelijke steun en liefde.

Emma en **Julia**, jullie zijn de mooiste, liefste en leukste meisjes die ik ken. Ik geniet intens van elke dag met jullie samen, jullie verwondering is het mooiste wat er is. Vanaf nu heeft mama op haar mama-dag ook ècht tijd voor jullie: mama's boekje is af!

Vincent, mijn liefste, waar moet ik beginnen? Van basiscursus Excel tot de omslag van dit boekje, van ratten wegen tot borrellocatie, er is geen hoofdstuk in dit proefschrift waar je niet aan hebt bijgedragen. Niet alleen praktisch, maar zeker ook mentaal. Dank voor je onvoorwaardelijke steun. Jouw liefde is veel belangrijker dan dit proefschrift.

CURRICULUM VITAE

Evelien Zonneveld-Huijssoon werd op 4 maart 1976 geboren te Deventer. In 1994 behaalde zij haar VWO diploma aan het Eckart College te Eindhoven. Nadat zij in 1995 haar propedeuse Gezondheidswetenschappen behaalde aan de Universiteit Maastricht begon zij in datzelfde jaar met de studie Geneeskunde aan deze universiteit. Tijdens deze studie heeft zij zich onder andere ingezet als student-lid van de Opleidingscommissie Geneeskunde. Zij bracht voor de klinische stages Kinderoncologie en Obstetrie, enkele maanden door in Debrecen, Hongarije (Orvostudomanyi Egyetem) en São Paulo, Brazilië (Universidade Federal de São Paulo). In 1999 behaalde zij cum laude het doctoraalexamen en deed in 2000, in het kader van haar wetenschappelijke stage, gedurende drie maanden onderzoek in de Perinatologie onder begeleiding van Prof. dr. R.A. Brace, University of California San Diego (Californië, USA). Voor haar laatste keuze co-schap Kindergeneeskunde en Primary Health Care reisde zij in 2001 voor drie maanden af naar Fortaleza en de binnenlanden van de provincie Céara in Brazilië. In datzelfde jaar slaagde zij cum laude voor haar artsexamen, waarna zij gedurende een jaar werkzaam is geweest als arts-assistent Kindergeneeskunde in het Sint Antonius Ziekenhuis te Nieuwegein.

Vanaf 2002 werkte Evelien op de afdeling Pediatrische Immunologie van het UMC Utrecht (locatie Wilhelmina Kinderziekenhuis) aan het onderzoek dat geresulteerd heeft in dit proefschrift. Tijdens deze onderzoeksperiode bracht zij enkele maanden door in het laboratorium van Prof. dr. S. Albani aan de University of California te San Diego (Californië, USA). Daarnaast was zij enkele jaren als docent verbonden aan het opleidingscentrum van het UMC Utrecht, waar zij onderwijs verzorgde voor de opleidingen Physician Assistant en Anesthesiemedewerker. In 2007 begon zij aan de opleiding tot kinderarts in het UMC Utrecht (opleider: dr. J. Frenkel). In het kader van haar perifere stage was zij van juli 2007 tot december 2009 werkzaam in het Catharina Ziekenhuis te Eindhoven (opleider: dr. H.J.L. Brackel). Recent is zij van de opleiding tot kinderarts overgestapt naar de opleiding tot klinisch geneticus (opleider: Prof. dr V.V.A Knoers). Sinds oktober 2011 is zij werkzaam als arts in opleiding tot klinisch geneticus op de afdeling Medische Genetica van het UMC Utrecht.

Evelien is getrouwd met Vincent Zonneveld en samen hebben zij twee dochters, Emma (2006) en Julia (2009).

LIST OF CONTRIBUTORS AND INSTITUTIONS

Salvatore Albani
Sanford-Burnham Medical Research Institute, Translational Research Laboratory, Inflammatory and Infectious Disease Center, La Jolla, California, USA
EUREKA Institute for Translational Medicine, Siracusa, Italy

Stephen Anderton
School of Biological Sciences, University of Edinburgh, Institute of Immunology and Infection Research, Edinburgh, United Kingdom

Rosario Billetta
Androclus Therapeutics, San Diego, California, United States of America

Marianne Boes
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Eveline Delemarre
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Carla Engels
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Negar Ghahramani
Androclus Therapeutics, San Diego, California, United States of America

Arno Hoes
Julius Centre for Health Sciences and Primary Care, University Medical Centre Utrecht, the Netherlands

Wilco de Jager
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Mark Klein
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Fiona van der Klis
Laboratory of Vaccine Preventable Diseases, RIVM, Bilthoven, the Netherlands

Eva Koffeman
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Wietse Kuis
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Paola Lanza
Androclus Therapeutics, San Diego, California, United States of America

Tho Le
Department of Medicine and Pediatrics, University of California San Diego, La Jolla, California, United States of America

Jenny Meerding
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Jan Jaap van der Net
Paediatric Immunology, Wilhelmina Children's Hospital, University Medical Centre Utrecht, Utrecht, the Netherlands

Berent Prakken
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands
EUREKA Institute for Translational Medicine, Siracusa, Italy

Gisella Puga Yung
Department of Medicine and Pediatrics, University of California San Diego, La Jolla, California, United States of America

Eyal Raz
Department of Medicine, University of California, San Diego, La Jolla, California, USA

Ger Rijkers
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Arash Ronaghy
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Sarah Roord
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Marion van Rossum
Section Immunology, Haematology, Oncology, Bone Marrow Transplantation and Autoimmunity, Department of Paediatrics, Leiden University Medical Centre, the Netherlands
Department of Paediatric Immunology, Emma Children's Hospital AMC, Amsterdam, the Netherlands
Department of Rheumatology, Jan van Breemen Institute, Amsterdam, the Netherlands

Elisabeth Sanders
Paediatric Immunology, Wilhelmina Children's Hospital, University Medical Centre Utrecht, Utrecht, the Netherlands

Maarten van Tol
Section Immunology, Haematology, Oncology, Bone Marrow Transplantation and Autoimmunity, Department of Paediatrics, Leiden University Medical Centre, the Netherlands

Patricia Vermeer-de Bondt
Centre for Infectious Diseases and Epidemiology, RIVM, Bilthoven, the Netherlands

Femke van Wijk
Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

Nico Wulffraat

Department of Pediatric Immunology, Centre for Molecular and Cellular Intervention (CMCI), University Medical Centre Utrecht, Utrecht, the Netherlands

LIST OF PUBLICATIONS

Zonneveld-Huijssoon E, Albani SA, Prakken BJ, van Wijk F. Heat shock protein bystander antigens for peptide immunotherapy in autoimmune disease. *Clin Exp Imm, review under revision*

Zonneveld-Huijssoon E, Van Wijk F, Roord STA, Delemarre E, Meerding J, De Jager W, Klein M, Raz E, Albani S, Kuis W, Boes M, Prakken BJ. TLR9 agonist CpG enhances protective nasal HSP60 peptide vaccine efficacy in experimental autoimmune arthritis. *Ann Rheum Dis, in press.*

Zonneveld-Huijssoon E, Roord STA, de Jager W, Klein M, Albani S, Anderton SM, Kuis W, van Wijk F, Prakken BJ. Bystander suppression of experimental arthritis by nasal administration of a heat shock protein peptide. *Ann Rheum Dis.* 2011;70:2199-206.

Ronaghy A, de Jager W, Zonneveld-Huijssoon E, Klein M, van Wijk F, Rijkers G, Kuis W, Wulffraat NM, Prakken BJ. Vaccination leads to an aberrant FOXP3 T cell response in non-remitting Juvenile Idiopathic Arthritis. *Ann Rheum Dis.* 2011;70:2037-2043.

Heijstek MW, Pileggi GC, Zonneveld-Huijssoon E, Armbrust W, Hoppenreijs EP, Uiterwaal CS, Kuis W, Wulffraat NM. Safety of measles, mumps and rubella vaccination in juvenile idiopathic arthritis. *Ann Rheum Dis.* 2007 Oct;66:1384-7.

Zonneveld-Huijssoon E, Ronaghy A, Van Rossum MA, Rijkers GT, van der Klis FR, Sanders EA, Vermeer-De Bondt PE, Hoes AW, van der Net JJ, Engels C, Kuis W, Prakken BJ, Van Tol MJ, Wulffraat NM. Safety and efficacy of meningococcal c vaccination in juvenile idiopathic arthritis. *Arthritis Rheum.* 2007 Feb;56:639-46.

Roord ST, Zonneveld-Huijssoon E, Le T, Yung GP, Koffeman E, Ronaghy A, Ghahramani N, Lanza P, Billetta R, Prakken BJ, Albani S. Modulation of T cell function by combination of epitope specific and low dose anticytokine therapy controls autoimmune arthritis. *PLoS One.* 2006 Dec 20;1:e87.

Brace RA, Vermin ML, Huijssoon E. Regulation of amniotic fluid volume: intramembranous solute and volume fluxes in late gestation fetal sheep. *Am J Obstet Gynecol.* 2004 Sep;191:837-46.

Huijssoon E, Woerdeman PA, van Diemen-Steenvoorde RA, Hanlo PW, Plötz FB. An 8-year-old boy with a Pott's puffy tumor. *Int J Pediatr Otorhinolaryngol*. 2003 Sep;67:1023-6.