ISS immune modulation,

vaccination, and the regulation of arthritis

Controlling arthritis through vaccination

About the cover Five year old Geoffrey A. Sarte is holding my mother's hand. My mother suffers from arthritis. I dedicate this degree and everything I have done in the lab to my mother Farah Ronaghy. Without her hard work to create a stable environment for me to study, I would have never gone to college let alone attain a PhD.

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Controle van artritis door vaccinatie

Proefschrift

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op donderdag 4 januari 2007 des middags te 2.30 uur

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As a young child, I heard stories of angels. I imagined them floating to earth from the heavens with silver tipped wings, silhouetted in golden rays of sunlight. As I grew older I stopped believing. Now, I believe again. These angels have no wings, no golden rays. You just have to be fortunate enough to recognize them when they appear in your life. I have been lucky to come across more than one in mine. To them, I am forever grateful.

Arash Ronaghy

to those I love

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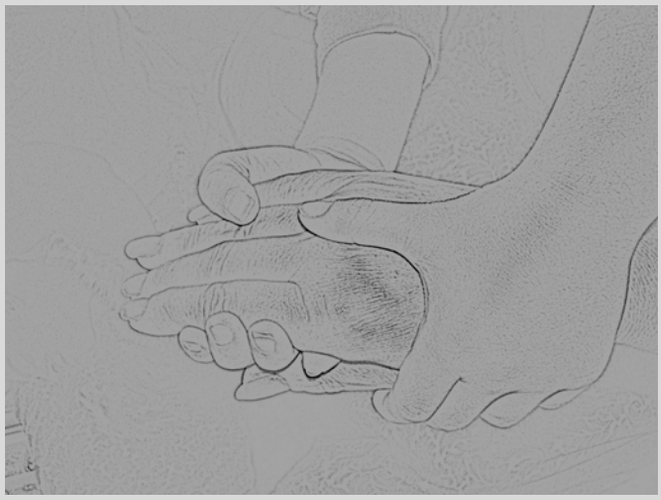

Chapter 1

General Introduction

Introduction

Immunity is generally defined as the inherited or acquired resistance to infection or cancer. The parts of the human body devoted to this function are collectively labeled as the immune system, and its reaction is called the immune response. The immune response consists of two main branches: innate and adaptive.

Innate Immunity

The first branch, called the innate immune response, is a more primitive and non-specific first line of defense shared by all species and it does not require prior exposure to the pathogen to be active. The cells involved in the innate immune response consist of granulocytes (eosinophils, basophils, and neutrophils), monocytes (including macrophages), dendritic cells (DCs), as well as natural killer (NK) cells. The innate immune system has two general functions. The first function is to sense and initiate the eradication of pathogens. The second function is to establish an adaptive immune response(1). Though the innate immune system is generally described as being non-specific, it is not completely so. Microorganisms possess molecules generated through metabolic pathways called microorganisms-associated molecular patterns (MAMPs). MAMPs, formerly known as pathogen-associated molecular patterns (PAMPs), are conserved throughout evolution as they are products of metabolic pathways essential for survival. Representative MAMPs include but are not limited to lipopeptides from the plasma membranes of mycoplasma as well as bacterial cell wall components such as LPS from gram negative and peptidoglycan from gram positive bacteria. The conservative nature of MAMPs has allowed the innate immune cells to evolve germline-encoded pattern-recognition receptors (PRRs) for MAMPs binding in order to carry out their dual function. PRRs are expressed constitutively in the host and detect the pathogens regardless of their life-cycle stage. Furthermore, they are expressed on all innate immune cells of a given type, and are independent of immunologic memory. Different PRRs react with specific MAMPs and result in distinct expression patterns, signaling pathways, and innate immune responses. The manner in which the innate immune cells recognize MAMPs are conserved from plants to fruit flies to mammals(2). PRRs can be functionally classified into signaling and non-signaling PRRs. Non-signaling PRRs include soluble factors (acute-phase proteins and lectins) as well as transmembrane proteins. Acute-phase proteins and lectins bind to invading organisms increasing their susceptibility to phagocytosis or complement recognition leading to protease cascade activation(1). Transmembrane proteins such as scavenger receptors bind, internalize, and transport microorganisms to lysosomal compartments without activating signaling cascades on innate immune cells. Cells of the innate immune system express PRRs that not only recognize microorganisms but also trigger pro-inflammatory cytokines such as TNF-alpha or IL-6 as well as type I IFNs. Among the PRRs exists a group of transmembrane receptors called Toll-like receptors (TLRs).

Toll-like receptors

Ten and twelve family members of TLRs exist in humans and mice, respectively. Their extra-cellular (binding) domain consists of a structure rich in leucine residues. The intra-cellular domain contains a common structure in the TLR and IL-1 receptor family members called Toll/IL-1 receptor homologous (TIR) domain that is essential for signal transduction. TLRs are potent activators of antigen presenting cells (APCs) and therefore possess sufficient adjuvant activity to subsequently skew the adaptive

immune response towards a Th1 subset. TLR4 recognizes lipopolysaccharide (LPS,) while TLR2, TLR1, and TLR6 recognize peptidoglycans, lipoproteins, or lipopeptides. Furthermore, TLR5 recognizes flagellin proteins from flagellated bacteria. While single or double stranded bacterial or viral nucleic acids are recognized by TLR 7 and TLR3 respectively.

TLR9 adjuvant activity is mediated through the recognition of unmethylated CpG motifs of bacterial or viral DNA. In the murine system TLR9 is expressed in conventional dendritic cells (cDCs), a subset of cDCs called plasmacytoid dendritic cells (pCDs), as well as B cells. However, human TLR9 expression is restricted to the latter two cell types. Unlike the murine system, in the human cDCs, CpGs are shuttled to the lysosome and undergo rapid degradation(3). Furthermore, upon CpG activation, human pDCs produce only IFN-alpha while our murine counterparts also secrete IL-12. When TLR9, located on the endosome, binds CpG DNA signal transduction molecules such as MyD88 and IFN regulatory factor-7 (IRF-7) are activated resulting in the production of type I IFNs (alpha and beta) as well as Nuclear Factor kappa B (NF-kappaB) mediated pro-inflammatory cytokines. The effect of TLR9 activation depends on the type of agonist. There are three distinct classes of synthetic oligonucleotides containing CpG motifs called Immunostimulatory DNA Sequences (ISS). These will be discussed below in the ISS section.

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Adaptive Immunity

The second branch, called the adaptive immune response, is a more sophisticated, specific, second line of defense present in vertebrates. Unlike the innate response, this response requires prior exposure to the foreign pathogen. Such a response is sometimes called a “memory response” alluding to the immune system’s ability to “remember” prior exposure to the pathogen and therefore fight against it more efficiently upon future encounters. The cells involved in this adaptive response include lymphocytes such as B and T cells. Vaccination is the most effective way to evoke a long-lasting protective (adaptive) “memory” response against pathogens without risking what may be a dangerous and potentially life threatening illness.

Manipulating the Immune Response through Vaccination

Smallpox (called *Variola* or *Variola vera* in Latin) is a highly contagious viral disease unique to humans and has been endemic in densely populated areas of China in ancient times (4). Vaccination with smallpox was termed *variolation* and dates back to the 10th century in China. The process of *variolation* involved blowing smallpox crusts from infected persons into the nostril of those who were not(2). This early form of immune intervention was brought from Constantinople to England in 1721 by Lady Mary Wortley Montague, wife of the British Ambassador to Turkey (5). In Britain, Europe, and the American Colonies the preferred method of *variolation* was rubbing pustular material from one afflicted with a mild case of smallpox (*Variola minor)* into a scratch between the thumb and forefinger of an individual not yet exposed. Subsequently, the more modern practice of vaccination with a less virulent form of the virus, in this case cowpox, (*Vaccinia* from the Latin word for cow: *vacca*) was demonstrated by Edward Jenner in 1796 (6).

Today vaccines are largely constructed in two forms: live attenuated or subunit based. Live, attenuated vaccines are able to induce a stronger and longer lasting immune response that mimics a natural infection (7). Initially, a live-virulent form of the smallpox was used as a vaccine with subsequent natural infection and death as

possible side effects (8;9). To avoid this, subunit or peptide based vaccines were created (10). These non-replicating vaccines are preferred for their safety especially in immunocompromised individuals. However, they may lack optimal immunogenicity by themselves and as a result require an adjuvant (11).

Adjuvant

Adjuvant comes from the Latin word "adjuvans", which means to help—particularly to reach a goal. Immunologists describe an adjuvant as a substance that enhances the immune response to an antigen. This is important when small, antigenic substances (haptens) are not able to induce immune reactivity by themselves and require an adjuvant to activate the immune system. Adjuvants are necessary to optimally activate and direct the innate and adaptive immune responses to potentially poor immunogens of a vaccine (11).

The most common adjuvants for human use today are still aluminum hydroxide and aluminum phosphate, although calcium phosphate and oil emulsions have some applications (11).

In addition, bacteria or their components may have “adjuvant like” effects on the immune response. Examples of microbial components used as adjuvants include lipopolysaccharide (LPS) as well as bacterial super antigens, such as staphylococcal enterotoxins A and B (12). The former induce monocytes to release IL-1 and TNF-alpha while the latter preferentially stimulate the proliferation of B and T cells. However, their effects are not long lasting (13). Recently, bacterial DNA sequences have emerged as important regulatory adjuvants of the immune response.

Immunostimulatory DNA Sequences (ISS)

Over the last decade the possible role of bacterial DNA for vaccine development has gained a lot of interest in the scientific community. Bacterial DNA contains unique unmethylated CpG motifs. The cytosine-phosphate-guanisine (CpG) sequence of this motif has an unmethylated cytosine which is suppressed or methylated in mammalian DNA. Synthetic oligodeoxynucleotides containing these CpG (CpG-ODN) motifs are called Immunostimulatory DNA Sequences (ISS)(14;15). These CpG motifs have a strong capacity to activate and/or regulate the immune system (14;15). When co-administered with an antigen, ISS activates antigen presenting cells (APCs) in a manner which ultimately result in the differentiation of Th1 cells, increased cytotoxic T lymphocyte (CTL) activity, IgG isotype switching, and suppression of IgE antibody secretion (Table 1).

ISS differ from the bacterial superantigens and LPS in two ways. First, they require coadministration with an antigen and they interact with intracellular targets. Also as DNA, ISS can persist in cells in an un-integrated state for a long time allowing for prolonged activation of the immune system(15).

There are three classes of CpGs with slightly different structures and biological activities. The first is called CpG-B, K-type CpG DNA, or conventional CpG DNA containing multiple CpG motifs and its activity is stabilized by a phosphorothioate backbone. This CpG-B type ISS has a potent ability to induce macrophage cytokine production as well as activate B cells. Its activity is dependent on its physical presence in the endosomal compartment of DCs. As a result, in the human system its activity is restricted to human B cells and human pDCs and not cDCs. In cDCs the CpG-B is transferred to the lysosomal compartment for rapid degradation. Interestingly, human cDCs activation can be induced if the CpG-B is modified to remain in the endosome via cationic lipid alteration(1). The second type of CpG is

called CpG-A or D-type CpG DNA containing a single CpG motif with a mixture of phosphorothioate-phosphodiester backbone. The region around the CpG motif is palindromic and phosphodiester linked with a poly-G tail at the 3 prime end. The CpG-A is localized to the endosome and results in a stronger ability to induce type I IFN production from pDCs but with a weaker ability to activate B cells and macrophages. The third type is called CpG-C or C-type CpG DNA containing multiple CpG motifs with a phosphorothioate backbone and a TCG dimer at the five prime end. The ISS used in the experiments of this thesis were of the CpG-B type. The role of ISS with regards to immunity and autoimmunity will be discussed later.

Preventing Chronic Inflammation and Autoimmunity

A strong immune inflammatory response initiated either by a natural infection or a subunit-adjuvant vaccine needs to be tightly regulated to prevent chronic inflammation. The process of immune regulation to minimize collateral damage to self (e.g., during the course of an immune response to microbial antigens) is generally termed immune tolerance. In this context, tolerance can be defined by any mechanism in which antigen recognition is not followed by an immune response devoted to its destruction. This process is essential for keeping the powerful immune response against microbial antigens at bay and preventing the immune system from responding to self antigens in a harmful manner. These mechanisms of tolerance can be either passive or active. Passive mechanisms include thymic deletion of potentially autoreactive cells. However, this process of thymic deletion is not 100% effective and will result in the escape of potentially autoreactive T cells from the thymus into the periphery. Activation of these cells, and subsequent self-injury, is prevented in various ways. First, they may physically be isolated from their autoantigen. For example, they may be located in certain immune privileged areas, such as the lens of the eye. Furthermore, they may possess T cell receptor (TCR) with low avidity for the self-antigen. Therefore, the binding of the TCR with the self antigen may not be strong enough to induce activation. This, in addition to a lack of secondary co-stimulatory signal may induce a state of anergy or deletion of these potentially pathogenic T cells. Moreover, in addition to these passive mechanisms of tolerance, more evidence points to a potentially dominant mechanism of active suppression. This process involves a subset of T cells called regulatory T cells (Tregs) (16-18).

Regulatory T Cells

The presence of T cells with a regulatory potential was first discovered in the early 1970s (19). These cells were $CD8^+$ T cells, with suppressive *in vitro* capacity. Interest in them waned, as it was difficult to understand their role *in vivo*. New interest in T cells with an immune regulatory function appeared with the discovery that a group of $CD4^+$ T cells, initially characterized by the high expression of the IL-2 alpha receptor (CD25) on their cell surface, played an important role in the maintenance of the immune homeostasis in animal models. It is now clear that $CD4^+$ Tregs are involved in immune regulation (20-31). They were first identified in animal models, but they are also part of the human immune repertoire (30;32-41). $CD4^+$ Tregs can largely be divided into two subtypes: naturally occurring $CD4^+CD25^+$ Tregs (nTregs) or induced Tregs (iTregs) in the periphery. Both are discussed below.

Furthermore, it is now recognized that other T cells types can have regulatory functions such as Cytotoxic ($CD8^+$) T cells, NK T cells, as well as gamma delta T cells subsets (30;31;42-48).

Naturally occurring $CD4^+CD25^+$ regulatory T cells

$CD4^+CD25^+$ Tregs are present in the periphery of every healthy individual and therefore referred to as naturally occurring Tregs (nTregs). nTregs were first discovered in mice. It was observed that mouse thymectomy on neonatal day three led to a multi-organ autoimmune disease(49). T cell precursors, including $CD4^+CD25^+$ Treg precursors, normally migrate from the bone marrow to the thymus where maturation occurs. Without the thymus, this process in hindered leading to a deficiency in T cells including Tregs resulting in autoimmune pathology. Sakaguchi and others have shown that depletion of $CD4^+CD25^+$ regulatory T cells resulted in severe systemic autoimmune disease in neonatally thymectomized mice (49;50). Furthermore, transfer of $CD25^-$ T cells into nude mice resulted in specific autoimmune diseases such as colitis, type I diabetes, gastritis and thyroiditis (25;49;51;52). Conversely, the coinjection of $CD4^+CD25^+$ T cells prevented these autoimmune pathologies. The removal of $CD4^+CD25^+$ T cells enhances the immune response to foreign antigens. Xenogeneic as well as allogeneic graft tolerance can be induced with the infusion of large doses of $CD4^+CD25^+$ T cells. Collectively, this suggests that $CD4^+CD25^+$ T cells help suppress the immune response.

However, suppressing the immune response too much may also be deleterious by increasing the risk to infections (53) as well as by decreasing anti-tumor activity (54;55).

$CD4^+CD25^+$ Tregs are present in the periphery of every healthy individual and therefore referred to as naturally occurring Tregs (nTregs). Also in humans, nTregs develop in the thymus, which may be the result of an escape from negative selection (56). They represent 1-3% of the fraction of $CD4^+$ T cells and constitutively express CD25, CTLA associated Antigen-4 (CTLA4), the glucocorticoid induced tumor-necrosis factor receptor (GITR), as well as the transcription factor FoxP3. The suppressive capacity of these nTregs are believed to be due to a cell-to-cell contact mechanism (42;57;58). Their activation is antigen specific. However, once activated they suppress $CD4^+$ and $CD8^+$ T cells in an antigen independent manner (59). The problem of working with T regulatory cells has always been the lack of specific markers. Markers like CTLA4 are also upregulated on activated non-regulatory T cells. However, FoxP3 has proven to be more specifically upregulated on $CD4^+CD25^+$ Tregs. The FoxP3 gene encodes a DNA-binding protein of the forkhead/winged-helix family and is involved in the development of $CD4^+CD25^+$ regulatory T cells. Mutation in FoxP3 has been associated with the rare multi-organ autoimmune disorder called immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX). While much of the above information has been extrapolated from experimental models, data on how human nTregs are influenced by environmental triggers such as a vaccine are still lacking.

Induced Regulatory T cells

In addition to the role of nTregs in maintaining tolerance to self-antigens, there is evidence that a distinct set of Tregs can be induced in the periphery after antigen encounter. A variety of protocols have been used to generate these induced Tregs (iTregs) (31;47;60;61). They are generally induced in a tolerogenic or anti-inflammatory environment producing such anti-inflammatory cytokines such as IL-10 and/or TGF-beta. Unlike the nTregs which require cell-to-cell contact, iTregs exhibit their function through the above mentioned cytokines. There are two types of iTregs: TR1 cells and Th3 cells. The former secrete high levels of IL-10 (47;60) while the latter secrete high levels of TGF-beta (31;61). This thesis will focus on the nTregs.

The Balancing Act

A healthy immune system can be expected to undergo activation as well as regulation depending on the type, dose, location, and local environment of the antigen. This balancing act is vital as dysregulation may result in either too much activation leading to chronic inflammation (such as chronic arthritis), or too little, leading to potential infections and increased cancer risk. In a healthy individual, nTregs function to downregulate the normal immune response once the inciting pathogen has been cleared away. In patients with autoimmune disorders, their pathology may be due either to lower numbers of Tregs or an inability of their Tregs to function properly. This imbalance may lead to chronic inflammatory diseases, such as Juvenile Idiopathic Arthritis.

Juvenile Idiopathic Arthritis

Juvenile idiopathic arthritis (JIA) is also called juvenile arthritis (JA), juvenile rheumatoid arthritis (JRA), or juvenile chronic arthritis (JCA). It is the most common rheumatic disease in children with an incidence of 1 in 1,000 (62-64). It is defined as chronic arthritic conditions lasting for a minimum of three months affecting children under the age of 16 years. JIA is often characterized by a waxing and waning course, with flares separated by periods of time during which no symptoms of active synovitis are noted (remission). This is especially the case in the Oligoarticular subtype (OA JIA) that is characterized by prolonged periods of medication-free remission of the disease. This has led to speculation that in subtypes of JIA with a favorable prognosis, a counter-regulatory immune response is operational (see more below).

Although the exact mechanism is still unknown, it is generally assumed that the joint destruction in JIA is due to an abnormal host response to a normal environmental trigger in a genetically predisposed individual. This trigger initiates a series of events that lead to sustained inflammation of the synovial lining eventually leading to severe functional disability in 49% of the patients (65). This sustained inflammation and resulting pannus is composed of activated macrophages and fibroblasts.

It is generally accepted that JIA has an immunological origin and, as mentioned above, may be due in part to the consequence of an abnormal response to a microbial trigger. Several lines of evidence suggest this. First, the presence of immune complexes attests to the previous exposure to bacterial or parasitic pathogens. Complement split products and the presence of autoantibodies such as rheumatoid factor or antinuclear antibodies also suggest autoimmune pathology in JIA. Furthermore, the HLA restriction to certain HLA-alleles suggests autoimmune T cell pathology. Finally, there is an accumulation of activated lymphocytes and monocytes in the joint.

Thus, in several aspects, JIA resembles an infectious disease with an autoimmune component. As no infectious organisms have been found, it is hypothesized that JIA is triggered by a transient exposure to bacteria or virus via a “hit & run” mechanism and perpetuated by an abnormal host response in predisposed individuals. As a result, the challenge is to understand how a “normal” trigger, such as foreign antigens, interacts with cellular components in genetically predisposed individuals to induce an abnormal synovial environment that may lead to chronic inflammation. Bacteria are not only an exogenous source of antigens that may cross-react with self-antigens and thus induce autoimmunity, they may also exert adjuvant effects on the ongoing immune response.

Bacterial components exert adjuvant effects that are not long lasting
Indeed, bacterial components cannot only induce an antigenic immune response, but also a non-specific adjuvant effect on the on going immune response. For example, LPS can activate monocytes to produce IL-1 and TNF-alpha. Furthermore, bacterial super antigens exert mitogenic effects on B and T cells. Both of these events may initiate chronic inflammation, but under normal circumstances they do not sustain it.

ISS is a different type of adjuvant.
Compared to LPS and bacterial super antigens, ISS are less immunogenic and interact with intra-cellular targets such as the TLR9 receptor on the endosome. Having an intra-cellular ligand allows ISS to be hidden from the immune system. The combination of a lower immunogenicity, and being hidden from the immune system, allows for the ISS to persist within the cell for a prolonged period of time. As a result, the Th1 priming effect of the ISS lasts much longer when compared to other adjuvants, resulting in a higher Th1 to Th2 memory T cell ratio. Once this is established, protein boosting that normally skews the immune response to a Th2 phenotype, is unable to do so if the immune cells were pre-exposed to ISS in the presence of antigen. As a result, bacterial ISS can initiate a stable delayed hypersensitivity reaction to antigens normally found on the bacteria of the skin and gastrointestinal tract.

Regulatory T cells in OA JIA
In JIA the joint damage is believed to be the result of a prolonged Th1 immune response. This inflammatory immune response includes activation of B cells, complement consumption and the production of pro-inflammatory cytokines such as TNF-alpha, IL-6, and IL-1beta (66-68). The synovial infiltrate of the JIA patients consists mainly of Th1 type cells and underscores the skewed Th1 type immune response. As mentioned above, some forms of JIA have a remarkably good prognosis, suggesting some kind of a functional counter-regulatory mechanism. Despite this predominance of a Th1 skewed immune response, an immune component is present in these patients that favor the downregulation of inflammation. This is especially true in the early stages of disease of the OA JIA with the presence of a Th2 component as measured by IL-4 and IL-10 (69-71).
In addition to Th2 T cells, other T cells with a regulatory capacity have been suggested to play a role in JIA. For example Thompson, *et al*. demonstrated increased expression of CCR5 and CCR4 of synovial fluid bearing lymphocytes. CCR5 is associated with a Th1 cytokine profile and is an activation marker while CCR4 is associated with a Th2 cytokine profile and is present on the surface of lymphocytes expressing higher levels of IL-4 than IFN-gamma. Therefore, CCR4 is preferentially expressed on and involved in the trafficking of Th2 like cells and suggested as a chemokine receptor for regulatory cells. Furthermore, human heat shock protein 60 (HSP60) specific T cells have been shown to play a regulatory role in JIA.

HSP and JIA

HSPs are intracellular molecules that are involved in cytoprotective and housekeeping functions (72). They can be released into the extracellular environment. This has created an interest in their potential role as intercellular signaling molecules (73;74). Furthermore, HSPs themselves are immunodominant (75-77) and are classified into six families based on their molecular weight: HSP10, HSP40, HSP60, HSP70, HSP90, HSP100 (72). Not only are HSPs highly conserved, ubiquitous, and essential for cell survival, but their expression is upregulated during times of environmental stress such as hypoxia, hyper-, and hypothermia (78). In recently diagnosed JIA patients, self HSP60 specific T cell responses correlated with a better prognosis. As a result, it was postulated that self HSP60 may induce T cells to suppress the inflammatory response in JIA.

Molecular Mimicry

The conserved, stress-induced upregulation, and immunodominant qualities of HSPs led to the idea that, during an infection, microbial and endogenous HSPs could initiate a potential cross-reactive immune response. This molecular mimicry hypothesis postulates the induction of auto-reactive lymphocytes through receptor mediated engagement via structurally similar human and microbial antigenic epitopes (78). This led some to suggest HSPs as potential disease-inducing antigens in experimental models of autoimmune disorders (79).

However, analysis of the HSP induced immune response in the same autoimmune experimental models showed that the T cell reactivity to self HSP can downregulate the disease process. Most of the evidence regarding the role of HSP-specific T cells in suppressing the immune response comes from to the adjuvant arthritis (AA) experimental model. As discussed above, pre-immunization with mycobacterial HSP60 prevents arthritis induction due to potential cross-reactivity for self HSP60 which can decrease chronic inflammation (80-82). Indeed, Anderton, *et al*., demonstrated that cross-reactivity between self and foreign HSP protected against AA (83), a protective quality that seems to be unique for HSP and not a general characteristic of conserved immunodominant antigens (84). These protective effects of self HSP60 specific T cells are not limited to the AA experimental model (85-90).

Immune reactivity to self HSP may be an essential part of the counter-regulatory feedback of the immune system. During a microbial infection, the immune system reacts by inducing proliferation of T cells specific to foreign antigens (including HSPs) required for optimal host defense. The resulting pro-inflammatory microenvironment induces upregulation in the expression of self HSPs. T cells responding to the conserved sequences of the foreign HSP may be the very same T cells that cross-react to self homologs and subsequently exert a regulatory function. Interestingly, there are clear similarities between JIA and AA. In addition to the histopathological resemblance, HSPs play a role in immune regulation of both diseases. The synovial lining cells of the JIA patients increase expression of endogenous human HSP60 resulting in the formation of anti-HSP60 IgG antibodies as well as T cells reacting to human and mycobacterial HSP60 in the blood and synovial fluid (91-93). T cell reactivity is not restricted to human HSP60 and reacts against *E. coli* HSP60 (GroEL) and DnaJ. Similar mechanisms may be in place in rheumatoid arthritis. However, their regulatory mechanism is unable to sufficiently downregulate the ongoing inflammation. Additional experiments are needed to better understand the cellular and molecular mechanism of action of HSPs.

Does ISS play a role in arthritis?
An adaptive immune response towards conserved microbial antigens may be acting as a counter-regulatory mechanism in human arthritis. With regards to the earlier mentioned ISS bacterial DNA with clear pro-inflammatory characteristics, to date there is no proof that ISS is directly involved in human arthritis. However, it has been suggested that bacterial DNA, which persists in the joint of patients with rheumatoid arthritis, may contribute to the chronic inflammation.

ISS and arthritis
Extrapolating from animal studies to humans may be overly simplistic. Despite this, all antigen induced experimental models require co-administration of a macrophage activating adjuvant such as complete Freund's adjuvant (CFA). ISS has been shown to mimic CFA in several animal models. Moreover, ISS exacerbates autoimmunity in a septic model of arthritis (94). Unlike the normal Th2 response to antigen alone, when the same antigen is exposed to the immune system in the presence of ISS, macrophages and other APCs secrete IL-12 which differentiates Th0 to Th1 memory cells. So, it is conceivable to believe ISS may exacerbate arthritis in genetically predisposed individuals. In summary, at disease onset the non-infectious ISS and monocyte precursors of the synovial lining find their way to the joint, initiating localized hypersensitivity reactions. Dead neutrophils release 0_2 radicals inside the confined synovium and facilitate the formation of ISS from human DNA. Despite the decreased immunogenicity of oxidized human DNA when compared to bacterial ISS, they may still raise the immunoinhibitory sequence ratio to a critical threshold sufficient to maintain a synovial pannus.

Table 1. Cellular and Humoral activation

Immune System		Cellular	Humoral
Innate	APC (Macrophage, DC)	Increase activity	IFNα, β, γ IL1, IL6,IL12, IL18 TNFα
Adaptive	CD4+ T cell CD8+ T cell	Th1 differentiation Increase CTL activityγ	IFNγ
	B cell	Express IgG Supress IgE	Secrete IgG Secrete IgE

IFN; Interferon, TNF; Tumor Necrosis Factor, IL; Interleukin, CTL; Cytotoxic T Lymphocyte

Aim of study

Vaccination is a powerful tool to enhance the immune response towards optimal protection not only in healthy individuals, but also in individuals with some type of immune pathology. One may question if ISS can be used as a potential adjuvant for vaccination in both healthy as well as arthritis prone individuals? Despite many scattered clinical studies the question still remains whether vaccination may hold the risk of developing an unregulated immune response in those who are arthritis prone, leading to subsequent exacerbation of their autoimmune disease. If vaccination may expose certain predisposed individuals to this risk, how does this risk weigh against the consequences of undergoing the natural infection?

In addition, modulating the type of immune response has powerful therapeutic benefits. For example, those afflicted with an autoimmune disease would benefit from a shifting of their immune responses towards a Th2 type phenotype or those with allergy towards a Th1 type. Thus, using adjuvants such as ISS to skew the immune response could complement antigen specific immune therapy. To achieve this goal, a better understanding is necessary of how the immune system is modulated by ISS.

Outline of thesis

In this study our goal is to gain a better understanding of how ISS modulates the immune response and how this response may influence chronic inflammation in those predisposed to arthritis. The first part of this thesis investigates the potential candidacy of ISS as a future vaccine adjuvant by investigating the Th1 promoting properties of ISS both when administered systemically as well as at mucosal sites. Furthermore, we studied the influence of ISS on the course of arthritis in an experimental animal model such as AA. The second part of this thesis explores the safety and efficacy of the MenC vaccine in the JIA population, addressing the issue of whether an immune response to a bacterial antigen may harbor the risk of exacerbating autoimmunity in susceptible individuals. Therefore, we also investigated the quality and quantity of the immune response elicited by the OA JIA as well as PA JIA after vaccination towards vaccine and arthritis associated antigens including their regulatory mechanisms.

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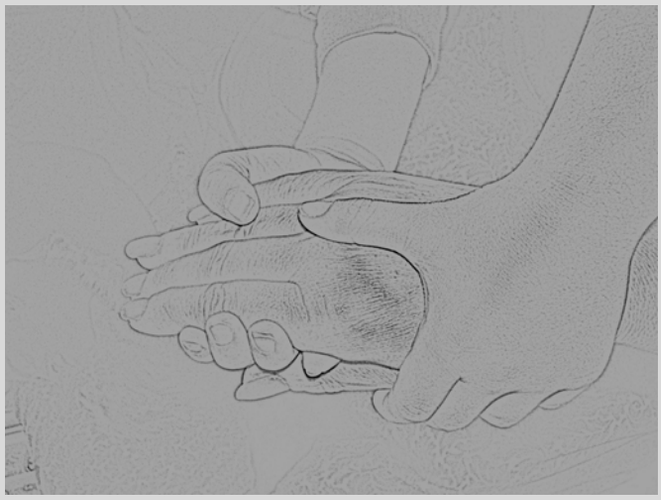

Chapter 2

Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants

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Immunostimulatory DNA sequences function as T helper-1-promoting adjuvants

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An adjuvant role for certain short bacterial immunostimulatory DNA sequences (ISSs) has recently been proposed on the basis of their ability to stimulate T helper-1 (Th1) responses in gene-vaccinated animals. We report here that noncoding, ISS-enriched plasmid DNAs or ISS oligonucleotides (ISS-ODNs) potently stimulate immune responses to coadministered antigens. The ISS-DNAs suppress IgE synthesis, but promote IgG and interferon-γ (IFN–γ) production. They furthermore initiate the production of IFN–γ, IFN-α, IFN-β, and interleukins 12 and 18, all of which foster Th1 responses and enhance cell-mediated immunity. Consideration should be given to adding noncoding DNA adjuvants to inactivated or subunit viral vaccines that, by themselves, provide only partial protection from infection.

Subunit or inactivated virus vaccines typically induce T helper-2 (Th2) immune responses, with high titers of neutralizing antibodies but without significant cell-mediated immunity. In contrast, intradermal and intramuscular vaccination with naked pDNA stimulates immune responses with a Th1 bias[1–4], with the expansion of $CD4^+$ T cells producing IFN-γ and cytotoxic $CD8^+$ T cells. The Th1 response to gene vaccination is maximal when the pDNA backbone contains short (6 base pairs) immunostimulatory DNA sequences (ISSs) with CpG motifs[5]. These sequences are 20 times as common in bacterial as in mammalian DNA (ref. 6). Simultaneous injection of ISS-deficient pDNA encoding a nominal antigen with a noncoding, ISS-enriched pDNA also fosters a Th1 response, indicating that the ISS-enriched pDNA exerts an adjuvant effect[5]. However, gene vaccines generally stimulate antibody production inefficiently when compared with matching protein vaccines. This deficiency probably results from the small number of transfected and/or antigen-producing cells, and from the inhibition of antigen synthesis by IFN-α and IFN-β induced by the ISSs in the pDNA backbone[5].

Given that the ISSs in pDNA have an immunostimulatory (adjuvant) effect in gene-vaccinated animals, we hypothesized that ISS-enriched pDNA or ISS-oligodeoxynucleotide (ISS-ODN) might also deliver the necessary stimulatory signals for Th1 induction to a coinjected protein antigen. In principle, protein/ISS-pDNA or protein/ISS-ODN coadministration could combine the advantages of the Th1 response achieved by naked DNA immunization with the high levels of antibody generated by protein vaccines. Experiments were therefore designed to determine whether the coadministration strategy could successfully improve upon the outcome of either gene or protein vaccination, as well as to establish the underlying immunostimulatory mechanisms of the DNA adjuvant[7].

Immunostimulatory DNA sequences enhance anti-β-gal antibody response

Mice were injected intradermally once with β-galactosidase (β-gal) protein, together with either genomic *Escherichia coli* or calf thymus DNA. Antibody isotype responses to the injected antigen were then assessed by enzyme-based immunosorbent assay (ELISA). Coadministered bacterial (*E. coli*) DNA enhanced IgG2a anti-β-gal antibody production, suggestive of a Th1 response, whereas calf thymus DNA had no effect (Fig. 1*a*). To identify DNA sequences that induced or enhanced the IgG2a response, mice were immunized with various pDNAs as adjuvants. The ampicillin resistance gene (*ampR*) contains two repeats of the 5'–AACGTT–3' ISS; the kanamycin resistance gene (*kanR*) contains none (Fig. 2). The coadministration of the noncoding kanR-based vector, pKCB, with β-gal induced no higher levels of IgG2α than β-gal alone; however, coinjecting the noncoding ampR-based vectors, pACB or pUC19, with β-gal generated high levels of IgG2a (Fig. 1*b*).

To determine the role of the 5'–AACGTT–3' ISS on anti-β-gal IgG2a production, the CMV promoter region (which contains other potential ISSs, Fig. 2), through the poly(A)$^+$ tail sequence of the parental pKCB vector was deleted, and the resultant vector underwent various modifications. A new set of vectors, the pKISS-0 through pKISS-3, was then created, which have zero to three repeats of the 5'–AACGTT–3' ISS, respectively (K for *kanR* gene and ISS for immunostimulatory DNA sequences). The coinjection of pKISS-0 with β-gal did not promote IgG2a production, whereas the coinjection of pKISS-1 through pKISS-3 with the antigen induced anti-β-gal IgG2a levels equivalent to those obtained with the codelivery of the ampR-based vectors pACB or pUC19 (Fig. 1*c*). The coinjection of β-gal with single-stranded ISS phosphorothioate ODN strongly enhanced the basal anti-β-gal

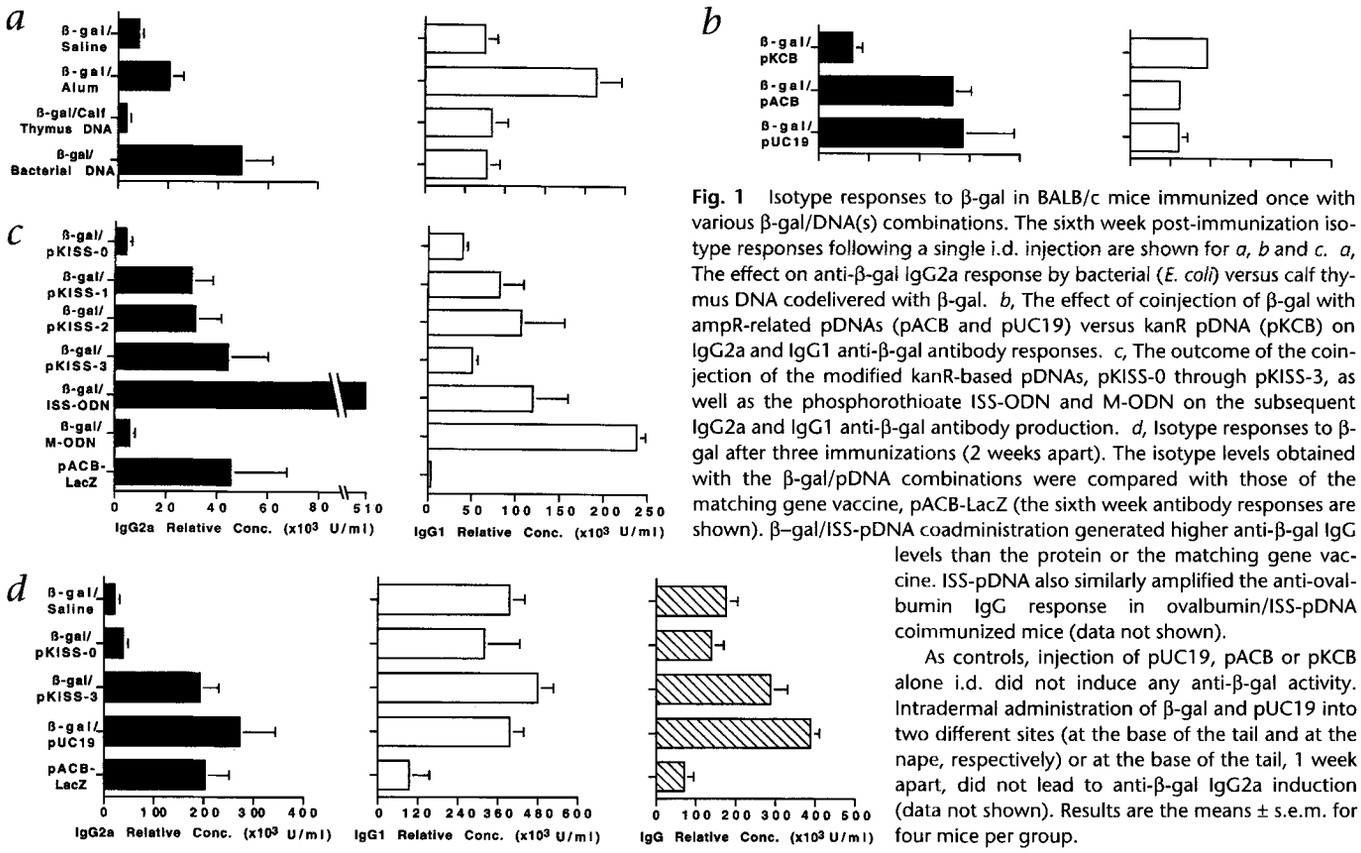

Fig. 1 Isotype responses to β-gal in BALB/c mice immunized once with various β-gal/DNA(s) combinations. The sixth week post-immunization isotype responses following a single i.d. injection are shown for *a*, *b* and *c*. *a*, The effect on anti-β-gal IgG2a response by bacterial (*E. coli*) versus calf thymus DNA codelivered with β-gal. *b*, The effect of coinjection of β-gal with ampR-related pDNAs (pACB and pUC19) versus kanR pDNA (pKCB) on IgG2a and IgG1 anti-β-gal antibody responses. *c*, The outcome of the coinjection of the modified kanR-based pDNAs, pKISS-0 through pKISS-3, as well as the phosphorothioate ISS-ODN and M-ODN on the subsequent IgG2a and IgG1 anti-β-gal antibody production. *d*, Isotype responses to β-gal after three immunizations (2 weeks apart). The isotype levels obtained with the β-gal/pDNA combinations were compared with those of the matching gene vaccine, pACB-LacZ (the sixth week antibody responses are shown). β–gal/ISS-pDNA coadministration generated higher anti-β-gal IgG levels than the protein or the matching gene vaccine. ISS-pDNA also similarly amplified the anti-ovalbumin IgG response in ovalbumin/ISS-pDNA coimmunized mice (data not shown).

As controls, injection of pUC19, pACB or pKCB alone i.d. did not induce any anti-β-gal activity. Intradermal administration of β-gal and pUC19 into two different sites (at the base of the tail and at the nape, respectively) or at the base of the tail, 1 week apart, did not lead to anti-β-gal IgG2a induction (data not shown). Results are the means ± s.e.m. for four mice per group.

IgG2a levels induced by β-gal alone; however, the coinjected mutated (M) ODN (in which each CG of the ISS-ODN was replaced with GG) did not. In contrast the coinjection of β-gal with ISS phosphodiester ODN did not alter the basal anti-β-gal IgG2a levels, probably because of the short half-life of the phosphodiester ODNs *in vivo* (data not shown). Immunization of animals three times with β-gal/ISS-pDNAs amplified their anti-β-gal IgG2a antibodies (9-fold for pKISS-3 and 12.5-fold for pUC19) in comparison with β-gal alone or with pKISS-0, the ISS-deficient pDNA (Fig. 1*d*). The coadministration of β-gal with the various ISS-pDNAs elicited higher anti-β-gal IgG antibodies than β-gal alone or the matching gene vaccine, pACB-LacZ (Fig. 1*d*).

ISS-DNAs enhance anti-hemagglutinin antibody response

The addition of pKISS-3 or ISS phosphorothioate ODN to a commercial, inactivated subunit human influenza vaccine upregulated by three and by eightfold, respectively, the IgG antibody responses to the viral hemagglutinins (HGNs) (Fig. 3). These results established that the adjuvant effect of ISS-DNAs (ODN or pDNA) also applies to common vaccines in clinical use.

Cytokine profile of b-gal/Iss-DNAs injected mice

T helper-1-type cells characteristically produce IFN-γ after antigen restimulation[8]. Protein/ISS-pDNA coimmunization induced this pattern of cytokine release (Table 1). Antigen-stimulated $CD4^+$ splenocytes from mice immunized with β-gal/pKISS-3 and β-gal/pUC19 generated high levels of IFN-γ, whereas $CD4^+$ cells from mice immunized with β-gal or β-gal/pKISS-0 synthesized only trace amounts. Compared with $CD4^+$ cells from gene-vaccinated mice, the T cells from protein/ISS-pDNA covaccinated animals produced higher levels of IL-4 (Table 1). However, the ratio of IFN-γ to IL-4 was consistent with a shift of the anti-β-gal response toward a Th1 phenotype.

ISS-DNAs suppress IgE antibody formation

Immunoglobulin E production is a consequence of extreme Th2 responses[8] and can lead to potentially fatal anaphylactic reactions to protein vaccines. To evaluate whether Th1 stimulation by antigen/ISS-DNAs (ODN or pDNA) coimmunization could inhibit the subsequent anti-β-gal IgE synthesis, mice were first primed with β-gal/pKISS-0, β-gal/pKISS-3, β-gal/M-ODN (phosphorothioate), β-gal/ISS-ODN (phosphorothioate), or saline. Mice were then boosted intraperitoneally 4 weeks later with β-gal in alum. The mice primed with β-gal/ISS-DNAs were "resistant" to IgE inductions as they did not generate any significant anti-β-

Table 1 Cytokine profile of *in vitro* β-gal-stimulated $CD4^+$ splenocytes from mice immunized with β-gal, β-gal/pDNA combinations or with the matching gene vaccine, pACB-LacZ

Immunization	mIL-4 (pg/ml)	mIFN-γ (ng/ml)
Naive	<10	<4
pKISS-3	<15	<10
β-gal	158 ± 60	9 ± 4
β-gal/pKISS-O	144 ± 57	12 ± 6
β-gal/pKISS-3	136 ± 46	54 ± 16
β-gal/pUC19	167 ± 51	95 ± 11
pACB-LacZ	16 ± 3	270 ± 108

Mice were killed 2 weeks after the last immunization. Only background levels of IFN-γ or IL-4 were detected in the supernatants of $CD4^+$ splenocytes unstimulated *in vitro* with β-gal. Results are the means ± s.e.m. of four mice per group.

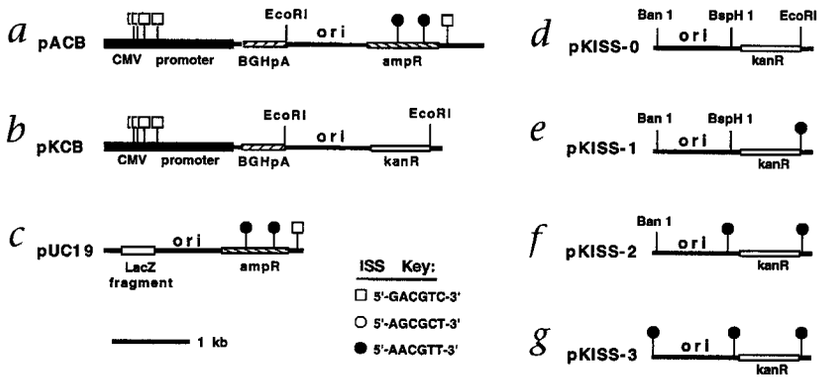

Fig. 2 Localization of the ISSs on the various pDNA(s) used in this study. The pACB is a pUC19-based pDNA and was previously described in detail[4]. The pKCB vector was constructed by replacing the 1542-bp ampR (*Bsp*HI–*Hin*dIII) with a 1042-bp kanR-containing fragment (*Bsp*HI–*Hin*dIII). The ISS-ODN flanked by *Eco*RI-compatible overhangs (sense, 5'–AATTG**AACGTT**CGC–3', antisense, 5'–AATTGC-GAACGTTC–3') was ligated into a unique *Eco*RI site of pACB, 3' to the BGHpA sequence. This resulted in the disruption of the *Eco*RI site, and the creation of a new Psp1406I restriction site (AACGTT). The ISS-containing region was then subcloned (*Bsp*HI–*Bam*HI) into the pKISS-O to create pKISS-1. The vectors pKISS-2 and pKISS-3 were constructed by ligation of the same ISS-ODN into pKISS-1 and pKISS-2, respectively, at a different *Eco*RI site.

gal IgE antibodies in response to a subsequent boosting with β-gal/alum, as opposed to mice primed with β-gal/pKISS-0 or β-gal/M-ODN, or saline-injected animals (Fig. 4). This inhibition of specific IgE induction is similar to that observed upon gene immunization[3].

ISS-DNAs activate macrophages to produce Th1-promoting cytokines

Specialized antigen-presenting cells (APCs) are susceptible to *in vivo* transfection with pDNA (ref. 9, 10) and dictate the specificity of immune responses after naked DNA immunization[11]. Transfection of fresh human macrophages with ISS-pDNA or with ISS-ODN, but not with ISS-deficient pDNA or M-ODN, increased IFN-α, IFN-β, IL-12, and IL-18 mRNA levels (Fig. 5, *a–d*). These cytokines of the innate response are established inducers of IFN-γ synthesis[12–15]. Incubation of human macrophages with IFN-α raised IL-12 and IL-18 mRNA levels, suggesting an amplifying role for IFN-α in ISS-induced stimulation (Fig. 5, *e* and *f*) and possibly in the induction of a Th1 response in this system. Furthermore, transfection of human peripheral blood mononuclear cells with ISS-ODN, but not with M-ODN, resulted in the production of IFN-γ (Table 2) as well as IFN-α, IFN-β and IL-12 by the transfected cells (Table 2 and Fig. 5). It is interesting that transfection of enriched peripheral human $CD4^+$ or $CD8^+$, as well as the human T cell lines CEM and Jurkat, under the same conditions, did not result in detectable secretion of IFN-γ. Taken together, these data suggest a key role for macrophages in the secretion of the IFN-γ inducers, and NK cells for the secretion of IFN-γ (ref. 16, 17).

Discussion

Compared with conventional protein vaccines, gene vaccines usually induce lower levels of antibodies, but stronger cellular immunity. Insofar as neutralizing antibodies are the first line of defense against viruses, bacteria and their toxins, gene immunization alone may not be an optimal approach for prevention of infectious diseases. However, cellular immune responses are a critical component of the host's response to infection and may be required for recovery. Unfortunately, neither inactivated virus nor subunit vaccines induce both vigorous cellular immunity and neutralizing antibodies. This limitation may have hampered the development of recombinant vaccines, as exemplified by the prominent failure of vaccines for HIV (ref. 18) and herpes simplex virus 2 (ref. 19). The present experiments show that coadministration of a standard protein vaccine with ISS-enriched DNA elicits antibody titers several times those of the matching gene vaccine. This combination additionally activates cell-mediated immunity, as assessed by production of IFNs α, β, and γ. The simple addition of the DNA adjuvant to a commercial, inactivated subunit influenza vaccine greatly increased its immunogenicity. Thus, the coadministration of the DNA adjuvant with influenza virus vaccine or with other viral antigens should enhance protection and could reduce morbidity and mortality of vaccinated hosts from invading pathogen(s). Moreover, in atopic diseases, the allergen/ISS-DNA coimmunization may elicit higher titers of blocking IgG antibodies, as well as Th1 immunity. Both effects should contribute to the suppression of immediate hypersensitivity[3] and the late-phase reactions upon reexposure to the allergen, by suppressing IgE synthesis and eosinophil recruitment, respectively[4].

Although intradermal and intramuscular gene immunization stimulates a Th1 immune response[1–3], gene immunization of mice by biolistic delivery into the skin with pDNA-coated gold microspheres induces immune responses with a Th2 bias[20,21]. Compared with intradermal or intramuscular gene immunization with naked pDNA, vaccination with gold microspheres uses 1/100th as much pDNA (ref. 22), and it might follow systemic dispersal of the delivered pDNA and the transfected cells. In these circumstances, the low dose of the delivered pDNA probably is insufficient to provide the local adjuvant effect that may be necessary to trigger Th1 immunity. On the other hand, the local production of IFNs α, β, and γ as well as IL-12 and IL-18 within the skin or muscle by higher doses of ISS-DNA may induce a specific cytokine milieu which promotes Th1 and cell-mediated immunity to the encoded antigen.

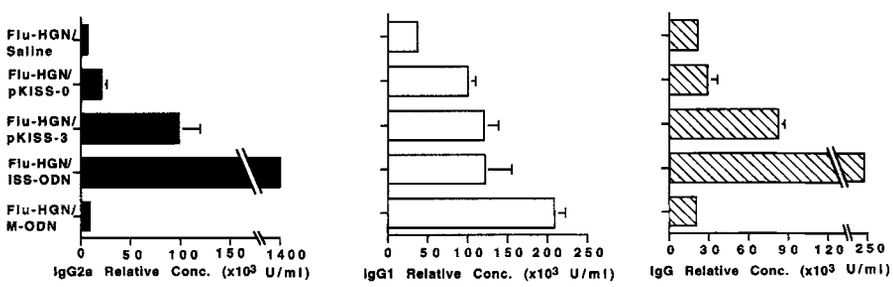

Fig. 3 The antibody response to influenza virus hemagglutinins (HGN) administration, alone or with various ISS-DNAs or ISS-deficient DNAs. ISS-ODN and pKISS-3 modified and upregulated the anti-HGN IgG2a and IgG responses. Results are the means ± s.e.m. for four mice per group.

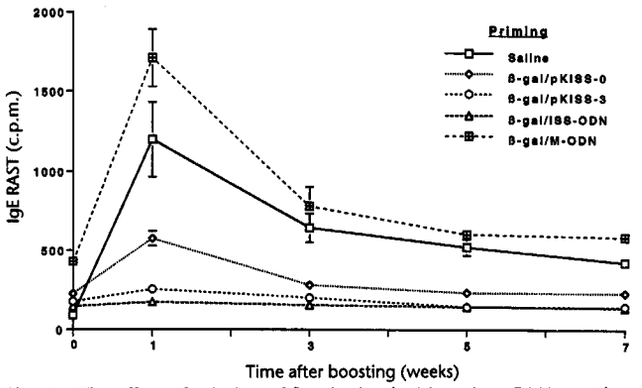

Fig. 4 The effect of priming of β-gal mixed with various DNAs on the subsequent anti-β-gal IgE response after i.p. boosting with β-gal in alum. Mice primed with β-gal/pKISS-3 and β-gal/ISS-ODN did not generate significant anti-β-gal IgE antibodies as opposed to the other groups. Results are the means ± s.e.m. for four mice per group. $P < 0.05$ for β-gal/pKISS-3 or the β-gal/ISS-ODN versus saline, β-gal, β-gal/M-ODN or versus β-gal/pKISS-3. The mice in these experiments displayed a strong anti-β-gal IgG response before i.p. boosting and higher titers post boosting (data not shown). The differences in the IgE levels between the β-gal/pKISS-0 and the β-gal/M-ODN may be attributed to some nonpalindromic CpG motifs[27] in the pKISS-0 vector.

Table 2 Production of cytokines involved in Th1 differentiation by hPBMCs untransfected (medium only) or transfected with ISS-ODN, M-ODN or polyinosinic-polycytidilic acid (pI:C)

	Cytokines produced (pg/ml) for conditions			
	Medium	ISS-ODN	M-ODN	pI:C
hIFN-γ	7 ± 4	222 ± 47	38 ± 15	140 ± 43
hIFN-α	<4	442 ± 40	37 ± 15	101 ± 32
hIL-12	29 ± 3	82 ± 8	48 ± 6	71 ± 11
hIL-2	11 ± 4	30 ± 10	22 ± 8	32 ± 4
hTNF-α	25 ± 12	4 ± 2	6 ± 3	4 ± 3
hIL-4	16 ± 3	13 ±2	15 ± 2	21 ± 2

Results are the means ± s.e.m obtained from PBMCs of eight healthy volunteers. Transfection of bulk BALB/c splenocytes, under the same conditions induced 1119 ± 227 pg/ml of IFN-γ for ISS-ODN and less than 5 pg/ml for M-ODN. The murine IL-4 levels for both ISS-ODN or M-ODN were below background (<4 pg/ml).

The mechanism by which gene vaccination induces a Th1 response[1-3] is still unclear. The data presented here support the assumption that this response is the consequence of the activation of the innate immune response by the ISSs in the pDNA backbone[5], rather than by the low dose of antigen intracellularly produced. The activation products of the ISSs, that is, type-I IFNs, together with IL-12 and IL-18, are established inducers of IFN-γ synthesis[12-15] and promote the differentiation of naive Th cells to Th1 lymphocytes. IL-18 (IFN-γ-inducing factor, or IGIF) is produced by monocyte- and macrophage-like cells, acts alone or synergistically with IL-12 to augment IFN-γ release by T cells and enhances NK-cell cytolytic activity[15]. Thus, the ISSs activate the precise cytokine network required to induce an initial burst of IFN-γ in an antigen-independent fashion (Table 2 and Fig. 6). In the presence of a protein antigen, this response promotes the differentiation of naive $CD4^+$ T cell toward Th1 cells, leading to a second burst of IFN-γ production, this time in an antigen-dependent fashion (Table 1 and Fig. 6). Thus, this basic mechanism of ISS activation can be harnessed to evoke a Th1 response to a coadministered protein antigen.

Notably, the ISSs were initially discovered in the mycobacterial genome as DNA sequences that selectively enhanced NK cell activity[16]. Based on empirical results, Freund utilized mycobacterial extract as a major constituent in his adjuvant formula[23]. The data of this study suggest that the potent immunostimulatory effects of Freund's adjuvant may depend on the ISS-enriched mycobacterial DNA (ref. 7, 16, 23) in addition to the cell-wall ingredients such as lipids and protein[24]. It is of interest that the *in vivo* administration of ISS-pDNA (that is, pUC19), in contrast to complete Freund's adjuvant, does not induce inflammatory reactions in the injected site [25]. Thus, the ISS-pDNA adjuvant can provide, in part, the immunostimulatory effects of the Freund's adjuvant without the severe inflammatory and toxic side effects attributed to the paraffin oil and the mycobacterial cell-wall products [24].

These data support the idea that higher organisms can combat an invading pathogen by recognition of particular polynucleotide sequences. These include foreign RNA (for example, double-stranded RNA) or DNA (that is, ISS). In contrast to bacteria, which utilize restriction enzymes to eliminate an invading pathogen (for example, phage), the recognition of these nucleic acid sequences activates innate immunity[26-29] and, subsequently, the adaptive immune response as well, in order to eliminate the "dangerous DNA" and its products. In this respect, the ISSs represent an immune "danger/alarm-like signal"[30] and trigger the release from transfected APCs and NK cells of a consorted set

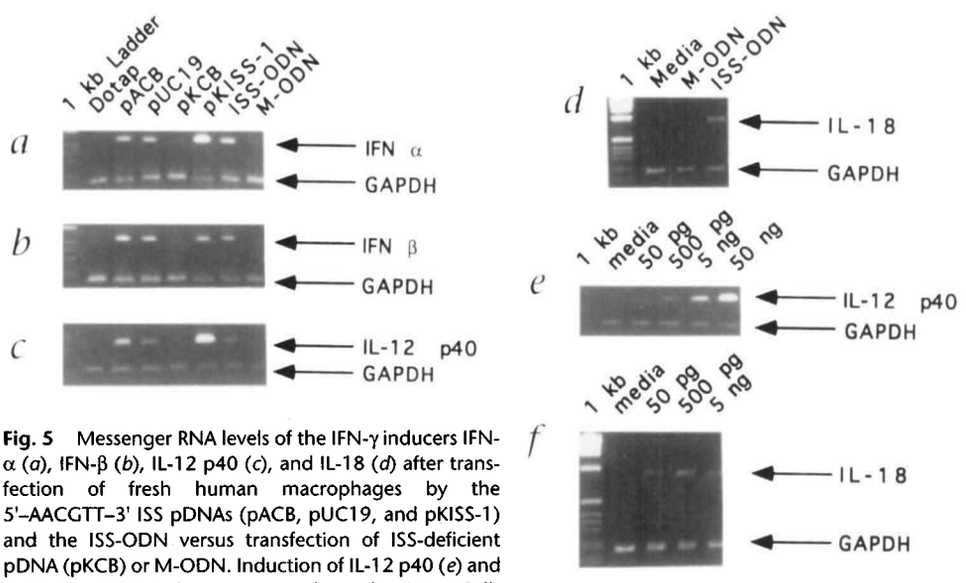

Fig. 5 Messenger RNA levels of the IFN-γ inducers IFN-α (*a*), IFN-β (*b*), IL-12 p40 (*c*), and IL-18 (*d*) after transfection of fresh human macrophages by the 5'-AACGTT-3' ISS pDNAs (pACB, pUC19, and pKISS-1) and the ISS-ODN versus transfection of ISS-deficient pDNA (pKCB) or M-ODN. Induction of IL-12 p40 (*e*) and IL-18 (*f*), mRNAs in human macrophages by IFN-α. Cells were incubated with the indicated amounts of hIFN-a (Biosource) for 3 h. RT-PCR of the GAPDH mRNA is shown as a control for the quantity and integrity of RNA. DNA molecular size markers (1-kb DNA ladder, Gibco BRL) are indicated in kilobases. The GAPDH PCR product was electrophoresed for 5 min before loading. The data shown represent one of three similar experiments performed on macrophages obtained from three different healthy donors.

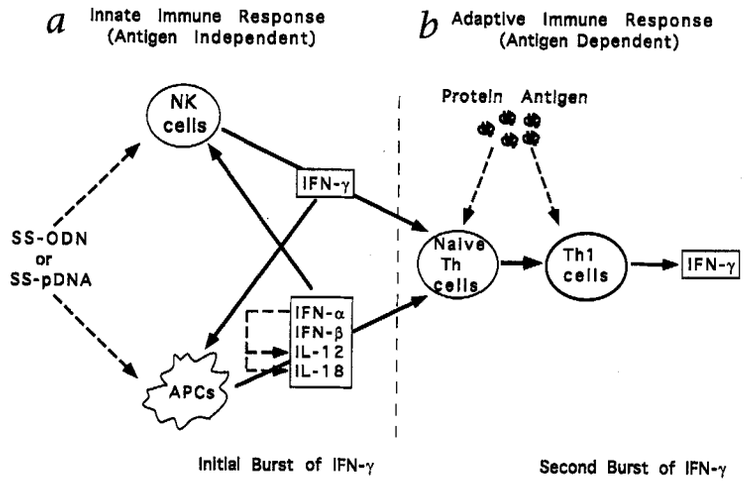

Fig. 6 The postulated instructive role of innate immunity activated by the ISSs on the subsequent Th1 differentiation[31] in this system (ISS-DNA/antigen coadministration). *a*, ISS-ODN or ISS-pDNAs trigger the release of IFN-γ and IFN-γ-inducers ("dangerkines") by natural killer cells (NK) and antigen-presenting cells (APCs) (for example, macrophages), respectively. These responses result in the initial burst of IFN-γ in the picogram/milliliter range (see Table 2) in an antigen-independent fashion. IFN-α has an amplification role in IL-12 and IL-18 synthesis (Fig. 4). The release of IL-12 from APCs results in further IFN-γ production by T cells[33]. *b*, In the presence of an antigen, the secreted innate cytokines prime naive Th cells to differentiate toward Th1 cells that produce additional IFN-γ in the nanogram/milliliter range (see Table 1) in an antigen-dependent fashion. Vectors for gene vaccination provide both the adjuvant (ISS) and the genetic information for the encoded antigen[5].

of cytokines (which may be designated "dangerkines") that inhibit pathogen replication, activate cell-mediated immunity and instruct the adaptive immune system to differentiate toward a protective Th1 response[31]. The DNA adjuvants achieve this effect by mimicking an intracellular pathogen without the risks posed by real infection.

In summary, the coadministration of ISS-DNAs with a conventional vaccine not only markedly augments antibody production, but also activates cell-mediated immunity. Protein/ISS-DNA combinations might thus constitute optimal vaccines with which to combat infectious pathogens and to desensitize against allergens.

Methods

Polynucleotide-based reagents. The pACB plasmid vector is a pUC19-based plasmid (ampR) containing the cytomegalovirus (CMV) promoter/enhancer sequences (807-bp), along with the CMV immediate early intron (824-bp) and a 560-bp bovine growth hormone poly(A)$^+$ signal (BGHpA). The pKCB vector was constructed by replacing the 1542-bp β-lactamase-containing fragment (ampicillin resistance) (*Bsp*HI–*Hin*dIII) of pACB with a 1042-bp kanamycin resistance-containing fragment (BspHI–*Hin*dIII). The pKISS-0 vector was constructed by *Eco*RI restriction digestion, followed by religation of the backbone at the *Eco*RI sites, thus removing the expression vector cassette [from the CMV promoter to the poly(A)$^+$]. The pKISS-1 vector was constructed by Psp1406I restriction digestion, followed by religation of the Psp1406I backbone, resulting in the removal of the expression vector cassette. The AACGTT sequences in the resultant vectors were confirmed by Psp1406I digestion. The pKISS-2 vector was made by ligation of double-stranded ISS-oligo (5'–3': sense, CATGAACGTTCGC; antisense, CATGGCGAACGTT) into the *Bsp*HI site of pKISS-1, disrupting the *Bsp*HI site at one end. The pKISS-3 vector was made by ligation of double-stranded ISS-ODN (5'–3': sense, GTGCGAAGCTTGAACGTTCGC; antisense, GCACGCGAACGTTCAAGCTTC) into the *Ban*I site of pKISS-2, disrupting both *Ban*I sites, and adding a *Hin*dIII site. The pACB-LacZ vector (encoding β-gal) has been described elsewhere[4].

Escherichia coli and calf thymus genomic DNA as well as polyinosinic-polycytidilic acid (pI:C) were purchased from Sigma Chemical Co. The genomic DNAs were sonicated and purified from endotoxin as described below.

For transfection of hPBMCs we used phosphodiester double-stranded ODNs. The sequences are ISS-ODN, 5'–TCATTGGAAAACGTTCTTCGGGGCG–3' (adapted from pUC19 sequence 2288–2312, containing the putative immunostimulatory 5'–AACGTT–3' palindrome); the mutated (M)-ODN, 5'–TCATTGGAAAAGGTTCTTGGGGGGG–3', were purchased from Integrated DNA Technologies Inc. (Coralville, IA).

For protein/DNA coimmunization we used phosphodiester and phosphorothioate, single-stranded ODNs. The sequences are ISS-ODN, 5'–TGACTGTGAACGTTCGAGATGA–3', M-ODN, 5'–TGACTGTGAAGGTTAGAGATGA–3' were purchased from Trilink (San Diego, CA).

Plasmid DNAs were prepared using Qiagen Maxi Kits (Chatsworth, CA). Endotoxin was removed by Triton X-114 (Sigma) extractions as described previously[3,4]. Endotoxin levels in all the nucleic acid preparations were determined using the Pyrotell limulus amebocyte lysate (LAL) assay (Assoc. Cape Cod, Woods Hole, MA)[3,4] and were below 2 ng/mg DNA. Before injection, all the polynucleotide-based reagents were precipitated in 100% ethanol, washed in 70% ethanol and dissolved in normal saline.

Transfection of hPBMCs and mouse splenocytes. Heparinized peripheral blood from eight healthy donors was diluted 1:2 with RPMI 1640. PBMCs were isolated by Ficoll Hypaque (Sigma) gradient, washed twice with RPMI 1640, resuspended at 2×10^6 ml in RPMI 1640/1% FBS (low endotoxin), in Teflon tubes and incubated for 14 h at 37 °C, in a 5% CO_2 incubator. Transfections of ISS-ODN, M-ODN and pI:C (10 μg each) were performed with DOTAP (Boehringer Mannheim). Twenty-four hours after transfection, supernatants from transfected and untransfected wells were collected. The levels of IFN-γ, IFN-α, IL-12, IL-2 and IL-4 were detected by commercial ELISA kits (Biosource, Camarillo, CA). Transfection efficiencies (90%) were confirmed by cytofluorometric analyses of human CEM lymphoblasts transfected with fluorescein-ISS-ODN. The same procedure was used for bulk BALB/c splenocytes. In another set of experiments hPBMCs from three different donors were fractionated to CD4$^+$ and CD8$^+$ subpopulations (CD4$^+$ and CD8$^+$ cell separation columns, R&D Systems, Minneapolis, MN). The enriched CD4$^+$ and CD8$^+$ subpopulations (>90% purity for each subset as confirmed by cytofluorometric analyses), as well as the human T cell lines CEM and Jurkat, were transfected according to the protocol mentioned above. Mouse IFN-γ was detected in the supernatants of transfected splenocytes as was previously described[3,4].

Immunizations protocols, antibodies and cytokine assays. Female BALB/c mice (6–8 weeks of age) were injected intradermally (i.d.) at the base of the tail once, or three times, 2 weeks apart, with 10 μg of β-gal (Calbiochem, San Diego, CA) or mixed with 50 μg of pDNA or 50 μg of ODNs in 50 ml normal saline. Influenza hemagglutinins (HGNs, 15 μg influenza virus vaccine, trivalent, types A and B, subvirion antigen, 1995–96 formula, Wyeth-Ayerst Laboratories, Philadelphia, PA) were injected alone, or with pKISS-0, pKISS-3, ISS-ODN (phosphorothioate) or with M-ODN (phosphorothioate) (50 μg for each DNA preparation) under the same conditions. Gene vaccinated mice (pACB-LacZ) were immunized i.d. with (50 μg) once or three times, at 2-week intervals. For IgE induction, mice were primed i.d. with 10 μg β-gal mixed with various DNAs (50 μg). These include β-gal/pKISS-0, β-gal/pKISS-3, β-gal/M-ODN (phosphorothioate), β-gal/ISS-ODN (phosphorothioate), or with saline. Four weeks after priming they were injected intraperitoneally with 2 μg β-gal in 3 mg alum as previously described[3] and followed for another 7 weeks. Anti-β-gal antibody assays (IgG, IgG1, IgG2a and IgE) were performed by ELISA or

radioimmunoassay as was previously described[3,4]. Anti-influenza HGN antibody screens (IgG, IgG1, IgG2a) were similarly performed using the original vaccine as the coated antigen (5 μg of HGN/1 ml of BBS).

Cytokine release (IFN-γ and IL-4) by β-gal-stimulated splenic CD4⁺ T cells from β-gal, β-gal/pDNAs and from pACB-LacZ (gene vaccine) immunized mice (three i.d. immunizations for each group) was performed as previously described[4].

Reverse transcriptase-PCR studies. Human (h) macrophages were isolated from the hPBMCs of three donors by fibronectin adherence, as previously described[32]. Transfections were performed as described above, except that the medium was RPMI 1640 supplemented with 5% low endotoxin FBS and that 2.5 μg of pDNA(s) or double-stranded ISS-ODN or double-stranded M-ODN were added to 2 3 10^5 cells per Teflon tube (Pierce, Rockford, IL). Three hours after transfection, mRNA was extracted using RNA STAT-60 ("B"; Tel Test, Inc., Friendswood, TX) and analyzed by RT-PCR. ODNs used in the RT-PCR assays were as follows: h**IFN**-α (430-bp PCR product, sense primer, 5'–TTTCTCCTGCCTGAAGGACAG–3', antisense primer, 5'–GCTCATGATTTCTGCTCTGACA–3'), h**IFN**-β (380-bp PCR product, sense primer, 5'–AAAGAAGCAGCAATTTTCAGC–3', antisense primer, 5'–CCTTGGCCTTCAGGTAATGCA–3'), h**IL-12 p40** (181-bp PCR product, sense primer, 5'–GGTGGTCCTCACCTGTGACA–3', antisense primer, 5'–GTGAAGCAGCAGGAGCGAATG–3') (positions 128 to 308 of the IL-12 p40 cDNA), h**IL-18** (IGIF) primers (866-bp PCR product, sense primer, 5'–CTTCCTCTCGCAACAAACTATT, antisense primer, 5'–GGCTCACCACAACCTCTACCTC) and h**GAPDH** as controls (190-bp PCR product, 5' primer, 5'–TGGTATCGTGGAAGGACTCATGAC; 3' primer, 3'–ATGCCAGTGAGCTTCCCGTTCAGC).

Synthesis of single-stranded DNA from mRNA was performed with a Superscript kit (Gibco BRL, Gaithersburg, MD). PCR was performed using *Taq* DNA polymerase (Boehringer Mannheim), buffer F of the PCR Optimization Kit (Invitrogen, San Diego, CA), 5 ng/ml of each primer per reaction, and the following PCR conditions: 94 °C for 1 min, 58 °C for 1 min, 72 °C for 30 s, 35 cycles and a final extension step of 72 °C for 7 min. The strength of the band of the GAPDH PCR product (housekeeping gene) was used as an internal standard. PCR analysis was performed by UV visualization of PCR products after size separation (electrophoresis) on a 1.5% agarose gel. Electrophoresis was performed in 0.5% TBE buffer in the presence of ethidium bromide (1 mg/ml agarose). Size determination was confirmed by comparison with the sizes of DNA markers using the 1-kb ladder (Gibco BRL).

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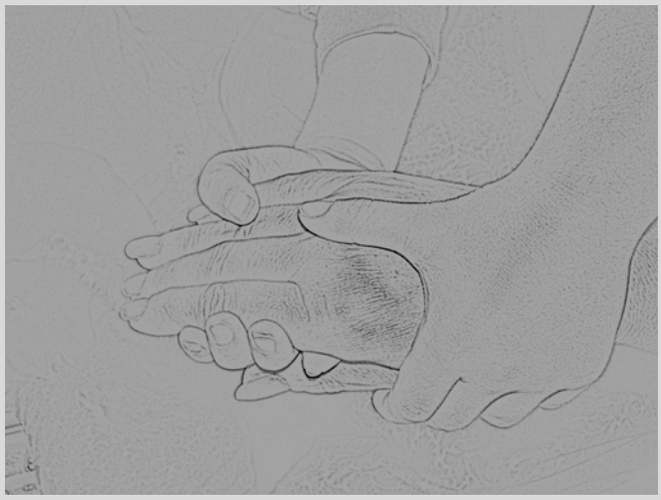

Chapter 3

Immunostimulatory DNA is a potent mucosal adjuvant

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Summary
Most proteins delivered to mucosal surfaces fail to induce mucosal or systemic immune responses. We demonstrate that a single intranasal (i.n.) coadministration of a model antigen (β-galactosidase, β-gal) with immunostimulatory sequence oligodeoxynucleotide (ISS-ODN) induces a mucosal IgA response equivalent to that induced by i.n. codelivery of β-gal with cholera toxin (CT). Furthermore, i.n. and intradermal (i.d.) delivery of the β-gal/ISS-ODN mix stimulates equivalent Th1-biased systemic immune responses with high level cytotoxic T lymphocyte (CTL) activity. In contrast, i.n. immunization with β-gal and CT results in a Th2-biased systemic immune response with poor CTL activity. Our data show that i.n. delivery of ISS-ODN provides effective adjuvant activity for the induction of both mucosal and systemic Th1-biased immune responses. This immunization approach deserves consideration in the development of vaccines against mucosal pathogens.

Introduction

The respiratory, gastrointestinal, vaginal, and rectal mucosa are sites where the majority of infectious agents are first encountered (1, 2). These surfaces are protected by secreted IgA (1–3). With intracellular pathogens, a CTL response is important for elimination of the infectious agent (2, 4). Natural infection often induces these protective immune responses (1, 2). In contrast, delivery of monomeric protein antigens via mucosal routes generally does not stimulate any immune response, and delivery by systemic routes (i.e., i.d. and intramuscular, i.m.) leads to serum antibody production but mucosal IgA and CTL activity are not induced (1, 2). To produce a more comprehensive immune response to protein antigens, the use of adjuvants, including the mucosal adjuvant CT, has been explored.

Immunostimulatory sequence oligodeoxynucleotides (ISS-ODN) have previously been shown to provide effective adjuvant activity for the induction of systemic Th1-biased immunity toward protein antigens coadministered via i.d. and i.m. routes (5–9). The immune response includes the induction of a Th1 cytokine profile (IFN-gamma but not IL-4), the production of high IgG2a and low IgG1 titers, and a CTL response (5–9). In this article we expand upon previous observations regarding the potent Th1-biased adjuvant effect of ISS-ODN and demonstrate that, in addition, it is as good a mucosal adjuvant as CT. We show that i.n. administration of β-gal with either ISS-ODN or CT leads to equivalent mucosal IgA responses. In addition, i.n. and i.d. codelivery of β-gal with ISS-ODN induces equivalent Th1-biased serum IgG subclass, splenic cytokine, and CTL responses, while i.n. β-gal/CT codelivery leads to a Th2-biased systemic immune response. In considering the potential application of ISS-ODN as a vaccine adjuvant against mucosal pathogens, our data suggest that i.n. antigen/ISS-ODN delivery is superior to i.d. delivery for the induction of protective immunity.

Materials and Methods

Immunization reagents.

β-Gal and CT (Sigma, St. Louis, MO), ISS-ODN, and mutated phosphorothioate oligodeoxynucleotide (M-ODN) (Trilink Biotechnologies, San Diego, CA) were used to immunize mice. The ISS-ODN used in these studies has the following sequence 59-TGACTGTGAACGTTCGAGATGA-39. The M-ODN has the sequence 59-TGACTGTGAACCTTAGAGATGA-39.

Immunization protocols

Female BALB/c mice 6–8 weeks of age were purchased from Jackson Laboratory (Bar Harbor, ME) and used in all experiments. Intranasal immunizations were performed with β-gal (50 μg) alone or mixed with 50 μg of ISS-ODN or M-ODN, or with CT (10 μg) in 30 μl of saline. Mice were anesthetized with Metofane (Mallinckrodt Veterinary Inc., Mundelein, IL) and 15 μl was delivered to each nare. Alternatively, mice received β-gal (50 μg) plus ISS-ODN (50 μg) in 50 μl of saline injected i.d. into the base of the tail, or β-gal (200 μg) plus ISS-ODN (50 μg) administered intragastrically (i.g.) by blunted needle in 400 μl of 0.2 M Na bicarbonate. Mice were fasted for 4 hours before i.g. immunization.

Bronchoalveolar lavage and fecal IgA extraction.

Bronchoalveolar lavage fluid (BALF) was obtained by cannulation of the trachea of sacrificed mice during week 7. The lungs were then flushed with 0.8 ml of PBS. The return was spun to remove cellular debris, and frozen at -70°C until IgA assay. Feces were collected at 2-week intervals and IgA was extracted according to a previously

published protocol (10). Briefly, three to six pieces of freshly voided feces were collected and subsequently dried in a Speed Vac Concentrator. After feces were dried, net dry weights were recorded, and the material was resuspended in PBS with 5% nonfat dry milk and protease inhibitors at a ratio of 20 μg/ml of feces to standardize for variability in the amount of fecal material collected (10). The solid matter was resuspended by vortexing for 2 hours followed by centrification at 16,000*g* for 10 min to separate residual solids from supernatant. Supernatants were then frozen at -70°C until IgA assay.

Immunologic assays.

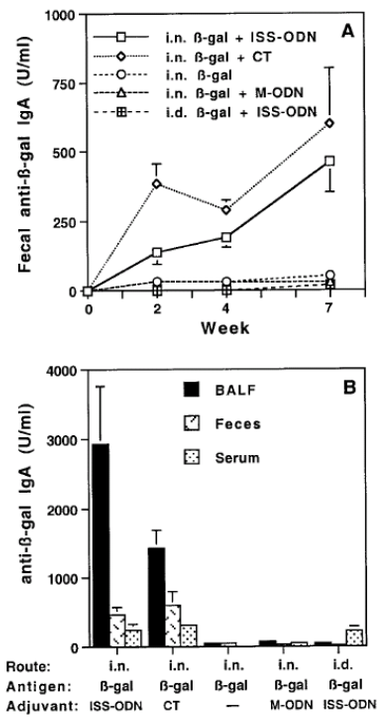
Serum, BALF, and fecal extraction fluid were used in ELISA assays for antigen specific immunoglobulin as described previously (8, 9). Results are expressed in units/milliliter based on pooled high titer anti-β-gal standards obtained from mice receiving multiple immunizations. The undiluted fecal IgA and serum IgG standards were given arbitrary concentrations of 2000 and 400,000 U/ml respectively. Samples were compared to the standard curve on each plate using the DeltaSOFT II v. 3.66 program (Biometallics, Princeton, NJ). Mouse spleens were harvested at week 7 for CTL and cytokine assays. For CTL assays, 7 X 10^6 splenocytes from immunized mice were incubated with 6 X 10^6 mitomycin C-treated naïve splenocytes in the presence of recombinant human IL-2 and class I H2d-restricted β-gal nanopeptide (T-P-H-PA-R-I-G-L) as previously described (9). After 5 days, restimulated cells were harvested and specific lysis of target cells measured (9). Splenocyte cytokine profiles were conducted by incubation of 5 X 10^5 splenocytes in 96-well plates in a final volume of 200 μl of supplemented RPMI 1640 with β-gal added at 10 mg/ml, at 37°C/5% CO2 as previously described (8, 9). Culture supernatants were harvested at 72 hours and analyzed by ELISA. A standard curve was generated using known amounts of recombinant IFN-gamma (PharMingen, San Diego, CA) and IL-4 (Genzyme, Cambridge, MA). Each culture supernatant was compared to the standard curve on the plate using the DeltaSOFT II v. 3.66 program. Statistical analysis of results was conducted using Statview computer software (Abacus Concepts, Grand Rapids, MI). A two-tailed Student *t* test was used to establish *p* values, and those <0.05 were considered significant.

Results and Discussion

ISS-ODN is an effective mucosal adjuvant.

Cholera toxin is the most potent known mucosal adjuvant (2). Therefore, the mucosal IgA response of mice immunized with i.n. β-gal/ISS-ODN and β-gal/CT were compared. As can be seen in Fig. 1, at 7 weeks post β-gal/ISS-ODN and β-gal/CT vaccination the mean anti-β-gal IgA levels were 462 and 599 U/ml in fecal material and 2935 and 1432 U/ml in BALF, respectively. Differences in mucosal IgA levels between i.n. β-gal/ ISS-ODN immunized and i.n. β-gal/CT-immunized mice were not statistically significant. To establish that a mucosal adjuvant was needed for the induction of mucosal IgA, we vaccinated mice i.n. with β-gal alone or with M-ODN. However, i.n. immunization without mucosal adjuvant resulted in no detectable IgA. We next evaluated whether contact with the respiratory mucosa was required for ISS-ODN to have mucosal adjuvant activity. Mice were therefore vaccinated with β-gal and ISS-ODN via i.d. and i.g. routes. These routes of immunization did not lead to measurable IgA in mucosal secretions (data for i.g. immunization not shown). To establish whether the IgA detected in fecal material and BALF of vaccinated mice

Figure 1. IgA responses



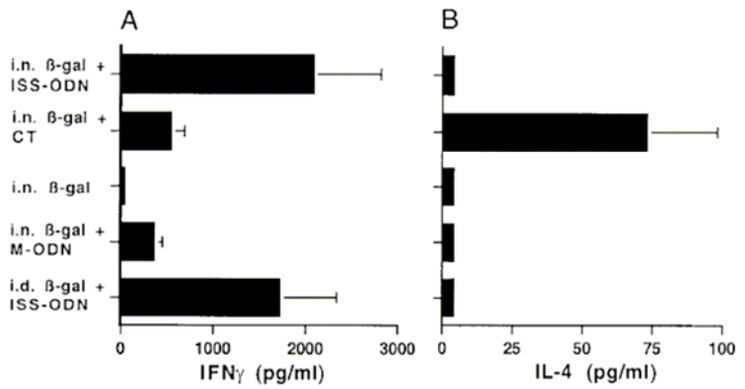
Mice received a single immunization with β-gal (50 μg) alone, with ISS-ODN (50 μg), M-ODN (50 μg), or CT (10 μg) via i.n. or i.d. routes. Results were obtained by ELISA and represent mean values for 4 mice per group. Error bars reflect the standard errors of the means. Results are representative of 3 similar and independent experiments. (A) Fecal IgA. Feces were collected at 2, 4, and 7 weeks and IgA extracted as described under Materials and Methods. There was no significant difference in anti-β-gal IgA levels between the i.n. β-gal/ISS-ODN and i.n. β-gal/CT vaccinated groups except at 2 weeks (p = 0.03). (B) BALF and serum IgA. BALF and serum were obtained at sacrifice during week 7 and compared to week 7 fecal IgA. There was no significant difference in the BALF anti- β-gal IgA levels between i.n. β-gal/ISS-ODN and i.n. β-gal/CT immunized groups.

was actively secreted by mucosal tissue or passively diffused from serum, anti-β-gal IgA levels in serum, fecal material, and BALF were compared. It should be noted that initial acquisition of BALF and fecal samples required an unmeasurable dilution of the IgA content of the material which does not occur when obtaining serum. Despite this fact, i.n. β-gal/ISS-ODN-immunized and i.n. β-gal/CT-immunized mice demonstrated higher levels of anti-β-gal IgA in feces and BALF then in serum, strongly suggesting that active secretion of anti-β-gal IgA from mucosal surfaces occurred in these mice (Fig. 1B). These results demonstrate that ISS-ODN and CT have equivalent mucosal adjuvant activity with a test antigen that has no capacity to induce mucosal IgA production when delivered alone. In addition, we show that i.d. delivery of β-gal with ISS-ODN does not lead to a mucosal IgA response. Taken together these findings show that ISS-ODN is an excellent adjuvant for the induction of mucosal immunity when codelivered with antigen via the nose.

Immunization with β-gal and ISS-ODN by the i.n. route induces a vigorous Th1-biased systemic immune response.

We next evaluated the magnitude and phenotype of the systemic immune response induced by i.n. β-gal/ISS-ODN immunization. Splenocytes were harvested from mice 7 weeks after vaccination and incubated with β-gal. Culture supernatants were assayed for the production of IFN-gamma and IL-4, cytokines classically associated with Th1 and Th2 immunity respectively (11, 12) (Fig. 2). Splenocytes from mice immunized with β-gal and ISS-ODN via the i.n. and i.d. routes produced a mean of 2084 and 1720 pg/ml of IFN-gamma, respectively (p value not significant), but no detectable IL-4. In contrast, i.n. vaccination with β-gal and CT led to splenocyte production of a mean of 542 pg/ml of IFN-gamma and 73 pg/ml of IL-4 (p = 0.05 for

Figure 2. Antigen-induced cytokine profiles.



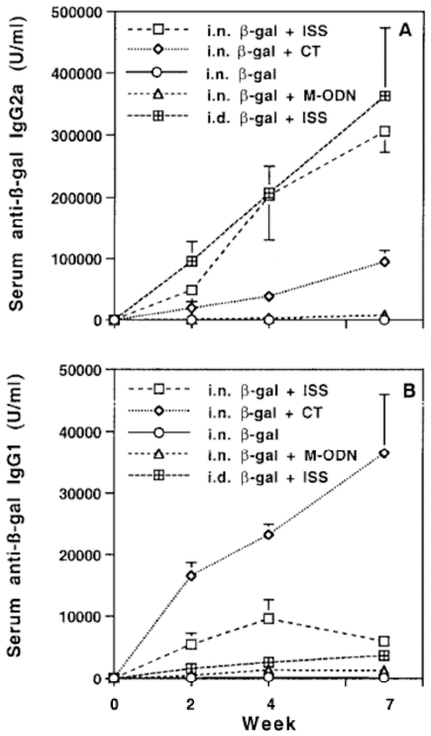
Mice received a single immunization with β-gal (50 mg) alone, with ISS-ODN (50 mg), M-ODN (50 mg), or CT (10 mg) via i.n. or i.d. routes. Splenocytes were harvested from sacrificed mice during week 7 and cultured in media with or without β-gal (10 mg/ml), and 72-h supernatants were assayed by ELISA. Splenocytes cultured without β-gal produced no detectable IFN-gamma or IL-4 (data not shown). Results represent the mean for 4 mice in each group and similar results were obtained in 2 other independent experiments. Error bars reflect standard errors of the means. (A) IFN-gamma levels. IFN-gamma levels were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice but statistically higher than in other immunization groups (P = 0.05 for i.n. β-gal/ISS-ODN versus i.n. β-gal/CT vaccinated mice). (B) IL-4 levels. IL-4 levels above background were detected only in mice immunized with i.n. β-gal/CT (P = 0.04 verses background).

both IFN-gamma and IL-4 when compared to i.n. β-gal/ISS-ODN vaccination). Intranasal immunization with β-gal alone or with M-ODN led to much lower or undetectable cytokine production from splenocytes. IFN-gamma is a switch factor for IgG2a production, while IL-4 is a switch factor for IgG1 (11, 12). Given the splenic cytokine profiles, it would therefore be predicted that i.n. β-gal/ISS-ODN coadministration would lead to higher IgG2a and lower IgG1 levels then i.n. β-gal/CT codelivery. Indeed, we found that i.n. and i.d. β-gal/ISS-ODN-immunized mice produced equivalent Th1-biased serum antibody responses, whereas i.n. β-gal/CT vaccination led to a Th2-biased IgG subclass profile. At 7 weeks post-i.n. and i.d. β-gal/ISS-ODN immunization mean serum anti- β-gal IgG2a levels were 306,144 and 362,850 U/ml, and anti-β-gal IgG1 levels were 5971 and 3676 U/ml, respectively (Fig. 3). These differences were not statistically significant. In contrast, i.n. vaccination with β-gal and CT induced mean serum IgG2a and IgG1 levels of 94,518 and 36,471 U/ml (p = 0.005 for IgG2a and P = 0.004 for IgG1 compared to i.n. β-gal/ISS-ODN immunization). Again, i.n. immunization with β-gal alone or with M-ODN led to poor or undetectable IgG responses. Cumulatively, these observations demonstrate that i.n. and i.d. delivery of antigen with ISS-ODN lead to equivalent Th1-biased cytokine and antibody profiles, whereas i.n. β-gal/CT co-administration leads to a Th2- biased systemic immune response. Considered in conjunction with the IgA data previously presented, we further demonstrate that production of mucosal IgA can occur in the context of both Th1- and Th2-biased systemic immune responses.

Codelivery of β-gal plus ISS-ODN by the i.n. route induces a strong splenic CTL response.

Although development of antigen-specific CTL activity is associated with Th1-biased immunity, not all Th1-biased immune responses include the development of cytotoxic T cells (2, 4). Therefore, we next evaluated the ability of i.n. codelivery of β-gal and ISS-ODN to induce a CTL response. As demonstrated in Fig. 4, mice immunized with β-gal and ISS-ODN by either the i.n. or i.d. route displayed vigorous splenic CTL activity. At an E:T ratio of 5:1, i.n. and i.d. codelivery of β-gal/ISS-ODN led to 52 and

Figure 3. IgG subclass profiles

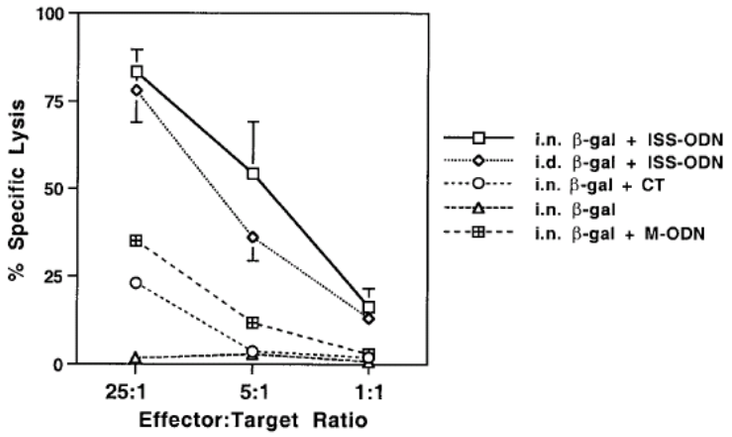


Mice received a single immunization with β-gal (50 mg) alone, with ISS-ODN (50 mg), M-ODN (50 mg), or CT (10 mg) via i.n. or i.d. routes. Serum was collected at 2, 4, and 7 weeks from immunized mice and assayed by ELISA. Results represent mean values for 4 mice per group, and error bars reflect standard errors of the means. Results are representative of 3 similar and independent experiments. (A) Serum IgG2a. Serum IgG2a levels were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice but statistically higher than in other immunization groups at 7 weeks (p = 0.005 for i.n. β-gal/ISS-ODN versus i.n. β-gal/CT-vaccinated mice). (B) Serum IgG1. Serum IgG1 levels were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice but statistically lower than in i.n. β-gal/CT-immunized mice at all time points (p = 0.003, p = 0.02, and p = 0.02 for i.n. β-gal/ISS-ODN versus i.n. β-gal/CT-vaccinated mice at 2, 4, and 7 weeks, respectively).

39% specific lysis of target cells, respectively. The difference was not statistically significant. However, i.n. β-gal/CT vaccination resulted in only 3% specific lysis at the same E:T ratio (p = 0.005 compared to vaccination with i.n. β-gal/ISS-ODN). Likewise, i.n. immunization with β-gal alone or with M-ODN led to poor or undetectable CTL responses. These results show that while i.n. and i.d. β-gal/ISS-ODN coimmunization leads to equivalent and robust CTL responses, i.n. β-gal/CT coadministration leads to a poor CTL response. In addition, the CTL assay results further demonstrate the dichotomy between the Th1- and Th2- biased systemic immune responses seen when β-gal is codelivered i.n. with ISS-ODN or with CT, respectively. In summary, our findings demonstrate that i.n. delivery of antigen with either ISS-ODN or CT leads to an equivalent and vigorous mucosal IgA response, whereas i.d. codelivery of antigen with ISS-ODN does not. However, i.d. and i.n. vaccination with β-gal and ISS-ODN induce equivalent systemic Th1-biased immune responses characterized by high levels of antigen-specific IFN-gamma but no IL-4 production from cultured splenocytes, high IgG2a and low IgG1 serum concentrations, and vigorous CTL responses. In contrast, i.n. codelivery of β-gal with CT leads to a Th2-biased systemic immune response characterized by low IFN-gamma but substantial IL-4 production from *in vitro* antigen-stimulated splenocytes, high IgG1 and low IgG2a serum concentrations, and a poor CTL response. The observation of equivalent mucosal IgA levels in the context of Th1-biased and Th2-biased systemic immune responses with i.n. β-gal/ISS-ODN and β-gal/CT immunizations respectively is consistent with other published results (18). Mariarosaria and colleagues recently demonstrated that oral delivery of tetanus toxoid with CT led to mucosal IgA production in conjunction with a Th2 systemic immune profile and that coadministration with oral IL-12 skewed the systemic

immune response toward a Th1 phenotype, whereas mucosal IgA production was unaffected (18).

Figure 4. CTL responses



Mice received a single immunization with β-gal (50 mg) alone, with ISS-ODN (50 mg), M-ODN (50 mg), or CT (10 mg) via i.n. or i.d. routes. Splenocytes were harvested from mice at week 7 and CTL responses were determined as outlined under Materials and Methods. Results represent mean values for 4 mice per group, and error bars reflect standard errors of the means. Results are representative of 3 similar and independent experiments. CTL responses were equivalent in i.n. and i.d. β-gal/ISS-ODN-immunized mice at all E:T ratios, but statistically higher than in i.n. β-gal/CT-immunized mice at E:T ratios of 25:1 and 5:1 ($p = 0.005$ and $p = 0.05$ for i.n. β-gal/ISS-ODN versus in β-gal/CT-immunized mice at E:T ratios of 25:1 and 5:1, respectively).

Taken together, these findings document that synthesis of mucosal IgA can occur in the context of both Th1- and Th2- biased systemic immunity. Mucosal IgA and CTL responses are known to provide protection against a number of infectious agents (1–3). HIV is but one example (4, 13, 14). There are a number of strategies available for the development of vaccines which induce these immune parameters. However, none at present appear globally applicable (15). Live attenuated vaccines produce robust immunity including mucosal IgA and CTL responses. Unfortunately, difficulty in attenuating many pathogens and the risk of iatrogenic disease limits the use and development live attenuated vaccines (1, 2, 13, 15). On the other hand, recombinant proteins from infectious agents are generally safe but induce relatively poor immune responses, and are not active when delivered to mucosal surfaces (1, 2). However, mucosal adjuvants can improve immune responses toward coadministered protein antigens substantially (1, 2, 15). Cholera toxin is an extremely potent mucosal adjuvant, but is inherently toxic and induces a Th2-biased immune response that includes the development of IgE and allergic sensitization toward the target antigen (16, 17). At present, such toxicity and other technical problems have kept many adjuvants from becoming available for use in humans (15). Alum is essentially the only adjuvant in clinical use today. It is relatively weak, does not work with a number of antigens, does not induce CTL activity, and, because it must be delivered systemically, does not induce mucosal IgA (15). A safe and effective mucosal adjuvant would be of great value in the development of better vaccines. ISS-ODN is a potent adjuvant which works with a wide range of protein antigens, and generally induces a Th1-biased immune response with CTL activity (5–9). In this report we have shown that both i.n. and i.d. administration of protein with ISS-ODN leads to vigorous Th1-biased systemic immune responses, whereas only i.n. delivery induces a mucosal immune response. Therefore, i.n. delivery of relevant antigens with ISS-ODN may well prove superior to i.d. delivery for the induction of protective immunity to mucosal pathogens. Our personal experience has been that ISS-ODN is easy to manufacture, stable, and without identified toxicity at immunogenic doses in mice and

primates (unpublished observations). Additionally, use of antisense phosphorothioate oligodeoxynucleotides in monkeys and human clinical trials has demonstrated no significant toxicity with daily doses of up to fivefold more per kilogram than those used in the present study (19). Moreover, we and others have shown that human and mouse immunocytes display similar immunologic responses to ISS-ODN, suggesting that our present findings might also be applicable to humans (8, 20). The data presented represent a proof of principle which shows that in addition to its systemic adjuvant activity, ISS-ODN is an excellent mucosal adjuvant, and suggests a novel approach for the development of vaccines against infectious agents.

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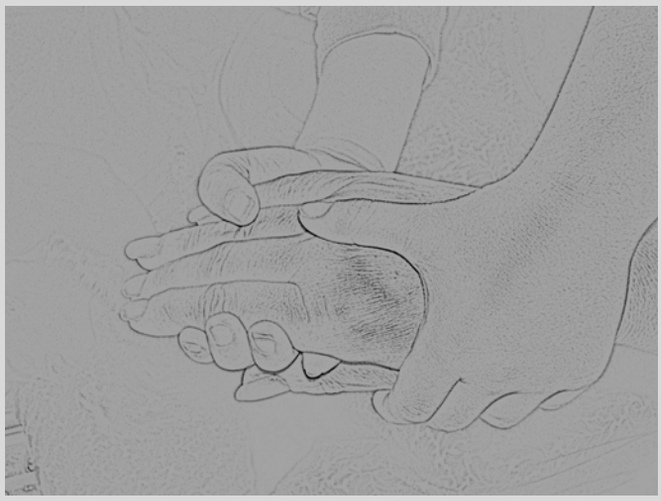

Chapter 4

Immunostimulatory DNA sequences influence the course of adjuvant arthritis

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Summary
Bacterial DNA is enriched in unmethylated CpG motifs that have been shown to activate the innate immune system. These immunostimulatory DNA sequences (ISS) induce inflammation when injected directly into joints. However, the role of bacterial DNA in systemic arthritis is not known. The purpose of the present experiments was to determine if ISS contributes to the development of adjuvant arthritis in Lewis rats after intradermal injection of heat killed *Mycobacterium tuberculosis* (Mtb). The results showed that Mtb DNA was necessary for maximal joint inflammation in adjuvant arthritis, but could be replaced by synthetic ISS oligodeoxynucleotides (ODN). The arthritis promoting effect of the Mtb DNA or of the ISS-ODN correlated with an increased Th1 response to Mtb antigens, as measured by the production of interferon-gamma and increased production of the osteoclast differentiation factor RANKL. The Mtb DNA did not enter the joints, but dispersed to the bone marrow and spleen prior to the onset of systemic joint inflammation. Thus, adjuvant arthritis is a microbial DNA-dependent disease. In this model, we postulate that massive and prolonged activation of macrophages, dendritic cells, and osteoclast precursors in the bone marrow may prime the joints for the induction of inflammatory Th1 immune responses to Mtb antigens.

Introduction

Mycobacterial DNA, compared to mammalian DNA, is enriched in palindromic sequences containing unmethylated CpG dinucleotides that can activate the innate immune system (1). Such immunostimulatory DNA sequences (ISS) stimulate the expression of co-stimulatory molecules (2), and the production of cytokines such as IL-12, TNF-α and interferon by macrophages, dendritic cells, B lymphocytes and natural killer (NK) cells (1). Consequent to this effect on innate immunity, ISS skews adaptive immune responses towards a strong and prolonged Th1 type of immunity (3,4).

Recently, Tarkowski and co-workers were able to link joint damage in a model of septic arthritis to the presence of ISS (5). They showed that direct injection of bacterial DNA or oligonucleotides containing ISS into the joints led to arthritis, whereas injection of mammalian DNA did not induce inflammation. An influx of monocytes and macrophages, and only a minority of CD4 positive T lymphocytes, characterized the resulting arthritis. Locally, in the arthritic joint, an increased expression was found of mRNA for TNF-alpha, IL-1alpha and IL-12 and for the chemokines RANTES and MCP-1 (6). Altogether, these results indicated that bacterial DNA was sufficient to provoke joint inflammation in septic arthritis, and raised the question whether ISS might also play a role in the pathogenesis of other forms of arthritis (6). To explore this issue, we turned to a T cell mediated model of experimental autoimmune arthritis, namely adjuvant arthritis (AA). Adjuvant arthritis is an extensively studied form of chronic arthritis with a close histopathological resemblance to rheumatoid arthritis (7,8). AA can be induced in susceptible animals such as Lewis rats by injection of heat-killed Mtb in incomplete Freund's adjuvant (IFA) in the base of the tail. Since the immunostimulatory properties of bacterial DNA were first discovered in Mtb (9), we reasoned that ISS might play a role in this model of autoimmune arthritis. Here, we show that, indeed, ISS are crucial for the induction of maximal joint inflammation in AA, and strongly stimulate both a Th1 response and the production of the osteoclast differentiating factor RANKL to mycobacterial heat shock protein 65 (hsp65). Surprisingly, days after immunization with heat killed Mtb, residual mycobacterial DNA was detected in the spleen and bone marrow, but not the synovium, of arthritic rats.

Methods

Animals

Male inbred Lewis rats (RT1. B^1) of 6-9 weeks of age and female Balb/c mice of 6-8 weeks of age were purchased from Harlan Sprague Dawley (Indianapolis, IN) and The Jackson Laboratory (Bar Harbor, ME) respectively. All animals were maintained in the University of California at San Diego Animal Facility, which is accredited by the American Association for the Accreditation of Laboratory Animal Care. All experiments were approved by the Animal Subjects Committee in accordance with USDA guidelines.

Reagents

Heat killed *Mycobacterium tuberculosis* (Mtb, strain H37Ra) and incomplete Freund's adjuvant (IFA) were obtained from Difco (Detroit, MI). Mycobacterial heat shock protein (hsp65) and β-galactosidase (β-gal) were obtained from Stressgen Biotechnologies Corp (Victoria, BC, Canada) and Sigma (St. Louis, MO) respectively. Phosphorothioate ISS-containing oligodeoxynucleotide (ISS-ODN) and control oligodeoxynucleotide (Cont.-ODN) were purchased from Trilink Biotechnologies (San

Diego, CA). The sequence of the ISS-ODN was 5'-TGACTGTGAACGTTCGAGATG-3', and the sequence of the Cont.-ODN was 5'-TGACTGTGAATGTTAGAGATGA-3'. Endotoxin levels of the ISS-ODN and Cont.-ODN were below the limit of detection (<1 ng/mg DNA) as measured by the limulus ameobocyte lysate assay (Bio-Whittaker, Walkersville, MD).

Induction and Clinical Assessment of Experimental Arthritis
Adjuvant arthritis was induced by a single intradermal injection (i.d.) at the base of the tail with 0.3 mg Mtb suspended in 100 µl IFA (CFA). Rats were examined daily for clinical signs of arthritis in a blinded fashion. Severity of arthritis was assessed by weight loss and scoring each paw from zero to four based on degree of swelling, erythema, and deformation of the joints (maximum score=16).

Deoxyribonuclease Treatment of Mtb
Mtb was incubated overnight at 37°C with 5 µg deoxyribonuclease I (DNase) /ml (Sigma) in the presence of 5 mM $MgCl_2$. After extraction, DNA depletion was confirmed by agarose gel electrophoresis, and ethidium bromide staining, Mtb (0.3 mg) was suspended in 100 µl IFA (DNase/CFA).

Immunization Protocols
Rats received a single i.d. injection with either Mtb, DNase treated Mtb, or DNase treated Mtb supplemented with either ISS-ODN (3, 10, 30 and 100 µg) or control-ODN (100 µg) in a final volume of 100 µl of IFA. Three µg ISS corresponds to the estimated concentration of DNA in 0.3mg heat killed Mtb. The animals were examined daily for clinical signs of arthritis as described above. Animals injected with ISS-ODN (100 µg) suspended in 100 µl of IFA served as a control.
Mice received a single i.d. injection of β-gal (10 µg) emulsified with either Mtb (50 µg), DNase treated Mtb (50 µg), or DNase treated Mtb supplemented with either ISS-ODN (10 µg) or Cont.-ODN (10 µg) in a final volume of 50 µl IFA. Injections of β-gal alone or β-gal with either IFA or IFA/ISS-ODN were used as controls. Four weeks after injection, mice were bled, and spleens harvested for cytokine assays.

Antibody and cytokine assays
Mouse β-gal-specific IgG2a and IgG1 antibodies were measured by ELISA and are expressed in relative units, as previously described (3). Antigen-specific mouse splenocyte cytokine profiles also were assessed by ELISA, as described earlier (3). Mouse spleens were harvested four weeks after injection, teased to single cell suspensions and resuspended in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS). Splenocytes were incubated at 5 x 10^5 cells per well in 96 well plates in a final volume of 200 µl of complete media with β-gal added at 10 µg/ml at 37˚C and 5% CO_2. Culture supernatants were harvested at 72 hours and analyzed for IL-5 and IFN-gamma (Pharmingen)by ELISA, according to the manufacturer's directions.
Rat inguinal lymph node cells (ILN) were harvested for antigen-specific IFN-gamma and RANKL assessment 55 days after induction of AA. Briefly, ILN were strained through a 70 um nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ) and resuspended at 1 x 10^6 cells per well in 96 well plates in a final volume of 200 µl Iscove's modified Dulbecco's Medium supplemented with 10% heat-inactivated FCS. Inguinal lymph node cells were cultured for 72 hours with hsp65 (10 µg/ml) and analyzed by ELISA as per kit instructions (IFN-gamma, Biosource International,

Camarillo, CA; RANKL, R&D Systems, Minneapolis, MN). The antibody against mouse RANKL cross-reacted with the rat protein.

Detection of Mtb DNA in rat tissues

Mtb in various rat tissues was detected by PCR. Two reactions for each organ for each time point were performed for each animal (n=4). Lewis rats were sacrificed 1, 3, 7, 10, 14, 17, 21, 29, and 36 days after receiving a single i.d. injection of 0.3 mg Mtb in IFA. For each time point, approximately 50 mm^3 of tail base (site of injection), spleen, inguinal lymph node, bone marrow of the tibia, synovium, kidney, and liver was harvested and frozen at $^{-}80°C$ until analyzed. Samples were boiled for 10 minutes in 0.5 ml-pyrogen free sterile water to lyse cells and release DNA.

The semi-nested PCR assay consisted of two rounds with each reaction (total volume 30 µl) performed as per AdvanTaq Plus PCR Kit instructions (Clontech, Palo Alto, CA). The template for the first PCR round consisted of 1 µl of a 1:20 dilution of DNA extract with a 0.3 µM concentration of forward primer (IDT, San Diego, CA) [5'-GATCCTGCGAGCGTAGGCGTCGGTGAC-3'] and reverse primer [5'-GATCTCGTCCAGCGCCGCTTCGGACCA-3'][6]. This primer-pair was designed to amplify a 123-bp region of a Mtb multi-copy insertion sequence (IS6110). The amplification parameters included 20 cycles each of denaturation at 94°C for 30 seconds, followed by a single annealing and extension step at 72°C for 30 seconds.

A second round of PCR amplification used the forward primer and an internal primer [5'-GCACCTAACCGGCTGTGGGTAGCA-3'] with identical conditions and one-thirtieth of the amount of the first PCR product as a template. The second round of amplification consisted of 27 cycles and yielded a 100-bp product. All samples were compared against the control gene, actin, with an amplification procedure of 35 cycles with identical conditions for each cycle. The PCR products were analyzed by electrophoresis on 1.5% metaphor agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator.

Dot Blot Hybridization

Nucleic acids were extracted from the injected areas (tail base) and from affected synovial tissues (ankle) by boiling in 1 ml of TE buffer at 100^0C for 10 min. Solid materials were removed by centrifugation at 14,000 for 10 min and 1 µl from each of supernatant was loaded on a Hybond N+ membrane (Amersham). After fixation of the nucleic acids onto the membrane with 0.2 M NaOH, the filter was hybridized with ^{32}P-labeled oligonucleotide complementary to the ISS-ODN (5'-TGACTGTGAACGTTCGA), washed with 2 x SSC containing 0.1 % SDS three times and exposed to X-ray film [Kodak X-omat Blue XB-1].

Histology

Excised rat joints were stained with hematoxylin and eosin. A synovial histology score was determined on the stained sections using a semi-quantitative scale that measures synovial inflammation (0-4), bone and cartilage erosions (0-4), marrow infiltration (0-4), and extra-articular inflammation (0-4) (maximum score=16).

Statistics

Two tailed unpaired student t tests were used to compare antibody levels, cytokine levels, clinical arthritis scores, and histology scores using Statview and Mathsoft computer software.

Results

Characterization of Mtb DNA treated with DNase

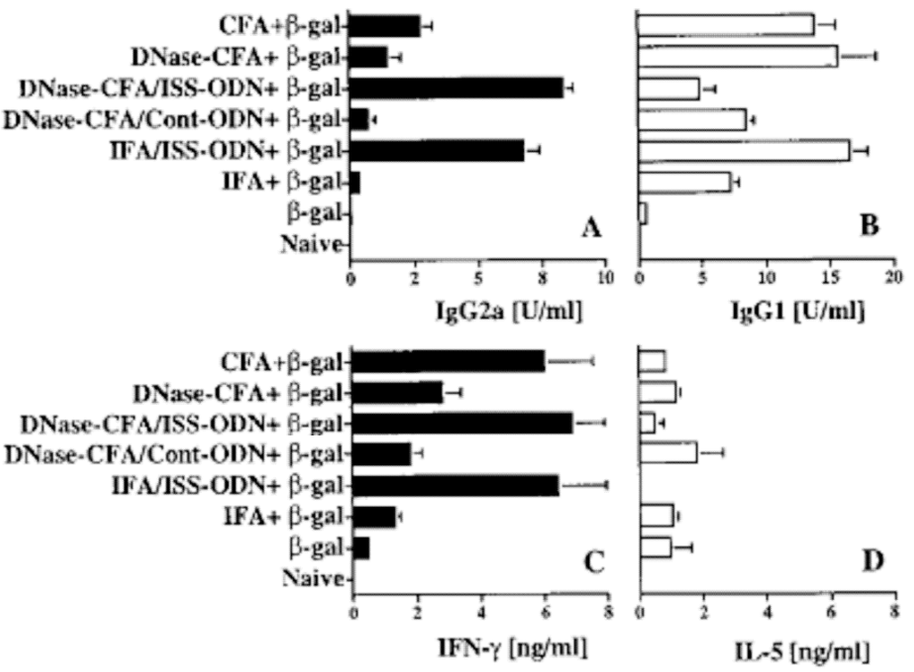
MTb extract was treated with DNase @ 37^0C overnight. The digest product, untreated Mtb DNA and a 10bp ladder standard were electrophorsed on a 20% polyacrylamide gel and stained with cyber green. The DNase treatment was complete, as no digest products were observed (< 10bp, data not shown).

DNase treatment inhibits the Th1 skewing properties of Complete Freund's Adjuvant

Complete Freund's adjuvant (CFA) has been reported to polarize immune responses to co-administered antigens towards a Th1 type. Before turning to the model of adjuvant arthritis, we questioned whether it is possible to abolish the Th1-skewing capacities of CFA by DNase treatment and subsequently restore it with oligonucleotides containing ISS sequences. Heat killed Mtb was treated with DNase and, subsequently, suspended in IFA to make DNase-CFA. To test immunogenicity of DNase-CFA *in vivo* we immunized Balb/c mice with β-galactosidase (β-gal) and the following adjuvants: IFA, CFA, DNase-CFA, DNase-CFA mixed with ISS-ODN, DNase CFA mixed with control ODN, and IFA mixed with ISS-ODN. The immunization consisted of a single intradermal injection of β-gal, emulsified with one of the mentioned adjuvants. Four weeks later blood and spleens were harvested. The antibody isotype responses to β-gal were assessed (Figure 1, upper panel). Compared to the antigen-CFA combination, mice immunized with β-gal and DNase treated CFA had reduced IgG2a levels ($p=0.05$). However, when DNase treated CFA was mixed with ISS-ODN, anti-β-gal IgG2a production was restored ($p<0.0001$).

In addition, we measured the antigen specific production of IFN-gamma and IL-5 by splenocytes after immunization with β-gal and the different adjuvants. The results mimicked the antibody data (Figure 1, lower panel). Mice immunized with β-gal, and DNase treated CFA showed reduced β-gal specific IFN-gamma levels, compared to the CFA positive control ($p=0.08$). In contrast, after immunization with β-gal and DNase treated CFA and ISS-ODN, a strong increase in IFN-gamma production was found, in comparison to β-gal DNAse treated CFA alone or with control ODN ($p<0.02$), indicative of a Th1-like response. A similar response was induced when β-gal was co-administered with IFA mixed with ISS-ODN, whereas β-gal co-administered with DNase CFA and control ODN did not lead to increased production of IFN-gamma ($p<0.03$). Thus, DNase treatment of Mtb reduced its Th1 polarizing adjuvant effect, which could be completely restored with ISS-ODN alone.

Figure 1. Th1-skewing capacities of CFA are dependent on the presence of ISS



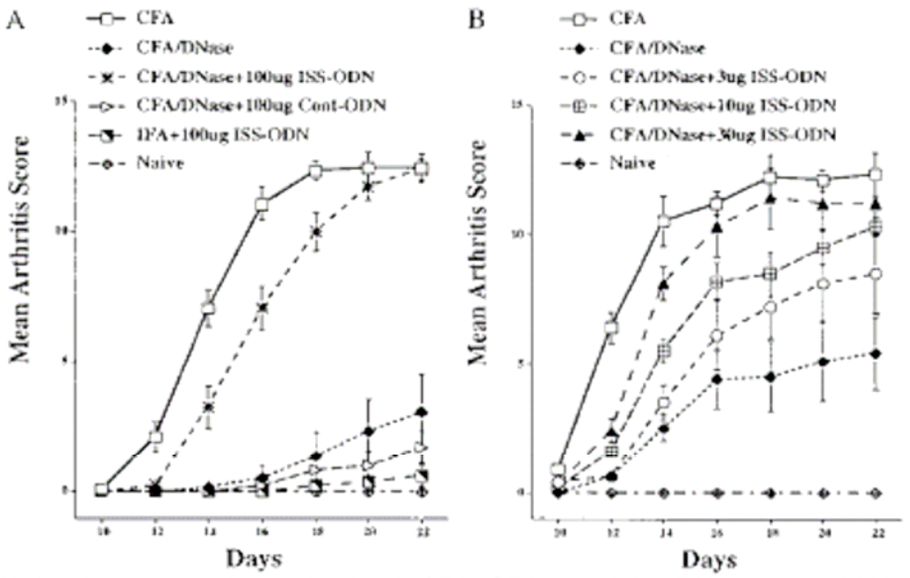
BALB/c mice were immunized with β-galactosidase (β-gal) alone or mixed with the following adjuvants: IFA, CFA, DNase treated CFA, DNase treated CFA mixed with ISS, DNase treated CFA mixed with control ODN, and IFA mixed with ISS-ODN. Four weeks later, blood and splenocytes were harvested to asses the (A) Anti-β-gal IgG2a, (B) Anti-β-gal IgG1, (C) anti-β-gal IFN-gamma, (D) and anti-β-gal IL-5 response. Results are the means ± SEM for four mice per group.

Immunostimulatory DNA sequences determine the severity of AA

Next, we determined whether the presence of Mtb DNA is necessary to induce adjuvant arthritis. We first treated heat-killed Mtb with DNase until no high mw DNA was detectable by electrophoresis and ethidium bromide staining. We injected Lewis rats with either emulsified heat killed Mtb, DNase treated Mtb, DNase treated Mtb supplemented with ISS-ODN (3, 10, 30 and 100 µg), or DNase treated Mtb with a control ODN. The results are shown in Figures 2A and 2B. DNase treatment of Mtb lead to a delay in the onset and a marked reduction in the severity of arthritis (mean maximum arthritis score 5) compared to the positive control (mean maximum arthritis score 12, $p<0.0001$). The addition of ISS-ODN (100ug) to DNase treated Mtb restored the severity of arthritis completely (mean maximum arthritis score 12), whereas the addition of control ODN was devoid of efficacy ($p<0.0001$). The clinical findings were also reflected in the histological scores (Figure 3). These experiments showed that the severity of joint inflammation in AA depends on the presence of Mtb DNA.

Systemic immunization with ISS, mixed with IFA alone, was not sufficient to induce arthritis in Lewis rats. Rats immunized with ISS and IFA showed normal weight curves (not shown), and did not display signs of arthritis up till 60 days after immunization (Figure 2 and 3). These results suggest that mycobacterial antigens, or other factors besides DNA, also are required for arthritis induction.

Figure 2. ISS present in CFA determine the clinical severity of adjuvant arthritis



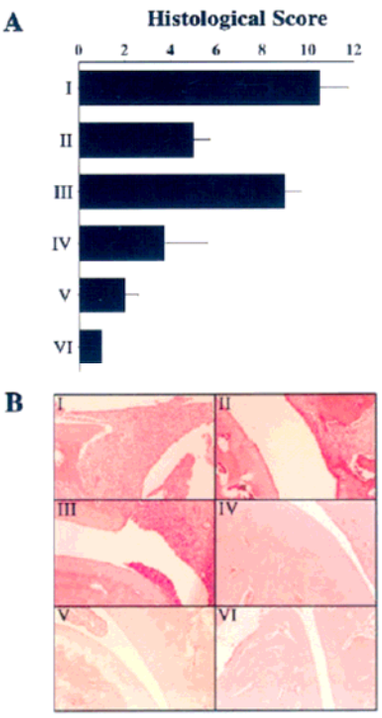
(A). Lewis rats were immunized with CFA, CFA containing DNase treated Mtb, CFA containing DNase treated Mtb mixed with ISS-ODN (100ug), CFA containing DNase treated Mtb mixed with control ODN, or with IFA mixed with ISS. The ordinate shows the mean arthritis scores (± SEM) at the indicated times after injection (N=16 for all groups except for CFA/DNase/Cont-ODN N=6, IFA/ISS-ODN N=4 and Naïve N=8). Results are means+SE. CFA *vs.* CFA/DNase $p<0.0001$, CFA/DNase *vs.* CFA/DNase+ISS $p<0.0001$, CFA/DNase+ISS *vs.* CFA/DNase+Cont $p< 0.0001$, and CFA/DNase+ISS *vs*. CFA $p=0.038$. (B) Dose response: Three doses of ISS-ODN (3, 10 and 30ug) were tested. (n=5 per group). There was no significant difference in the arthritis scores between CFA *vs.* CFA/DNase/ISS-ODN (3, 10 and 30 µg) at day 22.

Immunostimulatory DNA induces Th1 responses and the production of soluble RANKL to Mtb antigens

To determine the effects of Mtb DNA on antigen specific cytokine synthesis, we cultured inguinal lymph node cells (ILN) at day 55 after arthritis induction with medium alone, Mtb, DNase treated Mtb or purified Mtb hsp65. After 72 hours of culture, supernatants were collected and IFN-gamma and RANKL levels were assayed. Figure 4 illustrates the results of hsp65 stimulation. Similar results were found with Mtb extracts (not shown). Lymph node cells from rats immunized with whole heat killed Mtb produced IFN-gamma and soluble RANKL after *in vitro* activation with hsp65. In contrast, lymph node cells from rats immunized with DNase treated Mtb produced significantly less IFN-gamma and RANKL after *in vitro* restimulation with hsp65 ($p<0.05$). However, lymph node cells from rats immunized with DNase treated Mtb, which had been supplemented with ISS-ODN, produced high levels of IFN-gamma and RANKL after *in vitro* activation with hsp65, whereas lymph node cells from rats immunized with DNase treated Mtb, mixed with a control ODN,

produced IFN-gamma and RANKL at levels comparable to those produced by cells from rats immunized with DNase treated Mtb ($p<0.05$, DNase-CFA/ISS-ODN vs. DNase-CFA/Cont-ODN). We could not detect antigen specific IFN-gamma and RANKL production in naive rats. Other experiments demonstrated no significant production of IL-4 or IL-10 in any of the lymph node cultures. Thus, primed lymph node cells from rats immunized with a mixture containing ISS (either Mtb DNA or ISS-ODN) produced significantly higher levels of IFN-gamma and RANKL, compared to primed lymph node cells from rats immunized with Mtb without ISS. The levels of IFN-gamma and RANKL production by antigen restimulated lymph node cells correlated with the severity of arthritis found in the different treatment groups.

Figure 3. ISS determine the pathologic severity of adjuvant arthritis



On day 22, after injection with the antigens described in Figure 2, rats were sacrificed, and their ankles were decalcified and paraffin-embedded. Panel A shows the mean synovial histological scores ± SEM, and Panel B illustrates representative hematoxylin and eosin stained sections magnified 100 fold. (I) CFA, demonstrating marked synovial hyperplasia and cartilage erosion; (II) DNase-CFA, demonstrating modest erosion and synovial lining hyperplasia; (III) DNase-CFA mixed with ISS-ODN, demonstrating severe synovial inflammation with modest erosions; (IV) DNase-CFA mixed with control ODN, demonstrating mild synovial hyperplasia and edema without significant erosions; (V) IFA mixed with ISS, demonstrating normal cartilage and minimal synovial hyperplasia; (VI) Naïve, no synovial hyperplasia or erosions.

Disposal of Mtb DNA

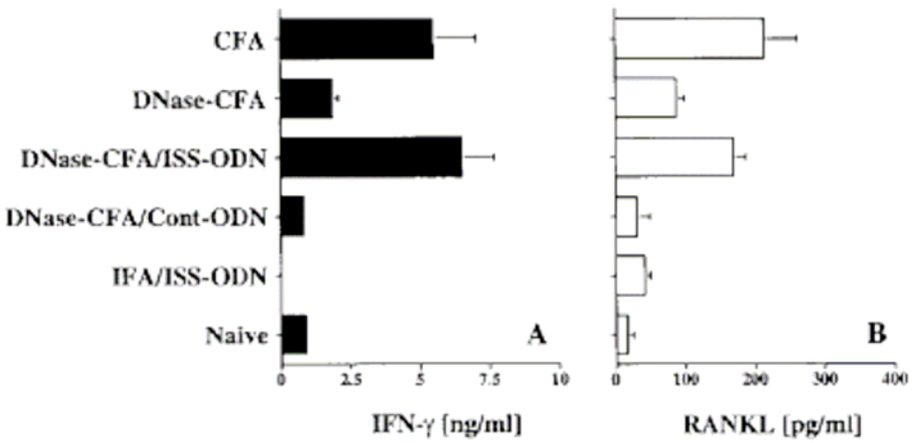
Since intra-articular injections of ISS induce joint inflammation, it was important to determine if Mtb DNA was present in the joints. At 1, 3, 7, 10, 14, 17, 21, 29 and 36 days after injection of Mtb into the tails of Lewis rats, PCR analyses were performed on tissue samples taken from kidney, liver, spleen, bone marrow, base of the tail, inguinal lymph node and synovium (Figure 5). Mycobacterial DNA was detected at the site of injection, in the spleen up till day 36, in the draining (inguinal) lymph nodes at day 10 and in the bone marrow at days 3, 7, 10 and 14. No Mtb DNA was detected in the liver and kidney, or synovium. Thus, after immunization with CFA, mycobacterial DNA disperses to bone marrow and lymphoid tissues, but not to the synovium. Hence, the arthritogenic effects of Mtb cannot be attributed to a local effect on the synovium, as was the case in the model of septic arthritis (5).

We then explored the presence of ISS-ODN in the affected synovial tissues (ankles). Rats were injected with CFA/DNAse/ISS-ODN (100 µg) and killed at day 1 and day 14 (two rats per time point). DNA was extracted from the injected area (base of the tail) and from the inflamed ankles, and was subjected to Southern blot analysis using ^{32}P labeled complementary ODN as a probe. As shown in Figure 5B, ISS-ODN was detected in the area of injection but not in the affected synovium.

Discussion

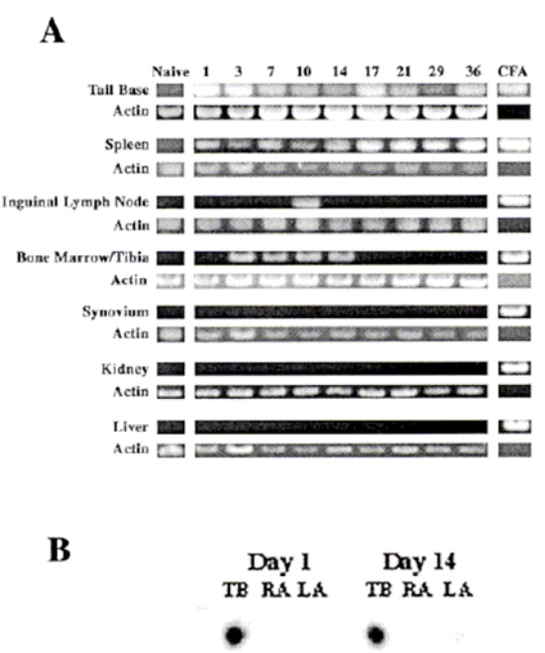
Our experiments show that the severity of AA in rats depends upon the presence of ISS in heat-killed Mtb DNA. The ISS polarize the immune response to mycobacterial antigens toward a Th1 phenotype. Indeed, the established Th1 stimulatory properties of CFA in mice are largely attributable to Mtb DNA. In AA, the Th1 response to Mtb antigens correlates with disease intensity. However, the combination of ISS and IFA

Figure 4. Bacterial DNA stimulates the production of IFN-gamma and RANKL in response to mycobacterial hsp65



Inguinal lymph node cells were (ILN) were harvested at day 55 after arthritis induction, and restimulated *in vitro* with purified Mtb hsp65 for 72 hours. Supernatants were harvested and IFN-gamma(Panel A) and RANKL (Panel B) levels were assayed by ELISA. Results are the means ± SEM for four rats per group.

Figure 5. Trafficking of mycobacterial DNA following arthritis induction



(A). PCR analysis. Rats were immunized with Mtb in oil to induce arthritis. At days 1, 3, 7, 10, 14, 17, 21, 29, and 36 following immunization tissue samples were taken from the base of the tail, spleen, inguinal lymph nodes, bone marrow of tibia, synovium, kidney, and liver to detect the presence of Mtb DNA using a semi-nested PCR. Actin was used as a control for each tissue. Note that Mtb DNA could be detected after immunization at the site of injection , and in the spleen until day 36. Mtb DNA in the inguinal lymph nodes was present at day 10, and in the bone marrow at days 3, 7, 10, and 14. No Mtb DNA was detected in the liver, kidney, or synovium. Results shown here were reproduced in four rats per organ per time point. (B) Southern blot analysis. Rats (n=2) were injected with CFA/DNase/ISS-ODN (100 µg) and killed at day 1 and day 14. Southern blot analysis was performed on DNA samples extracted from the injected area (base of the tail) and from the inflamed ankles using ^{32}P labeled complementary ODN as a probe. ISS-ODN was detected only in the area of injection but not in the affected ankle synovium. Data represent one experiment out of two, which yielded the same results. TB = tail base, RA = right ankle, LA = left ankle.

is not sufficient to induce arthritis. Collectively, the data suggest that ISS amplifies the course of arthritis by massive and prolonged activation of the innate immune system, especially in the bone marrow. This, in turn, may stimulate adaptive Th1 responses to various Mtb antigens (e.g., hsp65).Once established, AA is clearly a T cell mediated process, since the passive transfer of CD4+ T cells from affected rats to irradiated syngenic recipients induces disease, whereas serum transfer has no deleterious

effects (10). Moreover, arthritis can be triggered by a cross-reactive CD4 T cell clone that recognizes an epitope present on bacterial heat shock proteins and cartilage proteoglycans (11,12). However, the early induction of AA requires stimulation of innate immunity, as indicated by the ability of cyclo-oxygenase inhibitors, TNF-alpha antagonists, and chemokine blockers to prevent disease (13-15).

One way to explain these data is to propose that the activation of innate immunity by ISS primes the joints for the subsequent development of a Th1 autoimmune response. In this context, Sato et al., recently reported that the prior injection of ISS-ODN into mice exacerbated the severity of arthritis induced by immunization with collagen in CFA (16). Unlike most other bacterial products that activate innate immunity, ISS are poorly immunogenic and can persist for long periods within transfected cells. Tarkowsky et al. found that the direct injection of bacterial DNA into the articular cavity of rats induced severe inflammation (5). Both antigen-antibody complexes, and systemically administered antigens have been shown to traffic to the joints. Thus, the systemic dispersal of Mtb DNA, and its subsequent trapping in the synovium, could explain the potentiating effects of ISS in AA. However, from 1-36 days after injection of heat killed Mtb in the tail, we were unable to detect Mtb DNA sequences or ISS-ODNs within the joints. Instead, the DNA dispersed primarily to the bone marrow, the lymph nodes, and the spleen.

The activation of innate immune responses within the bone marrow may be particularly relevant to the induction of arthritis. In the collagen arthritis model, which requires Freund's adjuvant, the normally small channels between the bone marrow and synovial cavity enlarge prior to the onset of joint swelling (17). The resorption of subchondral bone is an established early event in adjuvant arthritis. Bone resorption requires the activation of osteoclast precursors within the marrow. The osteoclast activation, in turn, depends upon the increased production of RANKL (osteoprotegerin ligand/TRAIL) by T lymphocytes. Penninger and co-workers discovered that inhibition of RANKL signaling prevented bone and cartilage destruction in the adjuvant arthritis model, although leukocyte infiltration into the joints was still present (18). Our experiments showed that increased RANKL production by T cells is part and parcel of the immune system activation induced by ISS and antigen.

The deposition of Mtb DNA in the bone marrow not only argues against the direct involvement of Mtb DNA in the pathogenesis of AA, but also suggests that other Mtb related compounds (e.g., hsp65) could follow the same course. Activation of T cells in the bone marrow by Mtb related antigens might induce the local secretion of RANKL, which activates osteoclasts and thus makes the joint permeable and more susceptible to the influx of inflammatory cells and the subsequent development of arthritis.

Recently, there has been a revival of interest in the role of innate immunity in the pathogenesis of human rheumatoid arthritis and other inflammatory forms of arthritis of unknown etiology. The products of activated macrophages are abundant in inflammatory synovial fluids. Both bacterial DNA and peptidoglycans have been detected in the joints (19,20). Th1 type responses to bacterial heat shock proteins are readily measurable in synovial fluid T lymphocytes, whereas responses to other antigens are often weak or absent (21,22). Moreover, endogenous hsp65, as expressed in inflamed synovial tissue (23), can trigger innate immune responses through the LPS receptor TLR4, and CD14 (24).

The nine known Toll-like receptors (TLR) regulate the response of the innate immune system to bacterial products (25). Knockout experiments have demonstrated that the

murine response to ISS-ODN requires TLR9 (26). In addition to stimulation through TLR9, various Mtb products such as lipopeptide or hsp65 also can interact with TLR2 and TLR4, respectively (25,27). Thus, the activation of innate immunity by Mtb is multifactorial. Consistent with this interpretation, DNA digestion of mycobacteria did not totally abolish its arthritis-promoting properties. Future experiments will need to determine the role of different TLRs in the human response to microbial DNA, the expression of TLRs in cells from the bone marrow and joints, the fate of bone marrow derived macrophages exposed to bacterial products, and the interactions between the TLRs and various cytokines.

In general, Phosphorothioate ODN are much more potent than phosphodiester ODN or intact DNA. Thus, the lower efficacy of ISS ODN to induce AA could suggest that intact Mtb DNA is much more effective than the comparable amount of ISS ODN. This could reflect some intrinsic property of Mtb. Alternatively, the DNase treatment may have destroyed some other factor, which play a proinflammatory role in this system.

Bacterial DNA and ISS-ODN are much more potent activators of the innate and adaptive immune system in rodents, compared to primates (1). However, various interleukins have been demonstrated to synergize with ISS in the activation of human natural killer (NK) cells (28). The confined synovial spaces of patients with inflammatory arthritis can act as a sink for cytokines and other inflammatory mediators. In this setting, bone marrow derived macrophages, lymphocytes and NK cells could be more readily stimulated by bacterial DNA. Thus, although ISS are unlikely to be a direct cause of rheumatoid arthritis, they could play a significant role in disease progression and severity. Inhibitors of ISS binding to TLR9, or of ISS-induced signal transduction, therefore might be useful for the treatment of affected patients.

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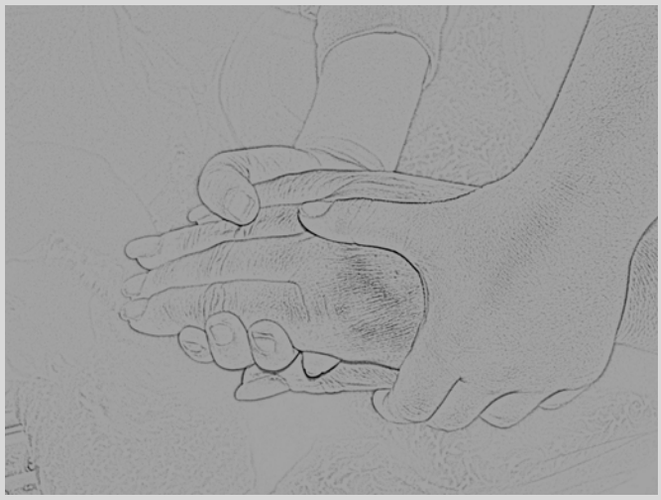

Chapter 5

Safety and efficacy of Meningococcal C Vaccination in Juvenile Idiopathic Arthritis

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Abstract
Background: It is uncertain whether vaccinations aggravate the course of autoimmune diseases such as Juvenile Idiopathic Arthritis (JIA). In addition, the immune response to vaccination may be hampered by immunosuppressive therapy for the underlying disease.
Methods: A multicentre cohort study was performed in 234 JIA patients between 1 and 19 years vaccinated with Meningococcal serogroup C (MenC) conjugate vaccine (NeisVac-C). Patients were followed for disease activity during one year, from six months before until six months after vaccination. IgG antibody titres against MenC polysaccharide and the tetanus carrier protein were determined by ELISA and Toxin binding inhibition assay, respectively. A serum bactericidal assay was performed for functional assessment of anti-MenC antibodies.
Results: No aggravation of all six core set criteria values for disease activity was seen after MenC vaccination. Moreover, no increase in the frequency of disease relapses was detected. Mean anti-MenC IgG concentrations of 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 on highly immunosuppressive medication. The four patients with a lower anti-MenC antibody response displayed sufficient bactericidal activity despite their highly immunosuppressive medication.
Conclusions: The MenC conjugate vaccine does not aggravate JIA disease activity or relapse frequency and results in adequate antibody levels even in patients on highly immunosuppressive medication. This means patients with Juvenile Idiopathic Arthritis can be vaccinated safely and effectively with the Meningococcal serogroup C conjugate vaccine.

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 on possible adverse effects hamper compliance.(5-12) The decreasing herd immunity poses patients with chronic autoimmune diseases at increased risks.(13;14)

Another concern is the potentially diminished efficacy in patients under immunosuppressive therapy.(15) In the UK, physicians were less likely to vaccinate children with Juvenile Idiopathic Arthritis (JIA) on higher levels of immunosuppressive therapy.(16) 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, may require boosters.(17)

In 2002, the Dutch health authorities initiated a nation-wide campaign in which all children between 1 and 19 years of age were vaccinated against Meningococcal serogroup C disease.(18;19) The guidelines for exclusion were unspecific 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.

Methods

Study design

A multicentre cohort study was performed in which patients served as their own controls. For each patient, the study period covered one year starting six months before MenC vaccination. Since most autoimmune reactions reported by others followed vaccination within one month, we defined this period as exposed period.(20-25) The remaining eleven months of the study period were defined as unexposed period.

Study population

All patients between 1 and 19 years of age with a diagnosis of JIA according to the criteria of the International League of Associations for Rheumatology (ILAR) were eligible.(26) Before the start of the national vaccination campaign with the conjugate MenC vaccine, patients from paediatric rheumatology outpatient clinics of the University Medical Centres of Utrecht, Leiden, Amsterdam, the Jan van Breemen Institute (Amsterdam) and the Juliana Children's Hospital (The Hague) were invited by mail to participate in this study. Written informed consent was obtained from patients or their parents. Approval by the medical ethical boards of the participating centres was acquired.

Of 538 invited patients, 277 replied (51.5%). Replying and non-replying patients were comparable for age, sex and JIA disease type. Twenty of the replying patients visited outpatient clinics elsewhere, ten patients moved elsewhere, eleven refused to participate and of two patients vaccination dates could not be retrieved. Thus, 234 patients from five centres in The Netherlands were enrolled (flowchart provided as supplement). The total study population consisted of 65% females. At the vaccination date, patients had a mean age of 11.1 ± 4.2 years (range 1.5-18.9) and a mean duration of JIA of 5.9 ± 3.5 years (range 0.2-16.0). Mean age at onset of JIA was 5.3 ± 3.7 years (range 0.5-15.9). The group of patients tested for relapse frequency (n=166) was statistically not different from the total cohort (n=234) with respect to demographic, disease and treatment characteristics.

Definition of medication groups

Patients were classified based on medication use at the time of MenC vaccination as defined in Table 1. Medication groups did not differ in age, gender, duration of JIA or age at onset of JIA. As expected, medication group 4 contained most patients with

severe forms of JIA (i.e. extended oligoarticular JIA and polyarticular JIA), whereas group 1 contained most persistent oligoarticular patients. Consequently, disease activity before vaccination was highest in medication group 4.

MenC conjugate vaccination

The NeisVac-C vaccine (Baxter, Vienna, Austria) contains the *Neisseria meningitidis* serogroup C polysaccharide (20 µg/ml) conjugated to tetanus toxoid (TT) (20-40 µg/ml). Patients received one 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 1st and December 26th, 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 serological 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 on disease activity.(27) Within this core set, the Physician Global Assessment (PGA) gives the overall impression of disease activity by a paediatric rheumatologist. PGA was measured on a 10 cm visual analogue scale (VAS) and converted to 0-3 scores. The Childhood Health Assessment Questionnaire (CHAQ) was used for determination of overall well-being (CHAQ well-being) and functional ability (CHAQ disability) both expressed on a 0-3 scale.(28;29) Active joints (AJ) were defined as all joints with swelling or with any two other signs of inflammation (heat, limited range of motion, tenderness or painful range of motion).(30;31) The presence of a limited range of motion (LOM) was defined for each joint as a loss of at least 5° in any articular movement with respect to the normal amplitude. Erythrocyte sedimentation rates (ESR) completed the core set of six criteria. A disease relapse was defined as a worsening of 40 percent or more in at least two out of six core set criteria without an improvement by more than 30 percent in more than one of the remaining.(32)

Disease activity parameters obtained by paediatric rheumatologists during at least one visit before and one visit after vaccination were compared. For the detection of disease relapses in a large subset of patients (n=166, all from UMCU), this assessment of core set criteria was extended to all available outpatient clinic visits and hospitalizations within the entire study period. Not every core set criterion was routinely evaluated, but PGA 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 by the next visit.

Serology

Blood samples of patients were drawn from 198 patients before and after MenC vaccination. Thirty-three out of 198 patients were excluded from serological analysis as their post-vaccination blood sampling was delayed to more than 12 weeks after vaccination. We analysed 141 pre- and 157 post-vaccination samples, of which 133 were paired.

Table 1. Base line characteristics of JIA patients*

		Patients tested <12 weeks after MenC Vaccination‡			
	Total enrolled N = 234	Group 1 N = 47	Group 2 N = 41	Group 3 N = 43	Group 4 N = 26
JIA subtype – no. (%)					
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)
RF (positive/total)	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 – no. (%)					
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)	46 (97.9)	23 (56.1)	25 (58.1)	11 (42.3)
Patients taking oral steroids – no. (%)	5 (2.1)	0	0	2 (4.7)	2 (7.7)
Mean dose (mg/kg/dy)	0.11 ± 0.08			0.14 (0.13)	0.08 (0.03)
Range (mg/kg/dy)	0.05 – 0.40				

*Plus-minus values are means ± SD. Numbers between brackets are percentages of patients of total per column. †PGA: physician's global assessment of disease activity (0-3). Scores 0: inactive, 0.1-1,4 mildly active, 1.5-2.4 moderately active and 2.5-3.0 severely active. ‡Group 1: no medication, group 2: NSAID monotherapy, group 3: MTX low dose (< 10mg/m^2/wk) (n=36) or sulfasalazin (n=7) +/- NSAID, group 4: MTX high dose (>10mg/ m^2/wk) (n=15), Infliximab (n=2), Etanercept (n=6) , cyclosporin A (n=1) or combination of MTX and sulfasalazin (n=2) +/- NSAID. NSAID: non steroidal anti-inflammatory drug, MTX: methotrexate. Infliximab and Etanercept are TNFα- antagonists.

Anti-MenC total IgG antibodies were quantified in sera by enzyme-linked immunosorbent assay (ELISA) with the use of the CDC 1992 reference serum assigned a value of 24.1 µg/ml of anti-MenC IgG.(33;34) The lower limit of antibody detection was 0.24 µg/ml. Sera with undetectable anti-MenC IgG levels were assigned a value of 0.23 for mathematical reasons. Low responders (LR) were defined by post-vaccination anti-MenC IgG levels of 2 µg/ml or less.(35;36)

The level of anti-tetanus toxoid (TT) antibodies was measured with a tetanus toxin binding inhibition (ToBI) assay at the Laboratory of Vaccine Preventable Diseases, Bilthoven, The Netherlands as described previously.(37) Lower limit of detection was 0.01 IU/ml.

Serum bactericidal assays (SBA) against the serogroup C strain (C11, phenotype C:16:P1.7^a,1) were performed with baby rabbit serum (Pelfreeze Biologicals, WI, USA) as an exogenous complement source. SBA titres were expressed as the reciprocal of the final serum dilution giving $\geq$ 50% killing at 60 minutes.(38) Post-vaccination bactericidal titres < 8 were considered to predict susceptibility to MenC infection.(39-44)

Both the toxin binding inhibition assay and serum bactericidal assay were performed on blood samples of the four low responders (LR, anti-MenC IgG ≤ 2µg/ml) and a random sample of 10 out of 153 high responders (HR, anti-MenC IgG > 2µg/ml).

Statistical analysis

To test the uniformity of the subset of 166 patients used for relapse analysis with the total cohort (n=234), χ^2 tests with expected frequencies of the total cohort for

distribution of categorical variables and one sample T-tests for means were used. Changes in paired pre- and post-vaccination values of core set criteria were detected by Wilcoxon Signed Ranks Test.
Risk of relapse was quantified by division of the number of detected relapses in the exposed or unexposed period by the amount of patient-months observed within that period. Observed patient-months were calculated by multiplication of the number of patients (n=166) with the duration of the observed period in months (exposed period n=1 month, unexposed period n=11 months). Relative risk of relapse (RR) was defined by division of the risk of relapse during the exposed period by the risk of relapse in the unexposed period. 95% confidence intervals were calculated by $e^{\ln RR \pm 1.96\sqrt{1/A1 + 1/A0}}$, in which A_1 represents the number of relapses in the exposed period and A_0 the number of relapses in the unexposed period. χ^2 tests were used to analyse seasonal variability of relapses. The MIXOR program (version 2.0) was used for logistic regression analysis of longitudinal data.(45)
Distribution of geometric mean concentrations (GMC) and titers (GMT) were extremely skewed. Therefore, we used non-parametric tests: Mann Whitney U test or Kruskall Wallis test for comparisons between two or multiple groups, Wilcoxon signed ranks test for paired variables and χ^2 test for ordinal variables. For comparison of patient characteristics and anti-MenC IgG GMC between medication groups, one way ANOVA was performed on (natural log transformed) data. In case of multiple tests, Bonferroni-correction on p-values was applied. Pearson's correlation coefficient was calculated on natural log transformed data of titres.
Statistical analysis was carried out with the SPSS version 12.0.2 (SPSS inc, Chicago, Illinois, USA). Two-tailed p-values of less than 0.05 were considered significant.

Results

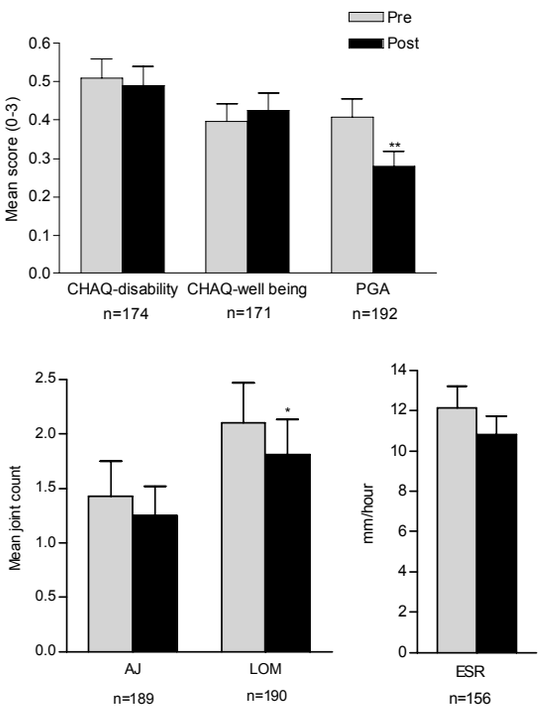
JIA disease activity

No worsening of disease activity was seen based on mean core set criteria values during six months after MenC vaccination compared to the period of six months before vaccination as measured in 234 JIA patients (Figure 1). Even a significant amelioration in PGA and LOM was observed after MenC vaccination but this was too small to be of clinical significance.

Relapses

We further analysed 747 visits (373 pre vaccination and 374 post vaccination) in a single centre subgroup of 166 patients. A total of 158 relapses were detected in 97 patients (Figure 2). 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 one 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 eleven months was 8.1%. The resulting relative risk 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 two, three or six months after MenC vaccination were similar (0.81 95% CI [0.48;1.38], 0.76 [0.52;1.12] and 0.52 [0.37;0.72]). Additional statistical analysis using a program for logistic regression 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).

Figure 1. Mean +/- SEM scores of core set criteria during six months before and six months after Men-C vaccination in 234 JIA patients.



Pre: 6-month period before vaccination, Post: 6-month period after vaccination, CHAQ: childhood health assessment questionnaire, PGA: Physician's global assessment of disease activity, AJ: arthritic joints, LOM: joints limited of motion, ESR: erythrocyte sedimentation rate. Statistically significant differences between pre- and postvaccination means are indicated as follows: **=p<0.005, *=p<0.05.

Efficacy of vaccination

Before vaccination, anti-MenC IgG GMC were comparable between medication groups (Table 2). The total group of JIA patients tested (n=157) showed a significant rise in anti-MenC IgG GMC from 0.4 µg/ml before vaccination to 28.9 µg/ml after vaccination (range 1.0-1820.5 µg/ml, p<0.0005). Anti-MenC IgG GMC were significantly lower in patients of medication groups 3 and 4 compared to GMC in patients of group 1 and 2 (Table 2). Four out of 157 tested patients (2.8%) had anti-MenC IgG levels ≤ 2 µg/ml after vaccination. Three of these low responders used low dose MTX, two of them in combination with Enbrel. The other low responder was on sulfasalazin treatment. None of the low responders was on steroids during vaccination. JIA was inactive in two low responders at vaccination date, while the other two had mild and moderately active disease. As the expected frequency of low responders was below five in each medication group, no conclusions can be drawn about the difference in risk of an anti-MenC IgG response ≤ 2 µg/ml between these groups.

The four patients within the study population that were on oral steroids all used MTX as well and thus belonged to medication group 3 and 4. Their mean anti-MenC IgG did not differ from the other patients in medication group 3 and 4 (p=0.63 and p=0.73). Anti-MenC IgG levels in patients with systemic onset JIA were comparable to levels measured in all other patients tested.

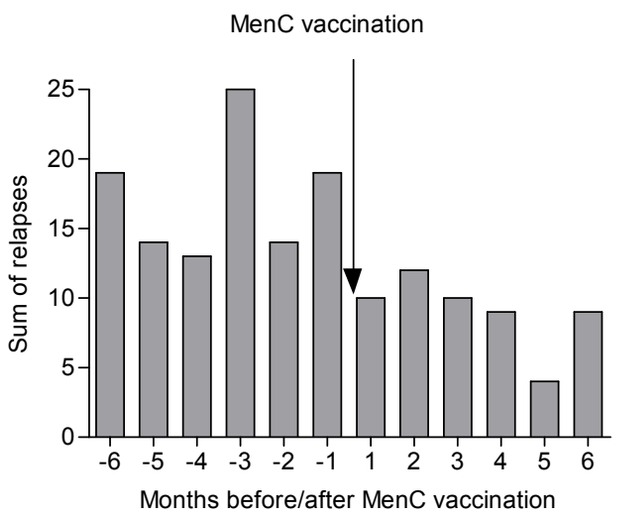
Table 2. Anti-MenC IgG concentrations in JIA patients categorized by medication group*.

	Group 1 N = 47	Group 2 N = 41	Group 3 N = 43	Group 4 N = 26	Significant differences between groups
Anti-MenC IgG – GMC					
Before vaccination	0.31	0.41	0.32	0.36	ns
After vaccination	41.00	46.93	17.53	16.28	Group 1 vs group 3 p=0.002 Group 1 vs group 4 p=0.01 Group 2 vs group 3 p=0.003 Group 2 vs group 4 p=0.012
Low responder – no. (%) (anti MenC IgG = 0.2mg/L)	0	0	2 (4.7)	2 (7.7)	

*For definition of medication groups, see Table 1. Differences between groups were determined by Kruskall Walllis test (p=0.001 for post vaccination MenC IgG) and Mann Whitney U test for skewed data. ns = non-significant. GMC; geometric mean concentration

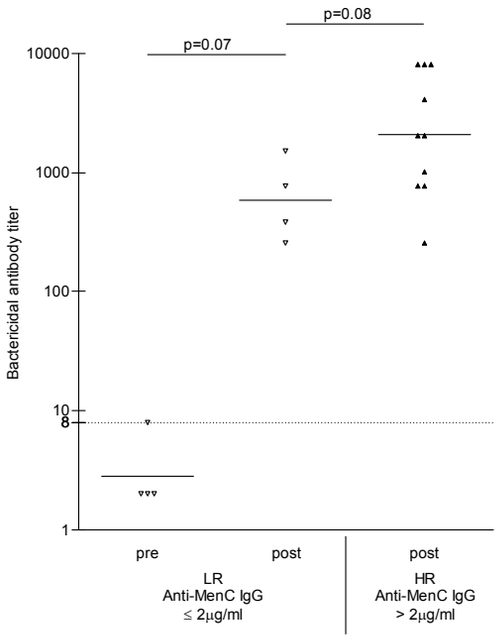
Patients with an anti-MenC response > 2 µg/ml (HR) showed a significant mean 17-fold rise in anti-Tetanus Toxoid (TT) antibodies after MenC vaccination ($GMTpostvacc_{HR}$: 14.95, p<0.001) whereas low responders (LR, anti-MenC ≤ 2 µg/ml) had only a 1.5-fold rise in anti-TT antibodies ($GMTpost_{LR}$:3.19, p=0.72) (data not shown).
Note that all tested patients including the four JIA patients with a low anti-MenC IgG response were able to mount SBA titres ≥ 8 (Figure 3). Although after vaccination a mean 206-fold rise in SBA GMT was observed in the four low responders, this did not reach significance (p=0.07) due to low numbers. The SBA titre raised by the low responders did not differ from the titre raised by the high responders (p=0.08).

Figure 2. Distribution of relapses in JIA patients before and after vaccination.



Total amount of relapses detected per month in a group of 166 JIA patients.

Figure 3. Serum bactericidal antibody titres in JIA patients before and after MenC vaccination.



LR: low responder (post-vaccination anti-MenC IgG ≤2 µg/ml), HR: high responder (post-vaccination anti-MenC IgG >2 µg/ml).

Discussion

This study shows that MenC vaccination is safe and effective in Juvenile Idiopathic Arthritis. In theory, molecular mimicry of components of the vaccine with self antigens combined with a specific bystander activation as well as a loss of regulatory mechanisms could account for aggravation of autoimmunity after vaccination.(46;47) The occurrence of arthritis after natural infection with *Neisseria meningitidis* in children and adults indeed suggests cross reactivity between non-self and self.(48-50) In earlier studies, nephrotic syndrome patients seemed to have increased relapse frequencies after MenC vaccination.(51) Patients with Idiopathic Thrombocytopenic Purpura also are at a particular risk of relapse after administration of life attenuated Measles, Mumps, Rubella vaccines (52). 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 one to six months. This is in line with previous studies by others in which rheumatoid arthritis and JIA patients tolerated influenza and Hepatitis B vaccination with no ill effects.(53-57)

As a double-blind placebo controlled study could not be performed in children for ethical reasons, the Dutch vaccination campaign against MenC yielded a unique study cohort. As 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 is limited for comparison of treatment groups.

A large proportion (48.5%) of invited patients did not reply to the invitation of participation. A low number of patients agreeing to participate is common in other vaccination studies. Studies on MenC or influenza vaccination yielded participant rates of 47%, 66% and 53%(58-60). Possible explanations for this low participant rate are the lack of information given by the authorities concerning vaccination of patients

with an autoimmune disease, parental concerns about a newly introduced vaccine and the necessity of two blood draws from the participating children in this study. As baseline characteristics of non-replying and replying patients were the same, selection bias is likely to have been minimal in this study.
As the majority of patients was 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 established earlier by other large studies (61;62).
The second aim of our study was to assess the influence of immunosuppressive medication used by JIA patients on efficacy of MenC vaccination. Overall, the anti-MenC IgG GMC levels we found in JIA patients (28.9 µg/ml) were in accordance with those observed in a mixed school entry (age 4,3 years) and school leaver (age 15.1 years) cohort (anti MenC IgG 29.1-51,6 ug/ml) and even higher than those observed by others in healthy adults (17.0 µg/ml) or in 12-18 months old healthy children (13.3 µg/ml) four weeks after a single dose of the NeisVac-C vaccine.(63-65) Because of this difference in anti-MenC IgG GMC measured between our JIA patients and healthy controls in the literature, we tested 12 healthy volunteers (age 21-50 years) ourselves before and six weeks after vaccination. Their anti-MenC IgG GMC (29.6 ug/ml, range 2.1-112.4) was not different from the total group of JIA patients reported here (p=0.631, 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 post-vaccination mean anti-MenC IgG GMCs in patients using DMARDs. MTX and, less consistently, TNFα 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 before.(66-71) As in our study only one patient was on anti-TNFα monotherapy (who 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 (Pearson's r=0.17), indicating that lower anti-MenC levels could not be explained by other immunodeficiencies. Taken together, these results indicate that patients taking highly immunosuppressive medication (like MTX) should be tested for their capacity to mount an adequate anti-MenC antibody response after vaccination.
The low response to the MenC part of the vaccine was associated with a low response to the conjugate protein TT. We assessed the ability of these low responders to raise sufficient bactericidal activity after a single dose of the vaccine by SBA.(72) Post-vaccination SBA titres in low responders (mean SBA= 736) were as high as in high responders and well above the earlier reported SBA titre in healthy 12-18 months old children (mean SBA= 564).(73) Importantly, all SBA titres after vaccination in patients with a low anti-MenC response were above the level predictive for protection. Therefore, all tested JIA patients seem to be adequately protected against Meningococcal type C disease after MenC vaccination, irrespective of the immunosuppressive treatment given.(74)

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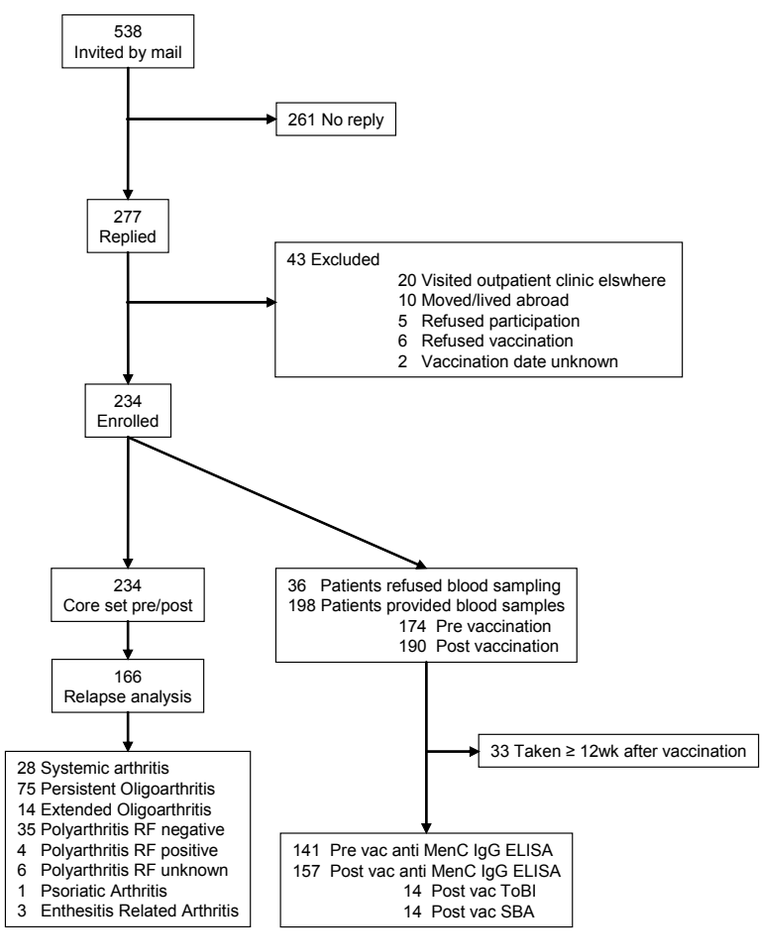
Supplement. Numbers of patients with Juvenile Idiopathic Arthritis assessed and enrolled in the study.



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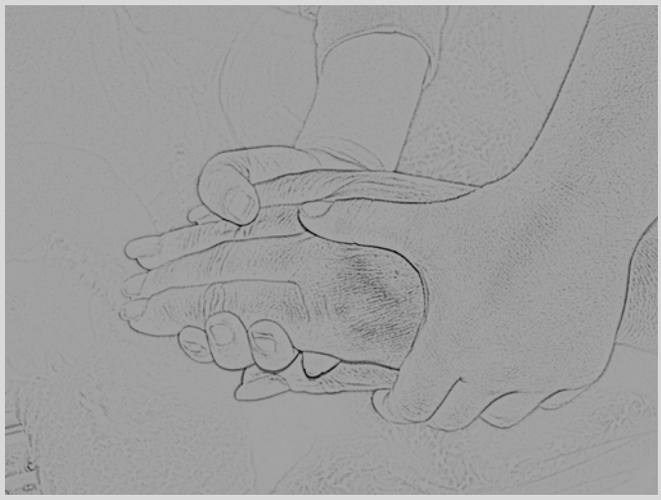

Chapter 6

Differential T and B cell immune responses towards vaccine antigens following vaccination against Meningococcal serogroup C in subgroups of Juvenile Idiopathic Arthritis

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Submitted

Summary
It is still under debate whether vaccination induces adequate immunity in children with autoimmune diseases undergoing immune-suppressive treatment. The meningococcal serogroup C (MenC) vaccination campaign of the Netherlands in 2002, allowed us to prospectively study B and T cell responses to MenC vaccine antigens before and after immunization in healthy adults (HC) and Juvenile Idiopathic Arthritis (JIA) patients. After vaccination, both groups demonstrated protective anti-MenC IgG titers and higher proliferative T cell responses towards the NeisVac-C vaccine and its carrier protein Tetanus toxoid (TT). Despite this, after immunization, polyarticular patients (PA JIA) had significantly lower anti-MenC IgG titers than the oligoarticular patients (OA JIA), or HC. Peripheral blood mononuclear cells (PBMCs) of JIA patient's had a higher in vitro proliferative response to the vaccine as compared to the HC, a difference which was due to the higher response in the PA JIA subgroup. Following vaccination, PA JIA PBMCs proliferated more vigorously and produced more interleukin-2 (IL-2) and tumor necrosis factor alpha (TNF-alpha) in response to the vaccine -when compared to the OA JIA or HC. No differences were found in plasma cytokine levels before and after vaccination. Thus, although JIA patients demonstrated an overall protective antibody and adequate T cell responses towards the vaccine, clear quantitative and qualitative differences are seen in PBMC from PA JIA patients. The vaccination induced immune responses in the OA JIA resembled the pattern found in HC, whereas the PA JIA immune response was aberrant. This may be the consequence of either immune-suppressive treatment, ongoing inflammatory disease, and/or an inherited defective capacity to respond to environmental antigens. These data warrant further studies especially in severe PA JIA patients.

Introduction

Juvenile Idiopathic Arthritis is one of the most common autoimmune diseases in childhood. It occurs in ten per 100,000 children per year(1) and is an important cause of childhood morbidity(2;3). JIA is an immunologically well defined autoimmune disease characterized by periods of remissions and flares(4). Untreated, JIA can be a crippling disease, leading to severe disability(5). The prognosis of children has improved significantly with new therapies eminently aimed at immune suppression. Especially the use of high dose methotrexate and the introduction of so-called biologicals that can block specific cytokine mediated pathways such as the TNF-alpha pathway has lead to a major improvement of quality of life(6;7). These immune suppressive treatments, however, can potentially lead to an increased risk of infections. Indeed, case reports in children with JIA and studies in adults with rheumatoid arthritis (RA) have demonstrated the clinical importance of this increased vulnerability to infections (8;9). Additionally, questions have been raised with regards to the efficacy of vaccination in individuals exposed to immune suppressive medications(10). As vaccinations are important in protecting children from hazardous infections, this may further increase the susceptibility to infections in children with JIA. Prospective studies on both the quantity and the quality of the immune response to vaccinations in patients JIA, however, are largely lacking.

An increase in incidence of meningococcal serogroup C (MenC) infections resulted in a nation-wide immunization campaign to protect children between 1 and 19 years of age in the Netherlands in 2002(11;12). The vaccine utilized, NeisVac-C, is composed of the group C polysaccharide of *Neisseria Meningitidis* conjugated with the carrier protein Tetanus toxoid (TT). This campaign offered a unique possibility to prospectively study the effects of the MenC conjugate vaccine on the T cell responses in JIA, and its subtypes, by monitoring vaccine related immunity. The MenC conjugate vaccine was safe and efficacious in a large cohort of JIA patients and did not affect the clinical course of JIA (Zonneveld-Huijssoon and Ronaghy et al, in press). For the purpose of this study, effect of the MenC conjugate vaccine on the T cell response, we focused on two main subtypes of JIA; oligoarticular (OA JIA) and polyarticular JIA (PA JIA). OA JIA typically has a mild disease course affecting four or less joints whereas PA JIA has a far more serious course often requiring immune suppressive treatment. The aim of this prospective follow up study is to compare the efficacy of the vaccine specific immune responses in both JIA subgroups: OA JIA and PA JIA.

Methods

Patients and healthy controls

All patients treated at our centre between 1 and 18 years of age with a diagnosis of JIA according to the criteria of the International League of Associations for Rheumatology (ILAR) were considered eligible(4). For this study we choose to select only patients with oligoarticular and polyarticular JIA as they are two well-defined subtypes with relatively comparable immune pathogenesis. Of the 234 eligible JIA patients, 26 representative OA JIA and 23 representative PA JIA patients were randomly selected for further immunological studies. Patient demographics are described in table 1. Twenty six patients with persistent OA JIA and 22 patients with PA JIA course (including 3 with extended OA JIA) were evaluated for this study. Twenty healthy adults (HC, average age 29.9 $\pm$ 5.5 years) that voluntarily received a Men C vaccination were included in this study as controls. Patients were seen at the

Pediatric Rheumatology Clinic at the University Medical Centre Utrecht, The Netherlands. Informed consent was obtained from the HC and either from parents (guardians) or from the children directly when they were 12 years or older. This study was approved by the medical ethical committee from the University Medical Center Utrecht.

Disease Activity
The Physician Global Assessment (PGA) measures the overall impression of disease activity given by a paediatric rheumatologist. PGA was measured on a 10 cm visual analogue scale (VAS) and converted to 0-3 scores.

Definition of medication groups
Patients were classified based on medication used at the time of MenC vaccination (Table 1): No medication (group 1), Non-Steroidal Anti-Inflammatory Drugs (NSAID) (group 2), low dose MTX (≤10 mg/m^2/wk) or sulfasalazin with or without NSAID (group 3), high dose MTX (> 10 mg/m^2/wk), or with such anti-TNF therapies as Infliximab, or Etanercept with or without NSAID (group 4).

Tabel 1. Patient characteristics

Patients enrolled Number (%)	OA JIA 26 (54)	PA JIA 22 (46)
Female/Male	17/9	14/8
Age on vaccination; mean yrs (±SD) Range	11.0 (4.0) 3.1 - 18.0	11.2 (2.4) 7.2 - 14.0
Rheumatoid factor positive/total typed (% positive)	3/7 (43)	10/20 (50)
Duration disease on vaccination date; mean yrs (±SD) Range	6.9 (3.6) 0.7 - 14.5	6.8 (3.5) 0.2 - 12.4
Age at onset of JIA; mean yrs (±SD) Range	4.1 (3.2) 0.9 - 14.5	4.4 (2.5) 1.1 - 11.8
PGA before vaccination; mean (±SD) Range	0.8 (0.9) 0 - 2.8	0.8 (1.0) 0 - 3.0
Medication; number of patients (%)		
No medication (group 1)	11 (42)	5 (23)
NSAID (group 2)	8 (31)	5 (23)
NSAID/MTX low*/Sulfasalazine (group 3)	4 (15)	5 (23)
NSAID/MTX high*/anti-TNFα (group 4)	3 (11)	7 (31)

PGA; Physician Global Assessment (0=inactive, 1=mildly active, 2=moderately active, 3=severely active). NSAID; Non-Steroidal Anti-Inflammatory Drugs, MTX; Methotrexate (*low dose ≤10 mg/m^2/wk, high dose >10 mg/m^2/wk), anti-TNFα; either Infliximab, or Etanercept.

MenC conjugate vaccination
The NeisVac-C vaccine (Baxter, Vienna, Austria) is composed of the *Neisseria meningitidis* serogroup C polysaccharide (20 µg/ml) conjugated to tetanus toxoid (TT) (20-40 µg/ml). Patients received one intramuscular dose of 0.5 ml NeisVac-C during

the Dutch national vaccination campaign. All patients were vaccinated irrespective of disease activity. Patients were vaccinated between May 20th and July 3rd 2002.
For in-vitro studies, the MenC conjugate in 0.7% $NaCl_2$ was used (kind gift of Robert Peterman, Charles Nye, and Shwu-maan Lee, Baxter, Illinois, USA).

Cell culture conditions
Heparinized blood samples were obtained by venipuncture from 43 JIA patients as well as from 20 healthy adult controls. Peripheral-blood mononuclear cells (PBMC) were isolated 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, MD, USA), and 10 v/v% heat-inactivated human AB 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.

Anti-MenC antibody titers
Anti-MenC mean total IgG antibodies were quantified in sera by enzyme-linked immunosorbent assay (ELISA) with the use of the CDC 1992 reference serum assigned a value of 24.1 µg/ml of anti-MenC IgG(13;14). The lower limit of antibody detection was 0.24 µg/ml. Sera with undetectable anti-MenC IgG levels were assigned a value of 0.23 µg/ml for mathematical reasons.

T Cell Proliferation
Antigen-specific T cell proliferative responses were studied by culturing PBMCs in triplicate for 120 h, in the absence or presence of 1 or 10 µg/ml NeisVac-C, 1.5 µg/ml Tetanus-toxoid (TT; National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands), as well as the control antigens Diphtheria-toxoid (1.5 µg/ml, DT; RIVM) or 10 µg/ml ovalbumin (OVA). The last 16 hours of culture were in the presence of thymidine (^3H, 1 µCi per well; ICN Biomedicals, Amsterdam, Netherlands). Incorporated radioactivity was measured by liquid scintillation and expressed as stimulation index (SI), calculated as the mean counts per minute of cells cultured with antigen divided by the mean counts per minute without antigen.

Multiplex Immunoassay
Antibody pairs and recombinant proteins used for the multiplex immunoassay were purchased from commercial sources. Calibration curves from recombinant protein standards were prepared using two-fold dilution steps in serum diluent (R&D Systems, Abingdon, United Kingdom) All assays were carried out directly in a 96 well 1.2 µm filter plate (Millipore, Billerica, MA, USA) at room temperature and protected from light as previously described(15).
Fluorescence intensities of the beads were measured in a final volume of 100 µl and blank values were subtracted from all readings. Measurements and data analysis of all assays were performed using the Bio-Plex Luminex 100 system in combination with the Bio-Plex Manager software version 4.0 using five parametric curve fitting (Bio-Rad Laboratories, Hercules CA, USA). The concentrations of cytokines induced with media alone were subtracted from those induced after in-vitro stimulation.

Data Analysis

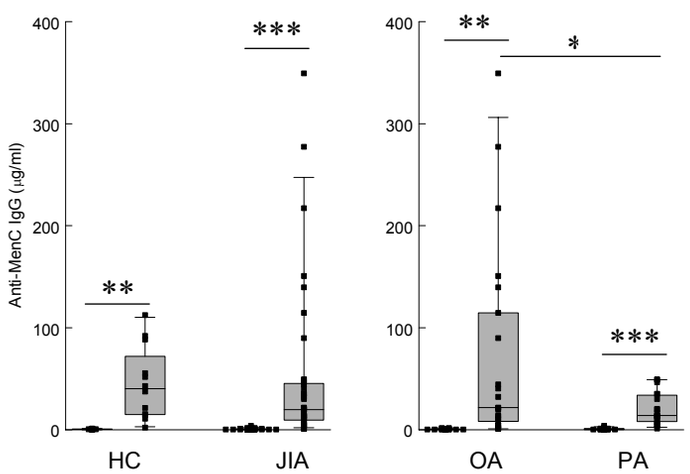
Paired samples T-tests were used to compare pre- and post- vaccination mean concentrations (MC) of anti-MenC IgG, as well as T cell proliferation expressed as SI. For comparison between HC, JIA, OA, or PA populations, an independent samples T-test was utilized. A probability (p) less then 0.05 was considered significantly different. Geometric means were used to generate color profile figures of plasma cytokine levels. All statistical analyses were performed using the statistical package for the social sciences (SPSS) software version 12.0.1 (SPSS, Chicago, IL, USA). All data are presented as mean ± SD.

Results

Efficacy of vaccination in PA and OA JIA

To asses efficacy, vaccine specific antibody titers were measured before and 4 to 6 weeks after vaccination. In the twelve HC, the anti-MenC specific IgG titers rose from a mean concentration (MC) of 0.6 $\pm$ 0.3 to 45.4 $\pm$ 36.0 μg/ml respectively (P=0.001), while, in line with our previous study in a larger cohort of JIA patients (Zonneveld and Ronaghy et al, in press) in the forty JIA patients the anti-MenC MC rose from 0.6 $\pm$ 0.7 to 49.1 $\pm$ 77.1 μg/ml (P < 0.0005). All HC as well as the JIA patients demonstrated a sufficient antibody response after vaccination (Figure 1, left panel) as total anti-MenC IgG titers $\geq$ 2 μg/ml have been shown to confer protection with neutralization of *Neisseria Meningitidis* by bactericidal assays(16). The JIA population was next divided into two subgroups; persistent OA JIA (N=22) and PA JIA (N=18). After vaccination, both the OA JIA (increase from 0.5 $\pm$ 0.4 to 71.7$\pm$ 98.3 μg/ml after vaccination, P=0.003) and PA JIA patients (pre 0.6 $\pm$ 0.9 rose to 21.5 $\pm$ 15.5 μg/ml post, P=<0.0005, Figure 1, right panel) obtained a protective antibody response. However, the post vaccination antibody titers were significantly lower in the PA JIA as compared to either the OA JIA (P=0.017), or the HC (P=0.048). As expected, medication groups 3 and 4 contained a higher number of patients with severe forms of JIA. Sixty three percent of JIA patients in medication groups 3 and 4 were of the PA JIA subgroup while 70% of medication group 4 was PA JIA. Furthermore, group 1 contained more persistent OA JIA (69%).

Figure 1. Pre and Post vaccination Anti-MenC IgG concentrations of healthy controls and of patients with JIA.



Box and whisker plots with median plasma anti-MenC specific IgG antibody titers before and after vaccination of HC, JIA (Left Panel); JIA patients subdivided into OA JIA and PA JIA (RightPanel).
Significant differences are indicated as follows: *=p<0.05, **=p<0.01, ***=p<0.0005.

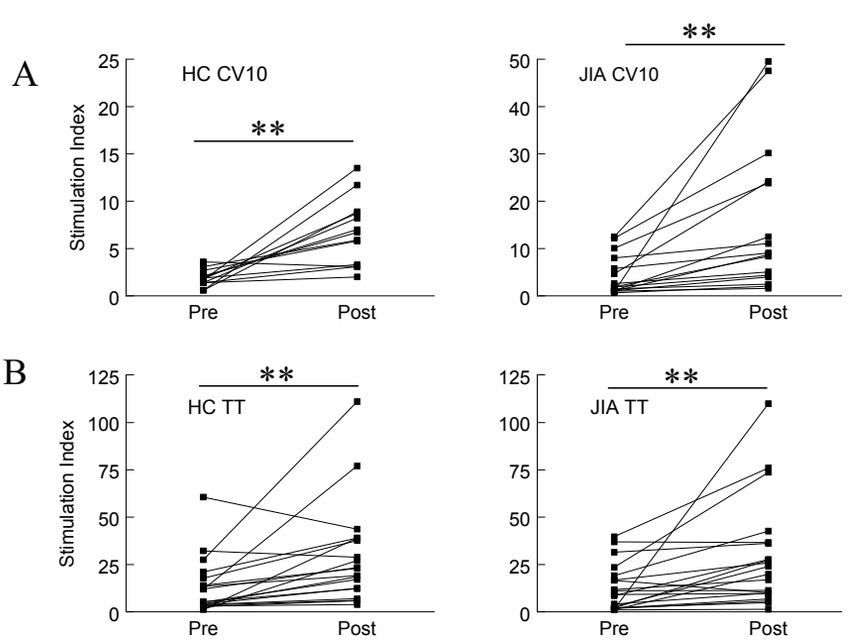
T cell responses to NeisVac-C and TT

The pre and post-immunization T cell proliferative responses, expressed as Stimulation Index (SI), to the whole vaccine increased significantly both in the HC (1.9 ± 0.9 rose to 6.8 ± 3.5, P=0.001) and the JIA patients (4.2 ± 4.2 to 15.3 ± 15.5, P=0.005, Figure 2A). Similarly after vaccination, the T cell proliferative responses to TT (included in the vaccine) increased significantly in the HC (12.9 ± 14.6 to 29.0 ± 26.5, P=0.008) and the JIA patients (10.9 ± 12.0 to 26.4 ± 27.0, P=0.006, Figure 2B). In PBMC of the JIA patients, these responses were higher than that of the HC before (P=0.048) and after immunization (P=0.011) for the vaccine but not for the TT (P=0.61 and 0.97 respectively). When analyzing the two subgroups of JIA, the OA JIA patients were found to have a significant increase in T cell proliferative response to TT (5.3 ± 5.7 to 13.9 ± 11.4, P=0.005, Figure 3) and an increasing trend in response to the whole MenC vaccine (2.3 ± 1.9 to 7.2 ± 7.5, P=0.066). T cell proliferative responses of PBMC from PA JIA increased significantly after vaccination for both the TT (18.1 ± 14.5 to 42.6 ± 10.5, P=0.049) as well as the whole vaccine (6.1 ± 5.1 to 23.4 ± 17.6, P=0.02, Figure 3). After vaccination, no increase in T cell proliferative responses to the control antigen, ovalbumin (OVA), was found in either the HC (0.98 ± 0.12 to 1.11 ± 0.14, P=0.55) or JIA patients (1.02 ± 0.07 to 1.17 ± 0.12, P=0.36).

In summary, both HC and JIA patients can mount significant proliferative responses to vaccine related antigens. However, post vaccination proliferative T cell responses against NeisVac-C and TT from the PBMC of PA JIA patients were more vigorous when compared to the HC (NeisVac-C, P=0.005; TT, P=0.089) and OA JIA (NeisVac-C, P=0.006; TT, P=0.005).

Figure 2. T cell proliferative responses against NeisVac-C and Tetanus of healthy controls and of patients with JIA.

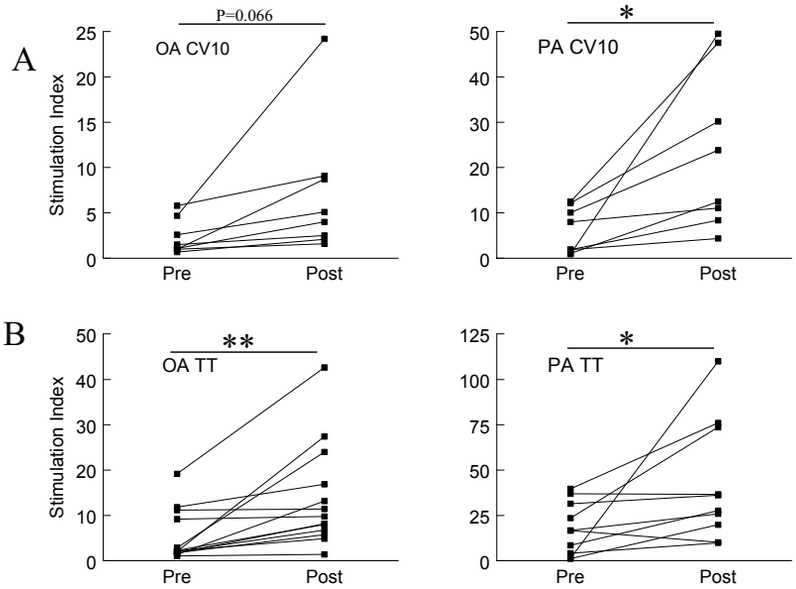
II



T cell proliferation responses (SI) to NeisVac-C (Panel A) and TT (Panel B) in HC as well as JIA patients using line plots before and after vaccination. Significant differences are indicated as follows: *=p<0.05, **=p<0.01, ***=p<0.0005.

Figure 3. OA and PA JIA T cell responses against NeisVac-C and Tetanus.

III

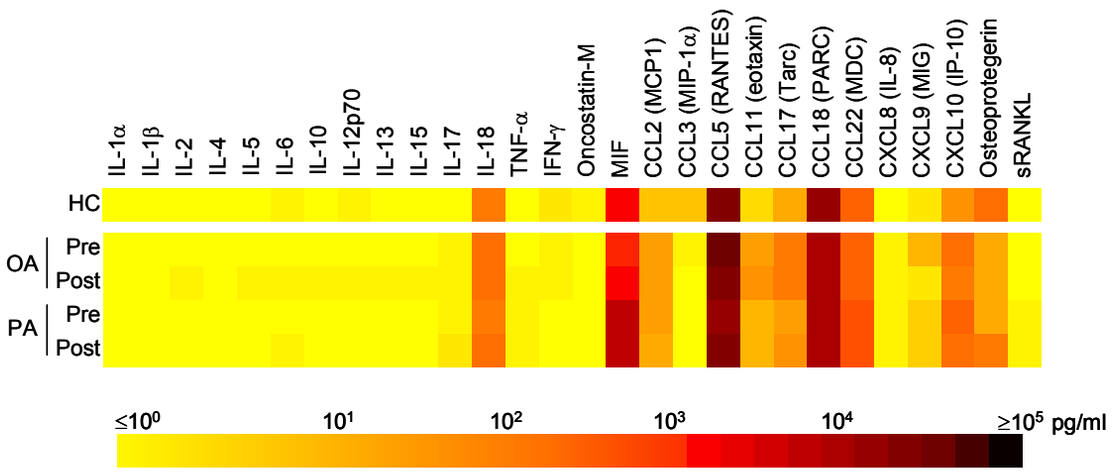


T cell proliferation response (SI) to NeisVac-C (Panel A) and TT (Panel B) before and after vaccination in OA JIA and PA JIA patients using line plots. Significant differences are indicated as follows: *=p<0.05, **=p<0.01.

Cytokine and Chemokine levels

To assess whether differences in the severity of systemic chronic inflammation may have influenced the antigen specific immune response, plasma cytokines were measured before as well as one month after vaccination in the JIA population. After vaccination, an increase in the pro-inflammatory cytokine IL-17 was noted in the PA JIA (pre=0.5 ± 0.9, post=3.0 ± 2.1 pg/ml, P=0.055) but not in the OA group (pre=1.3 ± 2.4, post=1.1 ± 1.3 pg/ml, P=0.90, Figure 4). However, the absolute levels of IL-17 remained low, and it is doubtful whether they may have influenced the B and T cell responses of the JIA presented in this study.

Figure 4. Cytokine and Chemokine concentrations in plasma of OA and PA JIA patients.



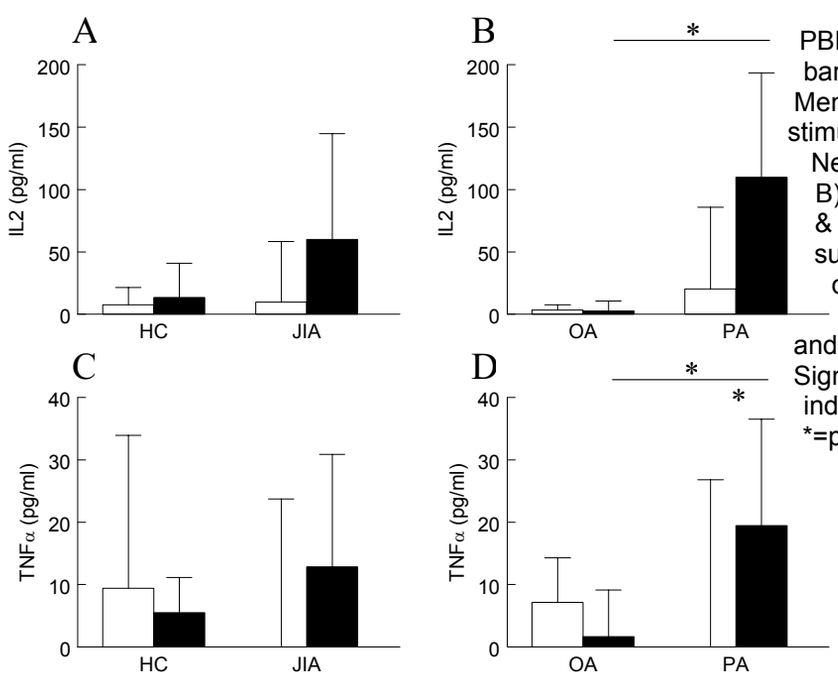
Plasma cytokine and chemokine concentrations expressed as geometric means were used to generate color profile figures. No significant differences were found in cytokine and chemokine concentrations in plasma of JIA patients pre versus post vaccination.

Vaccination leads to higher production of IL-2 and TNF-alpha in PA JIA

To assess differences in the quality of their immune responses before and after vaccination, the PBMCs of the HC and the JIA were stimulated in-vitro with NeisVac-C to measure IL-2 and TNF-alpha cytokine production. After vaccination, PBMC from JIA patients showed a more vigorous IL-2 production (9.7 ± 48.7 rose to 60.1 ± 84.4, P=0.093) whereas PBMC from the HC did not (7.6 ± 13.9 rose to 13.5 ± 27.5, P=0.288). This trend (Figure 5, Panel A) is largely accredited to the PA JIA population. Their vaccine induced IL-2 production increased from 20.3 ± 65.7 to 109.1 ± 84.0 after vaccination (P=0.065), while the OA JIA subgroup did not show any increase (3.4 ± 4.1 pre to 2.7 ± 7.9 post, P=0.788; Figure 5, Panel B). Post-immunization levels of the vaccine induced IL-2 production of the PA JIA patients were significantly higher than either the OA JIA (P=0.026) or the HC (P=0.03). Vaccination and in-vitro re-stimulation of PBMCs did not lead to increased production of TNF-alpha in either the HC (9.4 ± 24.5 pre to 5.5 ± 5.6 post, P=0.603) or the OA JIA (7.2 ± 7.1 pre to 1.7 ± 3.0 post, P=0.179, Panel C and D). However, after immunization, the JIA group as a whole demonstrated an increasing trend in vaccine induced TNF-alphą production (-0.6 ± 23.7 pre to 12.8 ± 18.1 post, P=0.118). This increase again was largely attributed to the PA JIA (-6.9± 26.8 pre to 19.6 ± 17.1 post, P=0.012). In addition, the PBMCs of the PA JIA patients produced higher levels of TNF-alphą when compared to the OA JIA (P=0.019). No significant differences were found in IL-10 concentrations in the supernatants of PBMCs of either the HC or JIA patients cultured with the vaccine. In conclusion, PBMCs from PA JIA patients show higher vaccine induced proliferation, IL-2, and TNF-alpha production as compared to HC or OA JIA patients.

Figure 5. Qualitative difference in the immune response of the HC and the JIA against the NeisVac-C vaccine.

V



PBMC taken before (white bars) and after (black bars) MenC vaccination were re-stimulated in-vitro with NeisVac-C. IL-2 (Panel A & B) and TNF-alpha (Panel C & D) cytokines in supernatants were quantified using a multiplex immunoassay and expressed as pg/ml. Significant differences are indicated as follows: *=p<0.05.

Discussion

With the increase in the use of immune suppressive treatment in children with JIA, questions have arisen regarding the efficacy and potential dangers of vaccination in these children. The MenC vaccination campaign in The Netherlands in 2002, offered the opportunity to prospectively study the efficacy of the NeisVac-C vaccine in JIA patients. We found that the HC as well as the JIA population as a whole had adequate anti-MenC IgG levels four to six weeks after vaccination. At closer analysis, we found significantly lower antibody levels in the PA JIA as compared to the OA JIA, or the HC. This difference may have been due to selection bias because in a larger cohort, a similar trend was demonstrated but this was not statistically significant (Zonneveld-Huijssoon and Ronaghy et al, in press). Despite this, the JIA-including the PA JIA, produced protective anti-MenC antibody MC $\geq$ 2 μg/ml as demonstrated by bactericidal assay (Zonneveld-Huijssoon and Ronaghy et al, in press). The lower anti-MenC IgG MC of the PA JIA may be a result of the stronger immunosuppressive treatment (table 1) as we and others have shown (Zonneveld-Huijssoon and Ronaghy et al, in press, (17;18)) . This is especially remarkable, since in the group of PA JIA patients studied here only 4 patients with high dose MTX and/or anti-TNF-alpha blockade were included. Thus it may be assumed that these results may even underestimate the antibody response in JIA patients treated with more severe immune suppression.

We aimed to further characterize the immune response in the JIA population by assessing the T cell responses after vaccination. As was the case with the antibody titers, both the JIA and the HC had a significant increase in the T cell responses towards both the NeisVac-C as well as the carrier protein TT. The JIA group had higher T cell proliferation levels than the HC towards NeisVac-C, which in part could reflect the more recent booster vaccinations with tetanus, administered around 9 years of age. Surprisingly, however, when looking at the responses within the JIA group, we found that the OA JIA group had similar proliferative responses towards the vaccine as the HC, whereas the PA JIA patients had significantly higher T cell proliferative responses to both the vaccine and TT. Furthermore, after vaccination, the PBMCs in the PA JIA produce higher levels of IL-2 after in-vitro stimulation with NeisVac-C as compared to the OA JIA patients or the HC as well as higher concentrations of TNF-alpha when compared to the OA JIA patients. The quantitative and qualitative differences in the T cell responses of the JIA group, as compared to the HC, are mainly attributed to the PA JIA subgroup. Thus the PA JIA subpopulation responded in a more pro-inflammatory fashion to the immunizing antigen. What may be the cause of this difference in the T cell mediated antigen specific immune response of the PA JIA patients following vaccination? It can be hypothesized that this may be the consequence of differences in disease severity, immune modulatory treatment, genetic background and/or inherent capacity for immune regulation. First, with regard to disease severity; we along with others have described that plasma levels of pro-inflammatory mediators are different in OA JIA and PA JIA(19). However, in the studied patient group large differences in severity of inflammation were not detected by assessment of systemic levels of pro- and anti-inflammatory plasma cytokines and chemokines before and after vaccination (data not shown).

Second, it can safely be assumed that both medication and disease activity can play a role in determining these differences in vaccine related immune responses between OA JIA and PA JIA, but the size of this study lacked the power to demonstrate this. Third, although JIA is a heterogenic disease, genetic differences may co-determine the way PBMC from JIA patients respond to environmental triggers, such as a

vaccination (20;21). Lastly, we as well as others recently showed a defect in the numbers and function of T regulatory cells in patients with PA JIA (22-24) . As regulatory T cells are strong suppressors of proliferative T cell responses, the increased proliferative responses in PA JIA may be a reflection of a defect in T regulatory cell function. If this is the case, indeed this subgroup of patients may have an increased risk in developing a (too) vigorous immune response to a normal environmental antigen. To our knowledge this is the first study that correlates a lack of T cell regulation *in vivo* with an exaggerated immune response to an antigen in patients with an autoimmune disease.
This study suggests that both the JIA and the adult HC population following vaccination develop adequate antibody and T cell proliferative responses to the NeisVac-C vaccine or its carrier protein TT. However, the immune responses of the PA JIA are quantitatively and qualitatively different as they develop an increased proliferative response following vaccination. Therefore, future studies will be needed to assess the safety and efficacy of vaccination in the severe form of PA JIA.

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Sponsors of this study had neither involvement in the study design, in collection, analysis and interpretation of data, in writing the report, nor in the decision to submit for publication.

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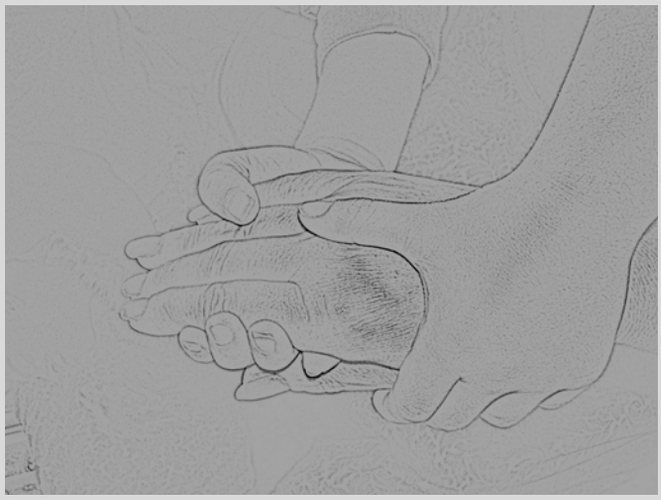

Chapter 7

Defective regulatory T cell response in polyarticular Juvenile Idiopathic Arthritis patients following vaccination.

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Submitted

Summary
There has always been an interest to better understand the pathology of arthritis. The Meningococcal group C immunization campaign in the Netherlands in 2002 allowed us to prospectively investigate if the higher T cell proliferation to NeisVac-C vaccine antigens in Juvenile Idiopathic Arthritis patients are due to an aberrant $CD4^+$ T cell counter-regulatory immune mechanism. PBMCs from JIA patients and healthy volunteers exemplified $CD4^+$, but not $CD8^+$ T cell proliferation towards vaccine (NeisVac-C) and the arthritis related auto-antigen (human HSP60). Furthermore, in the JIA population, vaccination and *in vitro* re-stimulation with NeisVac-C produced a higher trend in the percentage of the $CD4^+$ T cells that co-express TNF-alpha as opposed to IL-10. Moreover, re-stimulation of JIA or PA JIA PBMCs with either human or *E. coli* HSP60 also induced TNF-alpha production. Vaccination and *in vitro* re-stimulation with NeisVac-C, human HSP60, but not *E. coli* HSP60 induced $CD4^+CD25^{bright}$ T cells in the HC and OA JIA but not the PA JIA group, which was significantly lower than that of the HC after vaccination and re-stimulation with NeisVac-C. In addition, there were an increasing percentage of $CD4^+FoxP3^+$ T cells in only the HC. Remarkably, the percentage of induced $CD4^+FoxP3^+$ T cells was higher in the PA JIA than in the OA JIA after vaccination. Therefore, the HC seem better able to respond to vaccine-associated antigens after vaccination with the induction of an increased number of $CD4^+CD25^{bright}$ T cells and $CD4^+FoxP3^+$ T cells. Yet, overall the PA JIA group has the highest percentage of $CD4^+FoxP3^+$ T cells induced by the vaccine. After vaccination the percentage of $CD4^+FoxP3^+$ T cells induced with human or *E. coli* HSP60 tends to increase in the HC but not in the JIA. Collectively, this suggests that the $CD4^+CD25^{bright}$ T cells from the PA JIA group are not able to suppress the antigen specific immune response after vaccination and *in vitro* re-stimulation with either human HSP60 or the NeisVac-C vaccine. Furthermore, human HSP60 is better at inducing $CD4^+CD25^{bright}$ T cell suppression of the antigen specific immune response than the NeisVac-C vaccine.

Introduction

Vaccinations are one of the most important medical achievements. Immunization programs have lead to the eradication of smallpox, elimination of poliomyelitis and measles in parts of the world, and significant reductions in morbidity and mortality due to diphtheria, tetanus, and pertussis(1). The World Health Organization (WHO) estimated that vaccinations prevented 2 million child deaths in 2003(2). Despite such benefits, concerns still exist regarding a possible correlation between vaccines and the induction or exacerbation of autoimmune diseases(3). Such concerns mainly stem from anecdotal case reports(4;5) as only a few exceptional cases of association between autoimmune pathology and vaccinations have been documented. For example, the 1976-77 A/New Jersey/8/76 swine flu, measles-mumps-rubella (MMR), and the Meningococcal serogroup C (MenC) vaccines were associated with the Guillian-Barre syndrome (polyradiculoneuritis)(6), Idiopathic Thrombocytopenic Purpura(7-11), as well as nephrotic syndrome(12) respectively. On the other hand, numerous epidemiological reports attest to the general safety of vaccines(13-18). Furthermore, in most cases of vaccine-associated autoimmune pathology, the incidences of adverse events are lower than the complications of the natural disease the vaccines were designed to prevent(19;20). Though negative epidemiological reports are reassuring, concerns remain about the susceptibility of certain sub-populations. Genetically predisposed individuals may be at risk for developing or aggravating autoimmune diseases after vaccination without an overall increase of incidence(21). This could be the case in childhood autoimmune diseases such as Juvenile Idiopathic Arthritis (JIA) in which a temporal relationship exists between disease onset and the normal childhood vaccination schedule. JIA is a clinically well defined autoimmune disease characterized by periods of remissions and flares(22). Indeed, it has been suggested that disease flares in JIA could be attributed to vaccinations and other microbial triggers. A recent nation-wide Meningococcal group C immunization campaign in children between 1 and 19 years of age in the Netherlands in 2002 did not reveal any increase or aggravation of disease activity in patients with JIA following vaccination (23;24)(Huijssoon & Ronaghy *et al*, in press). However, a follow up study did reveal an increase in vaccine-specific T cell proliferation in JIA patients with a polyarticular disease course. (Ronaghy *et al* 2006, submitted). In these patients, who suffer from a more severe form of JIA, we questioned whether this increase in T cell proliferation was an indication of an aberrant counter-regulatory immune mechanism. Therefore, we set out to study the changes in the regulatory T cell response towards both a vaccine antigen (MenC) and a well-studied arthritis-related auto-antigen (HSP60) following vaccination. We focused on two subgroups of JIA; the subgroup of JIA with less severity and better prognosis, oligoarticular JIA (OA), and to the one of greater severity and poorer prognosis, polyarticular JIA (PA). We showed that the immune regulatory mechanisms in PA JIA quantitatively and qualitatively differ from both OA JIA and healthy controls.

Materials and Methods

Patients and healthy controls

All patients between 1 and 18 years of age with a diagnosis of JIA according to the criteria of the International League of Associations for Rheumatology (ILAR) were considered eligible(25). Patient demographics are described in table 1. Of the 234 eligible JIA patients, 26 representative patients with persistent OA JIA and 28 patients with PA JIA course (including 3 with extended OA JIA) were randomly selected for this study. Twenty healthy adults (HC, average age 29.9 $\pm$ 5.5 years) that voluntarily received a Men C vaccination were included in this study as controls. Patients were seen at the Pediatric Rheumatology Clinic at the University Medical Centre Utrecht, The Netherlands. Informed consent was obtained from the HC and either from parents (guardians) or from the children directly when they were 12 years or older. This study was approved by the local ethical committee from the University Medical Centre Utrecht.

Table 1. Patient characteristics

Patients enrolled	OA JIA	PA JIA
Number (%)	26 (48)	28 (52)
Female/Male	17/9	16/12
Age on vaccination; mean yrs (±SD)	10.9 (3.9)	10.7 (3.0)
Range	3.1 - 18.0	1.5 - 15.7
Rheumatoid factor positive/total typed (% positive)	0/0 (0)	2/21 (9.5)
Duration disease on vaccination date; mean yrs (±SD)	6.6 (3.6)	5.9 (3.7)
Range	0.7 - 14.5	0.2 - 12.4
Age at onset of JIA; mean yrs (±SD)	4.4 (3.5)	4.8 (3.0)
Range	0.9 - 14.5	1.1 - 11.8
PGA before vaccination; mean (±SD)	0.7 (0.9)	1.0 (1.1)
Range	0 - 2.8	0 – 2.5
Medication; number of patients (%)		
No medication (group 1)	10 (38.5)	5 (17.8)
NSAID (group 2)	9 (34.6)	7 (25.0)
NSAID/MTX low*/Sulfasalazine (group 3)	6 (23.1)	8 (28.6)
NSAID/MTX high*/cyclosprin A/anti-TNFα (group 4)	1 (3.8)	8 (28.6)

PGA; Physician Global Assessment (0=inactive, 1=mildly active, 2=moderately active, 3=severely active). NSAID; Non-Steroidal Anti-Inflammatory Drugs, MTX; Methotrexate (*low dose ≤10 mg/m^2/wk, high dose >10 mg/m^2/wk), anti-TNFα; either Infliximab, or Etanercept.

MenC conjugate vaccination

The NeisVac-C vaccine (Baxter, Vienna, Austria) is composed of the *Neisseria Meningitidis* serogroup C polysaccharide (20 µg/ml) conjugated to tetanus toxoid (TT) (20-40 µg/ml). Patients received one intramuscular dose of 0.5 ml NeisVac-C during the Dutch national vaccination campaign. All patients were vaccinated irrespective of disease activity. Patients were vaccinated between July 1st and May 26th, 2002.

For *in vitro* studies, NeisVac-C was dissolved in 0.7% $NaCl_2$ (kind gift of Robert Peterman, Charles Nye, and Shwu-maan Lee, Baxter, Illinois, USA).

Cell culture conditions
Heparinized blood samples were obtained by venipuncture from 54 JIA patients as well as from 20 healthy adult controls. Peripheral-blood mononuclear cells (PBMC) were isolated 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, MD, 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
Antigen-specific T cell proliferative responses were cultured in triplicate for 120 h, in the absence or presence of 1 or 10 µg/ml NeisVac-C, 1.5 µg/ml Tetanus-toxoid (TT;National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands), 10 µg/ml Human HSP60 (Stressgen, Victoria, British Columbia, Canada) and 10 µg/ml Escherichia coli *(E. coli)* HSP60 (Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands) as well as the control antigens Diphtheria-toxoid (1.5 µg/ml ,DT; RIVM) or 10 µg/ml ovalbumin (OVA). The last 16 hours of culture were in the presence of thymidine (^{3}H, 1 µCi per well; ICN Biomedicals, Amsterdam, Netherlands). Incorporated radioactivity was measured by liquid scintillation and expressed as stimulation index (SI), calculated as the mean counts per minute of cells cultured with antigen divided by the mean counts per minute without antigen.

Flowcytometry
PBMC were cultured for 72 hours as described above. Next, appropriately diluted phycoerythrin (PE)-, fluorescein isothiocyanate (FITC)-, cychrome (CY)-labeled anti-human monoclonal antibodies (mAbs) were used to stain cells with CD4 (RPA-T4, BD Biosciences, San Jose, CA) and CD25 (clone 2A3, BD Biosciences).
For intracellular cytokine staining PBMCs were cultured as mentioned above with medium or antigens. During the last 4 hours of culture, monensin (BD Biosciences) was added, except for FoxP3 staining. After staining with CD4 as well as CD25 the cells were fixed, permeabilized, and subsequently stained with anti-human mAbs as described by BD Biosciences: IL-4 (8D4-8), IL-10 (JES3-19F1), TNF-alpha (Mab11), and IFN-gamma (4S.133). Finally, cells were analyzed on a FACS-calibur using software (BD Biosciences).
Furthermore, some PBMCs were additionally mixed with 0.5 µM carboxyfluorescin diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, OR) for 5 min at 37°C, washed, and cultured as described above for 7 days in the presence or absence of the above mentioned antigens. For the analysis of the proliferative response of PBMC $CD4^+$ T cells, cells were stained and analyzed by FACS (BD Biosciences). Cell Quest software (BD Biosciences, San Jose, CA) was used for analysis

Multiplex immunoassay
Antibody pairs and recombinant proteins used for the multiplex immunoassay were purchased from commercial sources. Calibration curves from recombinant protein standards were prepared using two-fold dilution steps in serum diluent (R&D

Systems, Abingdon, United Kingdom). All assays were carried out directly in a 96 well 1.2 µm filter plate (Millipore, Billerica, MA, USA) at room temperature and protected from light as previously described(26).
Fluorescence intensities of the beads were measured in a final volume of 100 µl and blank values were subtracted from all readings. Measurements and data analysis of all assays were performed using the Bio-Plex system in combination with the Bio-Plex Manager software version 4.0 using five parametric curve fitting (Bio-Rad Laboratories, Hercules CA, USA). The concentrations of cytokines induced with media alone were subtracted from those induced after *in vitro* stimulation.

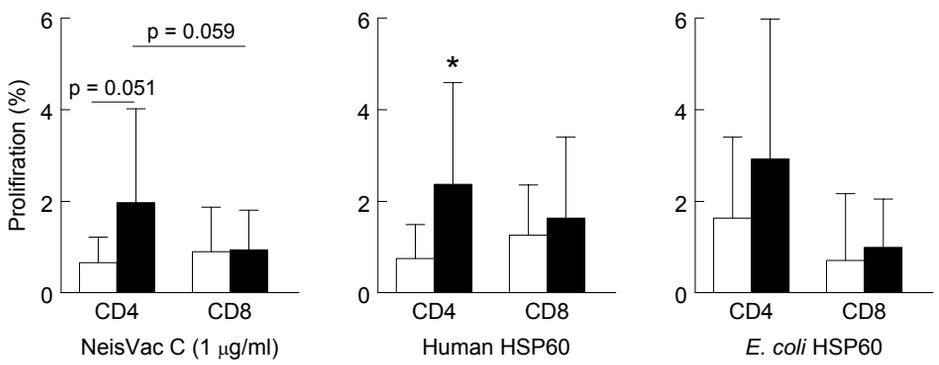
Functional assays.
For functional assays PBMCs were cultured for 96 hours as described above with either human HSP60 or the NeisVac-C vaccine. After culturing, $CD4^+CD25^{bright}$ cells with the highest two percent of CD25 expression were FACS sorted into anti-CD3 (OKT-1.5 µg/mL) precoated wells of a 96-well plate containing 3 x 10^4 APCs and co-cultured with $CD4^+CD25^-$T cells (effector cells) for another 96 hours. $CD4^+CD25^-$T effector cells were selected by FACS-sorting and APCs were harvested from PBMCs depleted of T cells using anti-CD3 beads and irradiated with 3500 rad. The last 16 hours of incubation were in the presence of thymidine (^3H, 1 µCi per well; ICN Biomedicals, Amsterdam, Netherlands). Incorporated radioactivity was measured by liquid scintillation and expressed as counts per minute (CPM). The proliferative capacity was calculated as the mean counts per minute of effectors cultured with the $CD4^+CD25^{bright}$ fraction divided by the CPM of the effectors alone.

Data analysis
Paired sample T-tests were used to compare 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, OA JIA, or PA JIA populations, an independent samples T-test was utilized. A probability (p) less then 0.05 was considered significantly different. All statistical analyses were performed using the statistical package for the social sciences (SPSS) software version 12.0.1 (SPSS, Chicago, IL, USA). All data are presented as mean ± SD.

Results
Increased CD4+ T cell proliferation towards the NeisVac-C vaccine and HSP60 in JIA patients following MenC vaccination
We previously documented an increased vaccine specific T cell proliferation in PA JIA following vaccination with MenC. (Ronaghy *et al*, submitted). Aiming to understand the mechanism underlying this increased proliferation, we first assessed which T cell subset was responsible for the T cell proliferation to NeisVac-C. Also, we questioned whether vaccination could influence the response to HSP60, a well-described auto-antigen in JIA. PBMCs of JIA patients were stained with CFSE, cultured and subsequently stained for $CD4^+$ and $CD8^+$, as described above. After vaccination, $CD4^+$T cells of JIA patients proliferated significantly more in response to NeisVac-C (pre 0.7 $\pm$ 0.6 rose to 2.0 $\pm$ 2.0 after vaccination, P=0.051) while $CD8^+$T cells did not (pre 0.9 $\pm$ 1.0 and post 0.9 $\pm$ 0.9, P=0.910, Figure 1 left panel). In addition, post-vaccination in JIA patients, $CD4^+$T cell proliferative responses increased against human HSP60 (pre 0.8 $\pm$ 0.7 rose to 2.3 $\pm$ 2.2 after vaccination, P=0.017, middle panel) but not against *E. coli* HSP60 (pre 1.6 $\pm$ 1.8 rose to 2.9 $\pm$ 3.7

after vaccination, P=0.201, right panel). $CD8^+$T cells did not show increased proliferation to either human HSP60 (pre 0.7 ± 0.9 and post 1.3 ± 1.1, P=0.184, middle panel) or *E. coli* HSP60 (pre 0.7 ± 1.5 and post 1.0 ± 1.1, P=0.284, right panel). There were no significant differences in the proliferative response of either the JIA $CD4^+$ or $CD8^+$ T cells to the control antigen diphtheria toxoid (DT). These results suggest that vaccination with MenC induces in PBMC from JIA patients $CD4^+$ T cell proliferation towards both a vaccine antigen (NeisVac-C) and an arthritis related auto-antigen (human HSP60). A similar increase in $CD4^+$, but not CD8+ T cell proliferation towards both NeisVac-C and Human HSP60) was found in HC (data not shown).

Figure 1. $CD4^+$ and $CD8^+$ T cell proliferative responses of JIA patients against NeisVac-C, human HSP60, and *E. coli* HSP60.



$CD4^+$ and $CD8^+$ T cell proliferative responses of the JIA (SI) against NeisVac-C, human HSP60, and *E. coli* HSP60 before (white bars) and after vaccination (dark bars). Significant differences are indicated as follows: *=p<0.05.

Higher production of TNF-alpha in response to HSP60 following vaccination in PA JIA

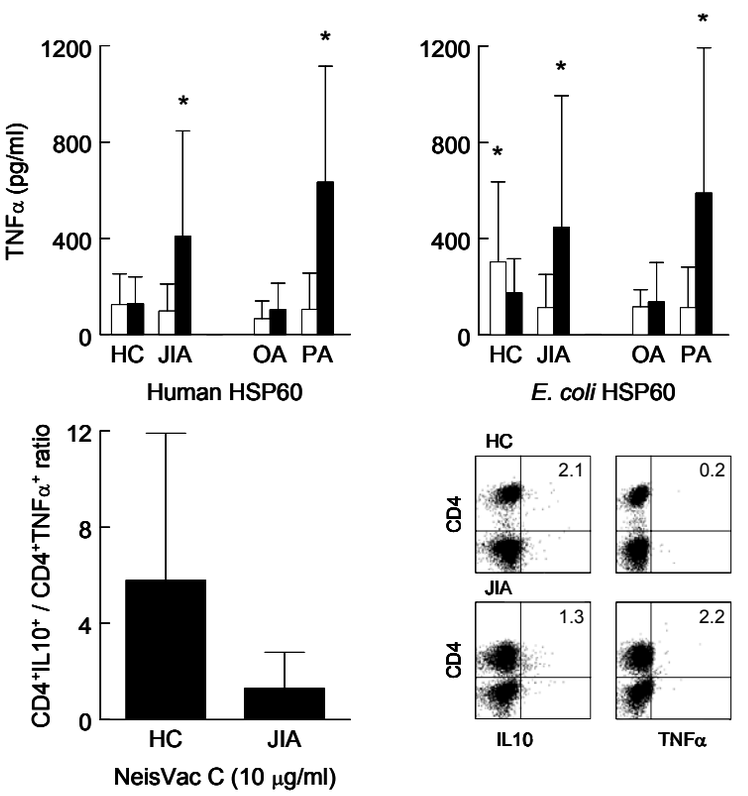
To determine the quality of the immune response towards HSP60 and NeisVac-C, we measured the antigen-specific cytokine production before and after NeisVac-C vaccination. PBMCs of the HC and the JIA were stimulated *in vitro* with NeisVac-C, human HSP60, and *E. coli* HSP60. Cytokine levels were measured in supernatants by multiplex immunoassay as described in the methods. After vaccination and *in vitro* re-stimulation of the PBMCs of the HC or JIA with either human or *E. coli* HSP60, no significant differences in cytokine production were detected in the supernatants for the following cytokines: IL-4, IL-5, IL-10, IL-17, and IFN-gamma. However, when comparing pre- and post vaccinations samples, clear changes in HSP-induced TNF-alpha production were found. Although vaccination and *in vitro* re-stimulation with human HSP60 did not increase TNF-alpha production in the supernatant of the PBMC of the HC (pre 124.4 ± 129.2 and 128.7 ± 111.2 post vac, P=0.897) a significant increase of HSP60-induced TNF-alpha production was detected in PBMC from JIA patients (pre 98.2 ± 113.0 rose to 408.6 ± 438.7 post vac, P=0.041). This increase in TNF-alpha production was largely accredited to the PA JIA (pre 104.5 ± 151.1 rose to 633.5 ± 483.0 post vac, P=0.041) and not to the OA JIA population (pre 65.9 ± 74.5 rose to 102.9 ± 111.3 post vac, P=0.153, Figure 2, Panel A). Furthermore after vaccination and *in vitro* re-stimulation with *E. coli* HSP60 the TNF-alpha production in the HC decreased (pre 302.8 ± 232.5 decreased to 173.9 ± 141.3

post vac, P=0.018) while it increased for in the JIA patients (pre 112.5 ± 138.2 rose to 446.7 ± 547.9 post vac, P=0.032). This increase was largely due to the TNF-alpha production of the PA JIA (pre 112.8 ± 168.5 rose to 588.7 ± 644.8 post vac, P=0.049) and not due to the production of TNF-alpha by the OA JIA population (pre 115.7 ± 71.3 rose to 136.2 ± 163.9 post vac, P=0.706, Figure 2, Panel B).

For the above mentioned cytokines, levels induced with media alone were subtracted from those stimulated with antigen. Thus, following NeisVac-C vaccination PBMC from PA JIA patients show higher antigen induced TNF-alpha production after *in vitro* re-stimulation with human or *E. coli* HSP60. This increase in TNF-alpha production is not seen in HC or OA JIA patients.
These findings are in line with what was previously documented, namely that after vaccination, PBMC from PA JIA patients show a more vigorous IL-2 and TNF-alpha production in response to *in vitro* activation with Neis-Vac-C whereas PBMC from the HC nor the OA JIA did not. (Ronaghy *et al*, submitted 2006).

Antigen-induced intracellular cytokine production following vaccination
Next, we measured antigen-specific intracellular cytokine production before and after vaccination in both HC and JIA patients as described above. Briefly; PBMC were cultured for 72 hours with NeisVac-C, HSP60 or medium and then stained for surface markers (CD4) and intracellular cytokines (IL-10, TNF-alpha). No significant differences were found in intracellular cytokine production in response to HSP60 following vaccination.

Figure 2. Qualitative difference in the immune response of the HC and the JIA against the NeisVac-C vaccine, human HSP60, and *E. coli* HSP60.

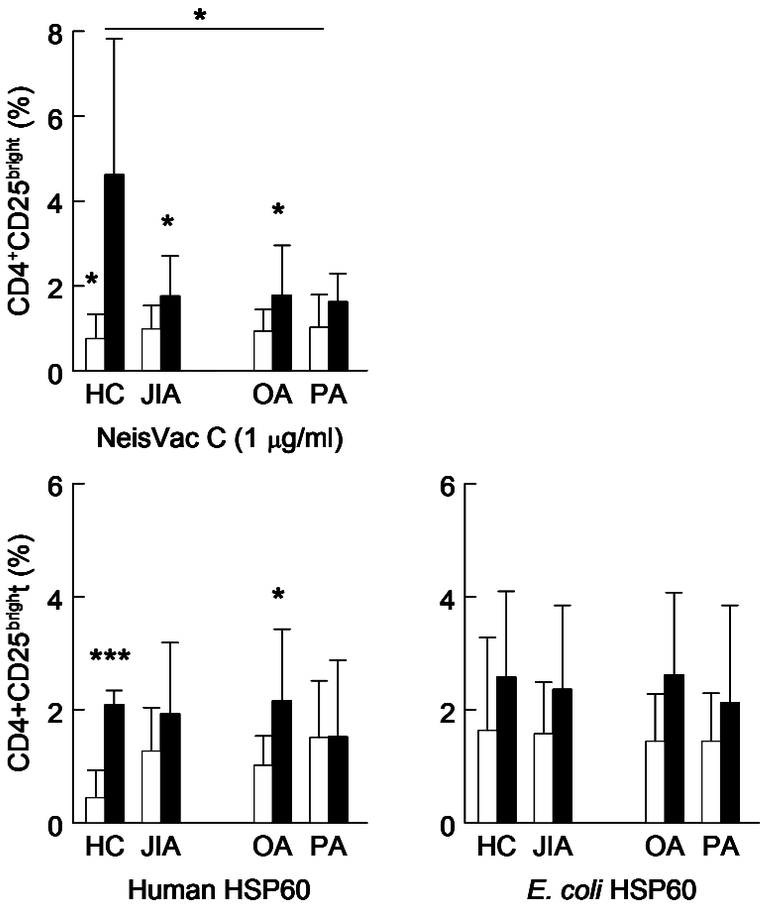


Supernatants from PBMCs of the HC and JIA were taken before (white bars) and after (black bars) MenC vaccination and *in vitro* re-stimulation with NeisVac-C, human HSP60, or *E. coli* HSP60 to asses antigen specific TNF-alpha cytokine production, expressed as pg/ml, using a multiplex immunoassay (upper panel).
The post-vaccine ratio of the percentage of $CD4^+IL\text{-}10^+$ T cells/$CD4^+$ TNF-alpha $^+$T cells of the HC and the JIA. After NeisVac-C vaccination, a representative FACS picture of one HC and JIA patient showing the percentage of the $CD4^+IL10^+$ T cells as well as $CD4^+$ TNF-α $^+$ T cells. Significant differences are indicated as follows: *=p<0.05 (lower panel).

In response to *in vitro* activation with NeisVac-C, after vaccination PBMC from HC displayed a higher ratio of the percentage of $CD4^+IL\text{-}10^+$ T cells/$CD4^+TNF\text{-}alpha^+$T cells (5.8 ± 6.3) as opposed to PBMC from JIA patient patients (1.3 ± 1.5; Figure 2). However, this trend was not significant (p = 0.18). Figure 2, shows representative FACS pictures of a HC and a JIA patient after vaccination.
Following *in vitro* activation with NeisVac-C, PBMC from the HC (upper row) have a higher percentage of the $CD4^+$ T cells that co-express IL-10 (2.1%) and a lower percentage of the $CD4^+$ T cells that co-express TNF-alpha (0.2%) when compared to PBMC from the JIA patient (1.3% and 2.2% respectively). *In vitro* activation with hsp60 showed a similar trend but without reaching significance. Also, no significant differences were seen after vaccination and *in vitro* re-stimulation with either of the control antigens OVA or DT in TNF-alpha production or the ratio of $CD4^+IL10^+/CD4^+TNF\text{-}alpha^+$.

NeisVac-C and HSP60 induce a higher percentage of $CD4^+CD25^{bright}$T cells in healthy individuals following vaccination.
We questioned whether the increased antigen specific T cell proliferation and TNF-alpha production in the PA JIA patients compared to OA JIA patients and HC might be related to differences in immune regulatory mechanisms induced by NeisVac-C vaccination. Therefore we measured the number of $CD4^+CD25^{bright}$ T cells expressed as a percentage of $CD4^+$ T cells in both the HC and the JIA (Figure 3). Vaccination followed by *in vitro* re-stimulation with NeisVac-C induced a higher percentage of $CD4^+CD25^{bright}$ T cells in the HC (pre 0.8 $\pm$ 0.6 rose to 4.6 $\pm$ 3.2 post, P=0.021), JIA (pre 1.0 $\pm$ 0.6 rose to 1.8 $\pm$ 0.9 post, P=0.018), the OA JIA (pre 0.9 $\pm$ 0.5 rose to 1.8 $\pm$ 1.2 post, P=0.034), but not the PA JIA (pre 1.0 $\pm$ 0.8 and 1.6 $\pm$ 0.7 post, P=0.459). Moreover, the percentage of $CD4^+CD25^{bright}$ T cells after NeisVac-C vaccination and *in vitro* re-stimulation was significantly higher in HC compared to the PA JIA (P=0.011).
Vaccination with NeisVac-C and re-stimulation with human HSP60 induced a higher percentage of $CD4^+CD25^{bright}$ T cells in the HC (pre 0.4 $\pm$ 0.5 rose to 2.1 $\pm$ 0.3 post, P<0.0005) and OA JIA (pre 1.0 $\pm$ 0.5 rose to 2.1 $\pm$ 1.3 post, P=0.022), but not the PA JIA (pre 1.5 $\pm$ 1.0 and 1.5 $\pm$ 1.3 post, P=0.980). The percentage of $CD4^+CD25^{bright}$ T cells following *in vitro* stimulation with human HSP60 of the PBMCs of the HC was not different from the JIA patients following NeisVac-C vaccination (P=0.319). Vaccination and re-stimulation with *E. coli* HSP60 did not induced a significantly higher percentage of $CD4^+CD25^{bright}$ T cells in either the HC (pre 1.6 $\pm$ 1.6 and 2.6 $\pm$ 1.5 post, P=0.122) nor the JIA (pre 1.6 $\pm$ 0.9 and 2.4 $\pm$ 1.5 post, P=0.126). Yet, there was again an increasing trend in the OA JIA (pre 1.5 $\pm$ 0.8 rose to 2.6 $\pm$ 1.5 post, P=0.065), and not in the PA JIA patients (pre 1.4 $\pm$ 0.8 and 2.1 $\pm$ 1.7 post, P=0.496). No significant differences were found in the percentage of $CD4^+CD25^{bright}$ T cells when comparing the HC with the JIA or when comparing pre- versus post-vaccination and *in vitro* re-stimulation with the control antigen DT.

Figure 3. Percentage of $CD4^+CD25^{bright}$ T cells induced after vaccination and *in vitro* re-stimulation with NeisVac-C vaccine, TT, human HSP60, or *E. coli* HSP60

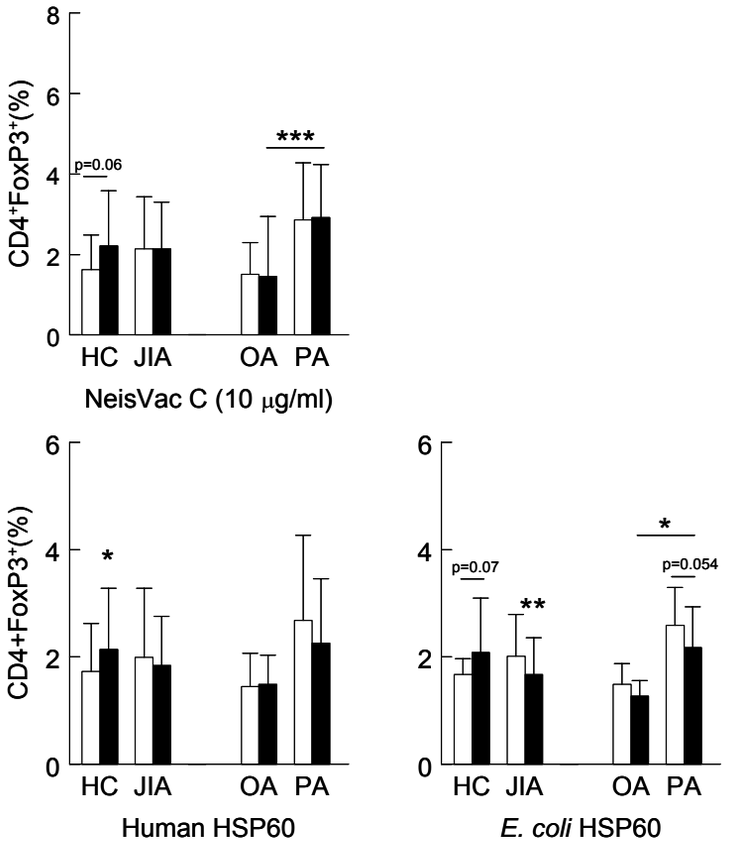


$CD4^+CD25^{bright}$ T cells expressed as a percentage of the total $CD4^+$ T cells are measured in the HC and JIA before vaccination (white bars) as well as after (black bars). Significant differences are indicated as follows: *=p<0.05, **=p<0.01, ***=p<0.0005.

Vaccination induces a higher percentage of $CD4^+FoxP3^+$ T cells towards vaccine antigens and HSP60 in the PA JIA

As expression of CD25 is not unique for T regulatory cells, we also measured expression of FoxP3, a transcription factor associated with T regulatory function. To assess differences of $CD4^+FoxP3^+$ T cells as a percentage of $CD4^+$ T cells induced by NeisVac-C vaccination, PBMCs from JIA patients and the HC were re-stimulated *in vitro* with either the vaccine, as well as human or *E. coli* HSP60 (Figure 4). After vaccination, *in vitro* re-stimulation with NeisVac-C vaccine induced a higher trend in the percentage of $CD4^+FoxP3^+$ T cells in the HC (pre 1.6 $\pm$ 0.9 rose to 2.2 $\pm$ 1.4 post, P=0.061), but not in either the JIA (pre 2.1 $\pm$ 1.3 and 2.1 $\pm$ 1.2 post, P=0.998), the OA JIA (pre 1.5 $\pm$ 0.8 and 1.5 $\pm$ 0.5 post, P=0.827), nor the PA JIA (pre 2.9 $\pm$ 1.4 rose to 2.9 $\pm$ 1.4 post, P=0.902). Remarkably, the percentage of induced $CD4^+FoxP3^+$ T cells was higher in the PA JIA than in the OA JIA after vaccination (P=0.008). Similar results were obtained following *in vitro* activation with another vaccine related antigen, TT (data not shown).

Figure 4. Percentage of $CD4^+FoxP3^+$ T cells induced after vaccination and *in vitro* re-stimulation with NeisVac-C vaccine, TT, human HSP60, or *E. coli* HSP60.



$CD4^+FoxP3^+$T cells expressed as a percentage of the total $CD4^+$ T cells are measured in the HC and JIA patients before vaccination (white bars) as well as after (black bars). Significant differences are indicated as follows: *=p<0.05, **=p<0.01, ***=p<0.0005.

Thus, compared to JIA, the HC seem better able to respond to vaccine-associated antigens (NeisVac-C or TT) after vaccination with the induction of an increased number of $CD4^+FoxP3^+$ T cells. Yet, overall the PA JIA group has the highest percentage of $CD4^+FoxP3^+$ T cells induced by either the vaccine or TT (included in the vaccine).

Vaccination and *in vitro* re-stimulation with human HSP60 induced a higher percentage of $CD4^+FoxP3^+$ T cells in the HC (pre 1.7 $\pm$ 0.9 rose to 2.1 $\pm$ 1.1 post, P=0.035), but not in the JIA (pre 2.0 $\pm$ 1.3 and 1.8 $\pm$ 0.9 post, P=0.562), the OA JIA (pre 1.4 $\pm$ 0.6 and 1.5 $\pm$ 0.5 post, P=0.753), nor the PA JIA (pre 2.7 $\pm$ 1.7 and 2.3 $\pm$ 1.2 post, P=0.474). Vaccination and *in vitro* re-stimulation with *E. coli* HSP60 induced a higher percentage of $CD4^+FoxP3^+$ T cells in the HC (pre 1.7 $\pm$ 0.9 rose to 2.1 $\pm$ 1.0 post, P=0.07), while in the JIA the percentage of *E.coli* HSP60 induced FoxP3 decreased (pre 2.0 $\pm$ 0.8 and 1.7 $\pm$ 0.7 post, P=0.009). This decreasing trend was also demonstrated in the PA JIA (pre 2.6 $\pm$ 0.7 and 2.2 $\pm$ 0.8 post, P=0.054), but not in the OA JIA (pre 1.5 $\pm$ 0.4 and 1.2 $\pm$ 0.3 post, P=0.108).

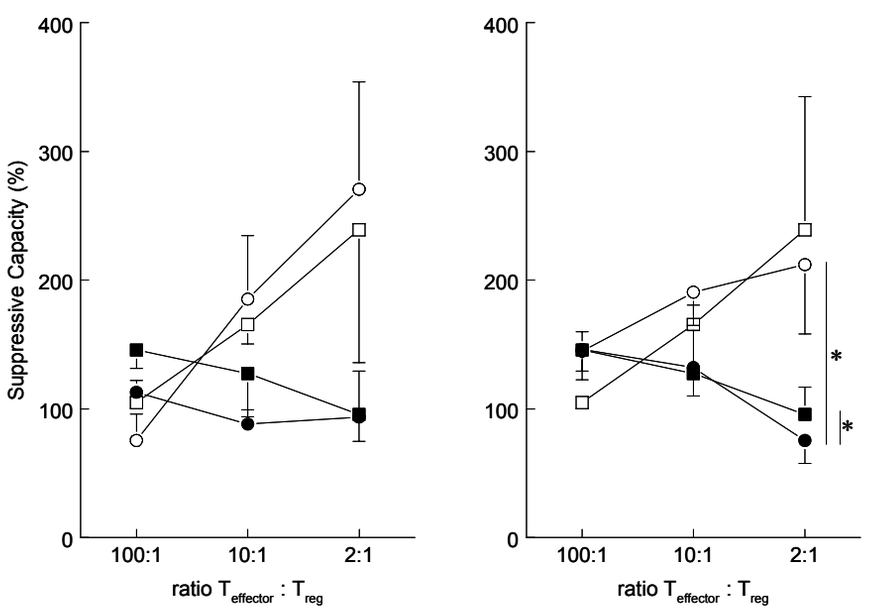
In summary, after vaccination with NeisVac-C the percentage of $CD4^+FoxP3^+$ T cells induced with human or *E. coli* HSP60 tends to increase in the HC but not in the JIA. In fact, in the JIA and the PA JIA after vaccination and *in vitro* stimulation with *E. coli* HSP60 these responses decreased. There were no significant differences in the percentage of $CD4^+FoxP3^+$ T cells induced after vaccination and *in vitro* re-stimulation with the control antigen OVA.

NeisVac-C and human HSP60 induced CD4^{+}CD25^{bright} T cells in PA JIA do not suppress the immune response

We questioned whether the antigen induced Tregs following vaccination were fully functional. Therefore, we tested the suppressive capacity of the induced CD4^{+}CD25^{bright} T cells of the PA JIA and the HC.

After vaccination, the induced CD4^{+}CD25^{bright} cells of the HC suppress T cell proliferation in response to *in vitro* re-stimulation with human HSP60 when comparing the 100:1, effector to regulatory T cell ratio expressed as a mean percentage ± SEM, (146.3 ± 38.1) with that of 2:1 (75.4 ± 39.7, P=0.015, Figure 5 Panel B) but not with the NeisVac-C vaccine *in vitro* stimulation (100:1, 112.8 ± 20.7 and 2:1, 93.7 ± 80.0, P=0.65, Panel A). Furthermore the PA JIA patients did not suppress their T cell proliferative response with *in vitro* re-stimulation to either the human HSP60 (100:1, 144.8 ± 31.5 and 2:1, 212.1 ± 76.2, P=0.279 Panel B) nor the NeisVac-C vaccine (100:1, 75.4 ± 29.1 and 2:1, 270.6 ± 117.9, P=0.198, Panel A). After *in vitro* human HSP60 re-stimulation, the T cell proliferative response of the HC were lower than that of the PA JIA (P=0.021, effector to regulatory cell ratio of 2:1). A similar trend was found after *in vitro* NeisVac-C vaccine re-stimulation (P=0.063, 2:1). No significant differences were found when comparing the pre vaccination versus post vaccination proliferative capacity for *in vitro* re-stimulation with either antigen. Collectively, this suggests that although the PA JIA patients display high numbers of putative T regulatory cells, they lack the immune suppressive capacity as their CD4^{+}CD25^{bright} T cells are not able to suppress the antigen specific immune response after vaccination and *in vitro* re-stimulation with either human HSP60 or the NeisVac-C vaccine. Furthermore, human HSP60 is better at inducing CD4^{+}CD25^{bright} T cell suppression of the antigen specific immune response than the NeisVac-C vaccine.

Figure 5. Percentage proliferative capacity of CD4^{+}CD25^{bright} T cells of HC and PA JIA patients after vaccination and *in vitro* re-stimulation with either NeisVac-C vaccine or human HSP60.



Proliferative capacity of the CD4^{+}CD25^{neg} T cells (effector) to CD4^{+}CD25^{bright} T cells (regulatory) ratio of 100:1, 10:1, and 2:1, expressed as a percentage of effectors alone, in HC (black) as well as PA JIA (white) patients after NeisVac-C vaccination alone (Squares) or with subsequent *in vitro* re-stimulation of either NeisVac-C (Circles, left panel) or human HSP60 (Circles, right panel). Only post-vaccine values are shown. Significant differences are indicated as follows: *=p<0.05.

Discussion

Human autoimmune diseases in general do not result from single gene mutations. It is more likely that a combination of genetic predisposition and environmental factors are needed to develop autoimmunity. An infection (or vaccination) could lead to a temporary release of self-antigens, which in combination with activation of innate immunity through Toll like receptors may lead to an enhanced pro-inflammatory immune response followed by more tissue damage. This may lead to a vicious circle of increasing immune activation and damage. Hence, the immune response towards a microbial antigen needs to be regulated to prevent excessive damage to our own tissues. Data from experimental models suggest this control lies with specialized subsets of T cells that specifically suppress an ongoing T cell response. These so-called regulatory T cells can either be directly derived from the thymus, (natural T regulatory cells) or induced following antigen encounter in the periphery (induced T regulatory cells). The data in experimental models have now been followed by various reports on the role and function of regulatory T cells in human autoimmune disease. Natural occurring T regulatory cells are present in various autoimmune diseases such as Multiple Sclerosis, Rheumatoid Arthritis, sarcoidosis, and Juvenile Idiopathic Arthritis (JIA). Considerable controversy still exists as conflicting reports have emerged. Most studies in human autoimmunity described a deficiency in either number or function of T regulatory cells, while in others the number of supposed T regulatory cells even seemed increased. Part of this controversy stems from the difficulty in identifying these T regulatory cells. Yet, none of these studies addressed how T regulatory cells respond *in vivo* to an immune response, triggered by an environmental antigen. This “plasticity” of the T regulatory response may very well be more important that mere cell numbers. We previously reported that in the self-remitting form of JIA, oligoarticular JIA, two types of T regulatory cells are present. First, we along with others described how the presence of naturally occurring $CD4^+CD25^+$ cells is correlated with a beneficial course in OA JIA. Moreover, the re-occurrence of $CD4^+CD25^+$ cells coincides with clinical success during the immune reconstitution following autologous stem cell transplantation for severe non-remitting JIA. Secondly, over the years we demonstrated the ability to induce adaptive T regulatory cells by an auto-antigen, human hsp60. Heat shock proteins (HSP) are highly conserved cellular proteins that are increasingly expressed during any form of cell stress. These so-called stress proteins can trigger both innate immune response through interaction with Toll like receptors (TLR) and induce an adaptive B and T cell response. The latter is associated with immune regulation in experimental models. Data from animal models and recent human studies have implicated T cell recognition of HSP60 as being part of a natural down regulatory response of the immune system. Furthermore, in JIA, a regulatory T cell response towards HSP60 is associated with a benign clinical course in OA JIA (27;28). It is assumed that during inflammation, e.g. as triggered by an infection, tissue damage leads to upregulation of HSP60, which in turn could activate HSP60-specific T cells instrumental in downregulating the immune response. Therefore, we postulated that vaccination may lead to changes in the antigen-specific regulatory T cell response for both the vaccine (NeisVac-C) and HSP60.

Indeed, in the present study we now for the first time have been able to determine how the immune system of JIA patients responds to an *in vivo* microbial trigger, namely vaccination with MenC. We found some remarkable differences between OA and PA JIA patients. Overall, the OA JIA patients seem to respond to vaccination in a fashion similar to the HC: an increase in $CD4^+$ T cell reactivity to vaccine and HSP60 epitopes, without an increased production of TNF-alpha. On the other hand, the PA JIA responded to vaccination with even higher levels of T cell proliferation and, importantly, increased production of TNF-alpha. Taken into account the pathogenic role of TNF-alpha in human arthritis, it seems likely that this type response holds an increased risk for enhancing inflammation. Secondly, the OA JIA and HC seemed capable of inducing $CD4^+CD25^+FoxP3^+$ T regulatory cells, specific for both NeisVac-C and HSP60. These induced T regulatory cells were functional and capable of *in vitro* suppression. On the other hand, although in PA JIA the absolute numbers of induced $FoxP3^+$ cells were even higher than those of the HC and OA JIA, we found little or no increase of these cells following vaccination in PA JIA. More importantly, the induced T regulatory cells of the PA JIA were not capable of suppressing T cell proliferation *in vitro*. Hence, overall, vaccination in PA JIA induced T cells with a potential risky phenotype (high T cell proliferation and TNF-alpha production), as the regulatory arm of the specific immune response displayed less plasticity in PA compared to JIA and HC. This reduced plasticity, and the apparent diminished *in vitro* suppressive capacity could be the consequence of an inherited deficient capacity to mount a regulatory response in PA JIA. It is also likely that the chronicity and height of inflammation in more severe forms of arthritis may exhaust the regulatory response and thus contribute to this limited regulatory capacity. Indeed, recently it was shown that high levels of TNF-alpha negatively affect T regulatory function through the upregulation of TNF receptor II on unstimulated T regulatory cells (29), This mechanisms may also play a role in the PA JIA as it was demonstrated in this study that their PBMCs expressed higher levels of antigen induced TNF-alpha *in vitro* as compared to the HC or the OA JIA. In summary, here we demonstrate that the PA JIA antigen induced regulatory response differs from that of the OA JIA or HC. It could be hypothesized that in a subset of patients with severe PA JIA this relative deficiency in T regulatory cell function may result in an inability to counter a pro-inflammatory response. This may be especially the case if such a response is strong, prolonged and more skewed towards a Th1 phenotype. Thus, in this particular subgroup of severe JIA, insufficient immunological feedback to certain triggers (MMR vaccination or prolonged infections) could indeed contribute to disease exacerbation. This is the first study that describes the flexibility of the T regulatory system following an *in vivo* environmental challenge in a human autoimmune disease. Future studies should focus especially on more severe subtypes of autoimmunity. This study underscores that enhancing T regulatory cell number and function seems a feasible therapeutic option in JIA and other human autoimmune diseases.

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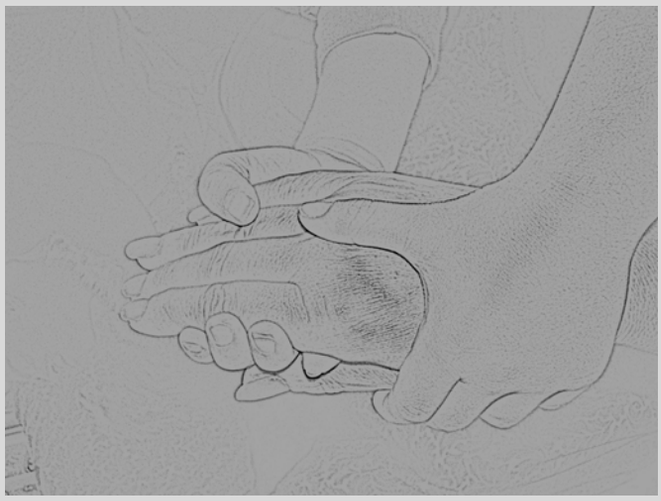

Chapter 8

General Discussion and Summary

Summary and discussion

Immunologists strive for a better understanding of how the immune system optimizes a protective immune response against an invading pathogen while minimizing collateral damage to self by avoiding chronic inflammation. Critical to this is a better understanding of the cellular mechanisms that influence the immune response. Among other cells, $CD4^+$ T cells are pivotal in initiating, orchestrating, and suppressing the adaptive immune response. Suppression of the immune response is attributed to a subpopulation of so-called regulatory T cells, among which include $CD4^+$ T cells that co-express high levels of CD25 called natural T regulatory cells. The balancing act between a healthy protective immune response to a foreign antigen and its regulation is tested with strong (induced) environmental triggers such as vaccines. Therefore, there is a great interest for a better understanding of (1) how vaccination, such as with the Meningococcal C Vaccine, influence the immune response in humans; (2) how ISS may influence such an immune response, and (3) how the use of Meningococcal C Vaccine and potential use of ISS in humans may affect an ongoing process of chronic inflammation in those predisposed. This is needed not only to facilitate further vaccine development, but it may also be instrumental for our understanding of the immune pathogenesis of certain autoimmune diseases. An environmental trigger that normally is kept at bay in a healthy immune system may contribute to perpetuation of inflammation in a genetically or otherwise predisposed individual.

Th1 Promoting effect of ISS

Previously, it was shown that certain bacterial components induced arthritis in experimental models (1-4). For example, arthritis is induced using syngeneic Lewis rats in an experimental model called adjuvant arthritis (AA) by injecting subcutaneously Complete Freund's adjuvant (CFA), a reagent that consists of heat-killed *Mycobacterium tuberculosis* (Mtb) suspended in oil. Furthermore, there are case reports that link Mycobacterium bovis to the induction of arthritis. This is exemplified by reports in the literature that discuss the rare complication of reactive arthritis following the BCG vaccination given as a therapeutic strategy for bladder cancer (5). Moreover, it was shown that short bacterial unmethylated DNA sequences containing the CpG motif called ISS, also found in Mtb, were able to stimulate a Th1 response in gene vaccinated animals (6). The above-mentioned studies did not show the stimulatory properties of non-coding DNA sequences containing ISS when co-administered with a model antigen.

In **Chapter two,** we showed that these bacterial DNA sequences containing the unmethylated CpG motif termed immunostimulatory DNA sequences (ISS) can potentiate Th1 responses to co-administered antigens. The ISS induced the production of IFN-gamma, IFN-alpha, IFN-beta, IL-12, and IL-18 all of which foster a Th1 response as well as stimulate cytotoxic T lymphocyte (CTL) activity.

This Th1 priming effect of ISS created an interest in its potential role as an adjuvant. Later it was shown that ISS exerts its effects by activating TLR9 on either plasmacytoid dendritic cells (pDC) or B-cells in humans. After activation through TLR binding the former secrete INF-alpha (7). TLR9 binding by ISS on pDC and B cells increases co-stimulatory molecule expression such as CD86 (8), chemokine receptor expression such as CCR7, production of Th1 type cytokines such as IL-6, IL-12, INF-gamma, CC, and CXC chemokines (9) as well as increase resistance to apoptosis (10;11). B cell activation through TLR9 induces plasma cell differentiation through a process that does not require but is enhanced with T cell help (12;13). This increases

the sensitivity of the B cell to antigen stimulation as well promote plasma cell differentiation and antibody production (14).
The next question we wanted to address was whether the Th1 promoting effects of ISS were limited to systemic administration. In **Chapter three,** we demonstrate that ISS co-administered with a model intranasally (i.n.) antigen can also provide effective mucosal adjuvant activity. This was evident with the induction of antigen specific mucosal IgA antibodies as well as a Th1 biased systemic response with high CTL activity. Mucosal immune responses via nasal administration to antigens co-administered with ISS have demonstrated a strong Th1 immune response to the antigen. This is the case even if the antigen was administered up to two weeks after intranasal ISS delivery. This finding allows for a greater therapeutic potential (15;16). It may even open the door for a novel, seemingly contradictory, indication for ISS, namely as an adjuvant for immune therapy in autoimmunity. From previous experimental work, we know that nasal immunization with HSP-derived peptides can have a beneficial effect on adjuvant arthritis (AA) in Lewis rats. A major concern is that the potential therapeutic immune response to HSP peptides may not be strong enough to regulate the inflammatory response in patients with severe RA or JIA. The question now is: will ISS enhance the tolerogenic response to hsp peptides in AA or will it stimulate the pro-inflammatory response? Therefore, it is crucial to investigate the safety and efficacy of antigen co-administration with ISS—a Th1 promoting adjuvant. To explore this, we turned our attention to the experimental model, adjuvant arthritis (AA). AA is a T cell driven systemic arthritis that is induced with heat-killed *Mycobacterium tuberculosis* (Mtb) in incomplete Freund's adjuvant (IFA). In **Chapter four,** we investigated the arthritogenic effects of ISS and in general (myco) bacterial DNA in this model. We found that AA depends in part on the presence of microbial DNA in Mtb, as DNase treatment lead to a strong amelioration of AA. Adding ISS to Dnase-treated Mtb in IFA restored the arthritogenic potential of Mtb. This clear arthritogenic potential of ISS was correlated with a Th1 skewing of the immune response as evident with higher levels of antigen specific IFN-gamma and increased production of osteoclast differentiation factor, receptor activation of NF-kappaB ligand (RANKL) following activation *in vitro* with microbial antigens. We speculated that ISS acted through a prolonged activation of the macrophages, dendritic cells, and osteoclast precursors priming the joint for a chronic Th1 driven inflammation to microbial antigens. These may suggest that ISS may hold a certain risk as an adjuvant in vaccination studies. However, it is important to note that an arthritis-related antigen was needed and that ISS alone was unable to induce arthritis. This is reassuring although caution is warranted due to the uncertainty of what effect ISS (or any type of bacterial antigen used for vaccination – see below) may have in predisposed individuals (i.e., patients with clinical or sub-clinical autoimmune diseases). This issue is addressed in **Chapters five, six and seven,** which will be discussed later.
To date, unlike the experimental model, human clinical trials with ISS show promising results and excellent safety. There are several explanations as to why the arthritogenic properties of ISS (or other vaccines) may not be seen in humans. First, Lewis rats are highly susceptible to autoimmunity. They are prone to develop an aberrant and prolonged Th1-driven immune response resulting in chronic inflammation. Whether a similar mechanism may play a role in humans is not known, as of yet no data are available on the effects of ISS in a population of rheumatoid arthritis patients.

Another important reason may be that rats have a different population of immune cells that express TLR9. In humans, TLR9 is expressed mainly on plasmacytoid dendritic cells and B cells. However, in the murine system it is also expressed in the mononuclear phagocytic cells. This discrepancy may explain the differences in athritogenic properties of ISS in rats or mice as compared to humans (17).

Allergy and ISS

The Th1 skewing properties of ISS have been utilized in therapeutic and prophylactic treatment of Th2 biased immune disorders such as allergy (18-20). Furthermore, in a Phase I clinical trial it has been shown that conjugation of ISS with ragweed allergen and administration to humans has proven to be effective with out any apparent adverse reactions (21;22). With this in mind, one may assume that ISS may be contraindicated in Th1-driven diseases such as in JIA and RA. Despite this, in the right context such as with mucosal administration with the proper antigen it is still conceivable that ISS may enhance the T regulatory response.
However, the question remains whether bacterial vaccines, in general, may hold a risk when used in individuals predisposed to autoimmunity. To answer this, we investigated both the clinical and the immunological consequences of immunization with a bacterial subunit vaccine (MenC vaccine) in patients with an autoimmune disease, namely JIA.

MenC Vaccination and JIA

The Th1 promoting effects of ISS, its effects in AA and its potential use in vaccines highlights issues of safety and efficacy in those predisposed to systemic chronic inflammation. We addressed this question in two ways. First, the initiation of a nationwide campaign in The Netherlands to vaccinate those under the age of 18 allowed us to prospectively investigate the safety and efficacy of MenC vaccination in individuals with JIA. In **Chapter five,** we found this vaccine to be both safe and effective in JIA. This was evident with no significant disease exacerbation within three months following vaccination. In addition, the total number of flares were lower (though not significantly) in the three months following, as opposed to three months prior to vaccination. Furthermore with regards to efficacy, one month after vaccination the JIA group as a whole demonstrated protective MenC-specific IgG antibodies > 2ug/ml, even in those using immunosuppressive medications. This is an important finding, although it does not exclude the potential for other vaccines to induce an exacerbation of JIA. The arthritogenic potential of a vaccine in those predisposed depends on the antigen, the adjuvant and its ability to promote a Th1 driven response. Vaccination in children with autoimmune diseases can be considered as a balancing act between sufficient protective immunity and the risk to aggravate autoimmunity. For the latter it is important that natural immune regulatory networks are not disrupted by the vaccination. This was addressed in **Chapters six,** and **seven**.

Vaccination and Tregs

Recently, when dealing with the safety and efficacy of a vaccine, there has been an interest in its influence on the T regulatory cell mechanism. In **Chapter six,** we demonstrate that patients with severe JIA, the poly-articular subgroup, have higher T cell proliferation to an environmental trigger (the MenC vaccine). Because of this, we investigated the regulatory T cells of this group. In **Chapter seven,** we show that the higher T cell proliferation in the PA JIA group corresponds to a dysfunction of the T

regulatory mechanism. Interestingly, though the numbers of Tregs are higher in the more severe PA JIA group, they do not function as well as healthy controls. This suggest that the hyperproliferation seen in the latter group may be the result of the lack of function of the $CD4^+CD25^+$ Tregs. This finding suggests that vaccinations and, by extension, infections may break tolerance in those predisposed to autoimmunity potentially leading to aggravation or even induction of the disease. It is conceivable that subunit vaccinations do not evoke a pro-inflammatory response that is strong enough to induce or exacerbate chronic inflammation. However, the risk may still exist, especially for live-attenuated vaccines known to induce more vigorous immune response. Therefore, it is important to evaluate the effects of every new vaccine in those predisposed to chronic inflammatory diseases both clinically as well as immunologically.

Implications for the Future

Reactivity of T cells to self HSP60 has been shown to induce T regulatory cells that may help to ameliorate chronic inflammation. These cells are necessary for the maintenance of a healthy immune system. Along this line of thinking, therapeutic strategies have been developed by using HSP60 peptides to further entice higher numbers and function of Tregs. The implications of this type of therapy in chronic inflammatory diseases are evident. They sidestep current adverse effects of blunt suppression of the whole immune system in treating various forms of arthritis. Theoretically, one could imagine that if TLR9 agonists such as ISS enhance the cellular immune response, co-administration of ISS with HSP peptides may enhance the anti-arthritogenic properties of HSP60-peptide therapy in humans by inducing Tregs if given in the right (mucosal) context. Interestingly, it was recently shown that TLR9 activation by ISS can induce $CD4^+CD25^+$Tregs (23-25). This deserves further exploration in an experimental model of arthritis such as AA.

Collectively, this thesis emphasizes the importance of the role of ISS as an adjuvant and its use in vaccinating healthy and JIA patients. MenC vaccination proved to be safe in the JIA population but caution should be exercised when considering vaccinating those with severe PA JIA, especially those treated with anti-TNF-alpha receptor agents. Future studies would need to address whether a natural downregulatory response to an environmental trigger is (absolutely or relatively) lacking in these patients. This can have profound influences on future vaccine development and on immune therapy for autoimmune diseases.

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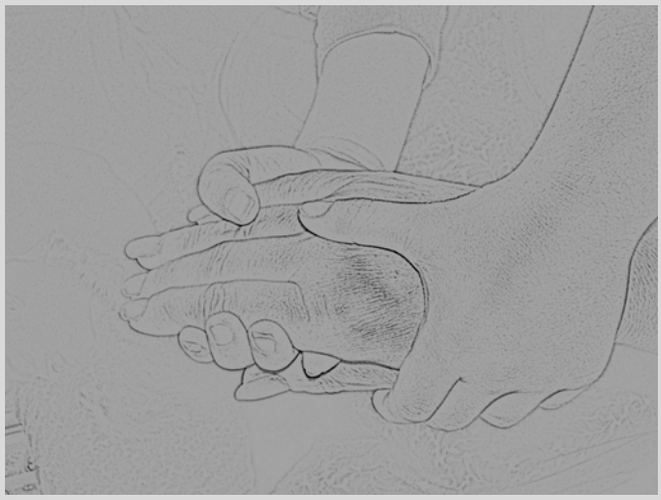


Nederlandse Samenvatting

Samenvatting en discussie

Immuniteit wordt over het algemeen gedefinieerd als de aangeboren of verworven bescherming tegen een infectie. Deze functie wordt uitgeoefend door het immuunsysteem en de reactie die veroorzaakt wordt noemen we de immuunrespons. Immunologen streven ernaar beter inzicht te krijgen naar hoe het immuun systeem ons beschermt tegen lichaamsvreemde, pathogene stoffen zonder het eigen lichaam te beschadigen. Als ons immuunsysteem een lichaamsvreemde stof zoals een bacterie of een virus onschadelijk probeert te maken, is het onvermijdelijk minieme schade te veroorzaken aan de eigen weefsels. Dit is een normaal fysiologisch proces dat inflammatie of ontsteking heet. Ontsteking, wat zich manifesteert als een warme, rode, gezwollen en pijnlijke plek, is noodzakelijk voor een gezond immuunsysteem. Het draagt zorg voor een snelle toename van het aantal immuun cellen op de plaats van de infectie, zodat de bacterie of het virus snel bestreden kan worden. Anderzijds, als de ontsteking te lang aanhoudt, kan het eigen weefsel excessief beschadigd worden. We spreken dan van een chronische ontsteking. Deze schade, veroorzaakt door eigen immuun cellen, kan even groot of zelfs groter zijn dan de schade veroorzaakt door het oorspronkelijke virus of bacterie.

Er zijn veel verschillende immuun cellen betrokken bij ontsteking. Zij kunnen onderverdeeld worden in twee groepen. De eerste groep betreft de cellen die toebehoren aan de ‘innate’ immuun respons. Deze respons is een primitieve en aspecifieke eerstelijns verdediging die bij alle organismen aanwezig is. De cellen die bij deze aspecifieke immuun respons betrokken zijn, reageren snel en op elke indringer. De tweede groep, de ‘adaptieve’ immuun respons, betreft een specifieke, meer gespecialiseerde verdediging, die enkel aanwezig is bij de gewervelde organismen. In tegenstelling tot de ‘innate’ immuun respons, is het voor deze immuun reactie noodzakelijk dat eerder contact met het lichaamsvreemde pathogeen plaats heeft gevonden. Deze reactie wordt ook wel een ‘memory’ respons genoemd, aangezien deze cellen zich het pathogeen ‘herinneren’ van het eerdere contact, en zo bij hernieuwd contact sneller een immuun respons op gang kunnen brengen. Beide responsen zijn noodzakelijk om een infectie te bestrijden. Dankzij de adaptieve immuun respons kunnen we beschermd worden door vaccinaties en immuun worden tegen bepaalde infecties, zoals de waterpokken (V*aricella*), na een eerste contact. Cellen die een rol spelen bij de adaptieve immuun respons zijn o.a. de B en T cellen. Dit proefschrift richt zich voornamelijk op de T cel responsen van het adaptieve immuun systeem.

Manipulatie van de Immuun Respons door Vaccinatie

De pokken (V*ariola* of *Variola Vera*) is een uiterst besmettelijke virale ziekte die alleen voorkomt bij de mens. Vaccinatie met de pokken wordt *variolatie* genoemd. In het verre Oosten hield het eerste *variolatie* proces in, dat korsten van geïnfecteerde personen in de neus van niet-geïnfecteerde personen werden geblazen. Deze vroege vorm van immuun interventie werd in 1721 vanuit Constantinopel naar Engeland gebracht door Lady Mary Wortley Montague, echtgenote van de Britse ambassadeur in Turkije. In Groot-Brittannië, het Europese vasteland en de Amerikaanse kolonies werd gekozen voor *variolatie* door middel van het aanbrengen van pus van een persoon geïnfecteerd met een milde vorm (*variola minor*) van de pokken, in een laesie op de huid tussen duim en wijsvinger van een nog niet-besmette persoon. Vervolgens werd in 1796 door Edward Jenner een modernere vorm van vaccinatie toegepast met een minder virulente vorm van een virus, in dit geval koeienpokken (V*accinia*, afkomstig van het Latijnse woord voor koe: vacca).

Tegenwoordig worden vaccinaties voornamelijk gecreëerd in twee verschillende vormen: een verzwakte, levende vorm van het virus of bacterie, ook wel ´geattenueerd vaccin´ genoemd. Een tweede vorm is een vaccin, gemaakt uit niet-infectueuze eiwitten van een bacterie of virus, genaamd 'subunit based vaccin'. Levende, geattenueerde vaccins kunnen een sterkere en langer durende immuun respons opwekken. Oorspronkelijk werd een levende, virulente vorm van pokken gebruikt als vaccin, met als mogelijke bijwerkingen een natuurlijke infectie en de dood. Om dit te vermijden werden subunit (op eiwit gebaseerde) vaccins gecreëerd. Deze niet-infectueuze vaccins verdienen omwille van veiligheids redenen de voorkeur, in het bijzonder bij immuun gecompromitteerde personen. Anderzijds stimuleren zij op zichzelf geen sterke beschermende immuun respons en vereisen daarom een adjuvant.

Adjuvant
Immunologen beschrijven een adjuvant als een substantie die de immuun respons op een antigen optimaliseert. Adjuvantia zijn nodig om de immuun responsen van het innate en adaptieve afweersysteem optimaal te activeren tegen de potentieel zwakke antigenen van een vaccin.
Daar kan aan toegevoegd worden dat bacteriën of hun componenten zelf een "adjuvant-achtig" effect kunnen hebben op de immuun respons. Recent zijn bacteriële DNA sequenties aangetoond met dergelijke adjuvant-achtige effecten.

Immunostimulatory DNA Sequenties (ISS)
Het laatste decennium is de interesse gegroeid onder wetenschappers naar de mogelijke rol van bacterieel DNA voor vaccinaties. Bacterieel DNA heeft unieke structuren die niet teruggevonden worden in het DNA van zoogdieren. Deze worden *Immunostimulatory DNA Sequences* (ISS) genoemd vanwege hun capaciteit om het innate en adaptieve immuunsysteem te stimuleren en zo beter virale en bacteriële infecties te kunnen bestrijden. T cel immuun responsen kunnen van het Th1- of Th2-type zijn. Indien het Th1-type van de T cel immuun respons ongecontroleerd voorkomt, kan dit leiden tot een pro-inflammatoire ziekte, zoals Juveniele Idiopathische Artritis (JIA). ISS veroorzaakt, in combinatie met een antigen, een sterke Th1 respons. Desalniettemin is er tot op heden geen bewijs gevonden dat ISS direct betrokken is bij humane artritis.

Preventie van Chronische Inflammatie en Auto-immuniteit
Een hevige inflammatoire respons, veroorzaakt door een natuurlijke infectie of een subunit-adjuvant vaccin, moet strikt gereguleerd worden om chronische ontsteking te vermijden. Het proces van immuun regulatie om secundaire schade aan lichaamseigen cellen te minimaliseren wordt immuun tolerantie genoemd. In deze context kan tolerantie gedefinieerd worden als elk mechanisme waarbij de herkenning van een antigeen niet leidt tot een immuun respons die tot zelf destructie leidt. Dit proces is van essentieel belang om de krachtige immuun respons tegen microbiële antigenen te weerhouden van een schadelijke reactie op het eigen lichaam. Een bepaalde subset van T cellen speelt een belangrijk regulatoire rol in het voorkomen van ongecontroleerde chronische ontsteking, genaamd de regulatoire T cellen (*Tregs)*.

Regulatoire T cellen
Tregs zijn betrokken bij de immuun regulatie en werden het eerst geïdentificeerd bij dieren, maar maken ook deel uit van het menselijk immuunsysteem. Ze kunnen ruimweg ingedeeld worden in twee subgroepen: de 'naturally occuring' (nTregs) of de 'induced' Tregs (iTregs).
Zoals hun naam doet vermoeden worden iTregs door bepaalde antigenen geïnduceerd en ze onderdrukken de immuun respons via hormonen van het immuunsysteem, cytokines genaamd.
In dit proefschrift gaat de aandacht naar nTregs, die aanwezig zijn in de periferie van ieder gezond individu. Depletie van deze cellen resulteert niet alleen in ernstige auto-immuunziekte bij muizen, maar ernstige auto-immuunziekte kan ook voorkomen worden als nTregs terug geïnjecteerd worden. Dit doet vermoeden dat nTregs de immuun respons helpen onderdrukken.
Anderzijds kan een te sterke onderdrukking van de immuun respons de kans op infecties doen toe nemen.
Humane nTregs brengen een uniek intracellulair proteïne tot expressie, FoxP3. Mutatie van FoxP3 is geassocieerd met een zeldzame menselijke auto-immuunziekte, IPEX. Aangezien nTregs normaal gesproken het immuunsysteem onderdrukken, is het logisch zich af te vragen wat er gebeurt met de nTregs qua hoeveelheid en functie indien het immuunsysteem gestimuleerd wordt door een infectie of een vaccin. Data over hoe humane nTregs worden beïnvloed door vaccinatie zijn nog steeds schaars.

'The Balancing Act'
Van een gezond immuunsysteem kan verwacht worden dat het zowel activatie als regulatie ondergaat. Disregulatie kan resulteren in enerzijds een hyperstimulatie van de immuun respons met eventuele chronische ontsteking (zoals chronische artritis) tot gevolg; anderzijds tot een hypostimulatie met een verhoogde kans op infecties en kanker. Bij gezonde personen onderdrukken nTregs de normale immuun respons zodra het veroorzakende pathogeen uit de weg is geruimd. Bij patiënten met een auto-immuun aandoening, kan de pathologie veroorzaakt zijn door een verlaagd aantal Tregs of een verminderde functie van hun Tregs. Deze disbalans kan leiden tot een chronische ontstekingsziekte, zoals Juveniele Idiopathische Artritis.

Juveniele Idiopatische Artritis (JIA)
Juveniele Idiopatische Artritis is de meest voorkomende reumatische aandoening bij kinderen. Het wordt gedefinieerd als een chronische artritis, die minimaal 3 maanden duurt, bij kinderen onder de 16 jaar. JIA wordt vaak gekarakteriseerd door perioden waarin de symptomen verslechteren (flare) en verbeteren (remissie). JIA kan opgedeeld worden in verschillende subtypes, maar enkel de ernstige polyarticulaire JIA (PA JIA) en de minder ernstige oligoarticulaire JIA (OA JIA) zullen in dit proefschrift besproken worden. Het minder ernstige verloop van OA JIA heeft geleid tot de speculatie dat de gunstigere prognose veroorzaakt kan zijn door optimaal functionerende nTregs.
Hoewel het exacte mechanisme nog steeds niet bekend is, wordt over het algemeen verondersteld dat de gewrichtsdestructie bij JIA veroorzaakt wordt door een abnormaal sterke Th1 immuun respons, ten gevolge van een omgevings trigger, bij een genetisch gepredisponeerde persoon. Deze trigger kan van bacteriële of virale aard zijn, of zelfs een vaccin. Een vaccin zou een reeks van gebeurtenissen op kunnen wekken die leiden tot een chronische ontsteking van het gewricht en de

productie van pro-inflammatoire cytokines: TNFα, IL-6 en IL-1β. Desondanks zijn bij OA JIA regulatoire T cellen aanwezig (nTregs) die de ontstekingsreactie onderdrukken. Bovendien is aangetoond dat T cellen die reageren tegen het Human Heat Shock Protein 60 (HSP60) een regulatoire rol spelen bij JIA.

HSP en JIA
HSPs zijn intracellulaire proteïnen die in bijna iedere cel aanwezig zijn en die betrokken zijn bij cruciale intracellulaire functies. Voorts kunnen HSPs vrijkomen in de extracellulaire omgeving als signaal moleculen. HSPs zijn in staat zelf een sterke immuun respons te induceren en worden daarom immunodominante eiwitten genoemd. Ze worden geclassificeerd in zes families, afhankelijk van hun moleculaire gewicht: HSP10, HSP40, HSP60, HSP70, HSP90, HSP100. HSPs zijn sterk geconserveerd tijdens de evolutie tussen verschillende species, ook tussen bacteriën en mensen. Ze worden opgereguleerd ten tijde van een infectie, zuurstoftekort en bij temperatuursschommelingen.

Molecular Mimicry
De geconserveerde, stressgeïnduceerde opregulatie en de immunodominante eigenschappen van HSPs hebben geleid tot de hypothese dat bacteriele HSPs tijdens een infectie specifieke T cellen zouden kunnen activeren, die op hun beurt zouden kunnen kruisreageren met menselijke HSPs en vervolgens een onderdrukkende functie op de immuun respons kunnen uitoefenen. Deze ‘molecular mimicry’ hypothese suggereert dat de inductie van auto-reactieve lymfocyten veroorzaakt wordt door structureel gelijkwaardige humane en microbiële antigenen.

Doel van het onderzoek
Vaccinatie is zowel bij gezonde personen als bij personen met artritis een krachtig instrument om de immuun respons te versterken. Men kan zich afvragen of ISS bij een van beide groepen gebruikt kan worden als een adjuvant voor vaccinatie. De vraag blijft of vaccinatie een risico inhoudt om een ongereguleerde immuun respons te veroorzaken bij diegenen die een aanleg voor artritis hebben, wat vervolgens zou leiden tot exacerbatie van hun auto-immuunziekte. En hoe weegt dit risico op tegen de consequenties van het ondergaan van de natuurlijke infectie?
Bovendien heeft het moduleren van het type immuun respons therapeutische voordelen. Zo zouden bijvoorbeeld personen met een auto-immuunziekte baat hebben van een shift van hun immuun responsen naar een Th2 phenotype en personen met allergieën naar een Th1 phenotype. Dus, het gebruik van adjuvantia zoals ISS om de immuun respons te sturen, zou een antigen specifieke immuun therapie kunnnen complementeren**.** Om dit doel te bereiken is het van belang een beter begrip te krijgen van de manier waarop het immuunsysteem gemoduleerd wordt door ISS.

Inhoud van het proefschrift

Het doel van dit onderzoek is beter te begrijpen hoe ISS de immuun respons moduleert en hoe dit chronische ontsteking kan beïnvloeden bij diegenen, die gepredisponeerd zijn voor artritis.

Het eerste deel van het proefschrift beschrijft het onderzoek naar ISS als mogelijk vaccinatie-adjuvant, zowel na systemische als mucosale toediening. Verder wordt de invloed van ISS op het verloop van artritis bestudeerd in een experimenteel diermodel, Adjuvant Artritis (AA).

Het tweede deel van het proefschrift beschrijft het onderzoek naar de veiligheid en effectiviteit van het MenC vaccin bij JIA patiënten, waarbij bekeken werd of de immuun respons ten opzichte van een bacterie een verhoogde kans geeft op exacerbatie van auto-immuniteit bij vatbare individuen.

De kwaliteit en kwantiteit van de door vaccinatie opgewekte immuun respons bij zowel OA JIA als bij PA JIA patiënten werd onderzocht, zowel ten opzichte van vaccinatie als van artritis geassocieerde antigenen, inclusief hun regulatoire mechanismen.

In **hoofdstuk 2** wordt aangetoond dat de bacteriële, ISS bevattende, DNA sequenties een Th1 respons induceren ten opzichte van mede toegediende antigenen. Hierdoor werd de interesse gewekt voor een potentiële rol van ISS als adjuvant.

In **hoofdstuk 3** demonstreren we dat, indien ISS intranasaal (i.n.) wordt toegediend in combinatie met een model antigeen, dit een beschermende Th1 immuun respons kan opwekken op mucosaal niveau alsook via nasale toediening van ISS in combinatie met antigeen. Dit impliceert dat ISS ook oraal of nasaal toegediend zou kunnen worden ter preventie van respiratoire en urogenitale infecties.

Dit zou zelfs de deur kunnen openen voor een nieuwe, op het eerste gezicht contradictoire, indicatie voor ISS: ISS als adjuvant voor immuun therapie bij auto-immuniteit. Uit voorgaand experimenteel onderzoek is gebleken dat nasale immunisatie met HSP peptiden een arthritis suppressief effect heeft op Adjuvant Arthritis (AA) bij Lewis ratten. Toevoeging van ISS kan mogelijk de anti-inflammatoire reactie optimaliseren. Anderzijds kan de intrinsieke pro-inflammatoire respons artritogeen zijn.

Om dit beter te onderzoeken hebben we gekozen voor het dierexperimentele model Adjuvant Artritis (AA). In **hoofdstuk 4** wordt het artritogene effect van ISS en het bacteriële DNA in dit model onderzocht. Het bleek dat AA deels afhankelijk is van de aanwezigheid van het microbiële DNA in Mycobacterium Tuberculosis (Mtb). Dit kan betekenen dat het gebruik van ISS als adjuvant bij vaccinatiestudies een zeker risico inhoudt. Anderzijds is het belangrijk op te merken dat een (artritis-gerelateerd) antigeen nodig was en dat ISS op zichzelf niet in staat was om artritis te induceren. Hoewel dit geruststellend is, blijft voorzichtigheid geboden, daar het precieze effect van ISS (of een ander type bacterieel antigeen gebruikt voor vaccinatie – zie later) bij gepredisponeerde personen (bijv. patiënten met klinische of subklinische auto-immuunziekten) onzeker is. Dit wordt behandeld in **hoofdstuk 5, 6 en 7** en zal later besproken worden.

Tot nu toe hebben humane klinische trials met ISS, in tegenstelling tot in het experimentele model, veelbelovende resultaten en een uitstekende veiligheid getoond. Er zijn verschillende verklaringen mogelijk waarom de artritogene eigenschappen van ISS (of andere vaccinaties) niet bij de mens gevonden worden. Een eerste verklaring is dat Lewis ratten, die gebruikt worden voor het experimentele model van auto-immuunziekten, uiterst gevoelig zijn voor auto-immuniteit. Of een

gelijkaardig mechanisme ook bij de mens een rol speelt, is niet bekend. Tot op vandaag zijn er geen data beschikbaar van het effect van ISS bij patiënten met Reumatoïde Artritis. Een andere belangrijke mogelijkheid is, dat in de rat een andere populatie van immuun cellen aan ISS bindt dan in de mens. Niettemin blijft de vraag of bacteriële vaccinaties, over het algemeen, een risico inhouden indien ze gebruikt worden bij personen, die gepredisponeerd zijn voor auto-immuunziekten. Om dit te beantwoorden onderzochten we zowel de klinische als de immunologische gevolgen van immunisatie met een bacterieel subunit vaccin (MenC vaccin) bij patiënten met een auto-immuunziekte, namelijk JIA.

MenC vaccinatie en JIA

Het Th1 stimulerende effect van ISS, diens effect in AA en diens potentieel voor het gebruik bij vaccinaties, benadrukken het belang van veiligheid en effectiviteit bij gepredisponeerden voor systemische chronische inflammatie. We hebben deze vraag op twee manieren benaderd.

Allereerst gaf de landelijke campagne in Nederland om iedereen onder 18 jaar te vaccineren tegen MenC ons de mogelijkheid om prospectief de veiligheid en effectiviteit van het MenC vaccin te onderzoeken bij patiënten met JIA.

In **hoofdstuk 5** werd dit vaccin zowel veilig als effectief bevonden bij JIA patiënten. Bovendien werden, met betrekking tot de effectiviteit, bij de gehele JIA groep een maand na vaccinatie waarden van beschermende MenC-specifieke IgG antilichamen hoger dan 2µg/ml aangetoond, zelfs bij degenen die immuunsuppressieve medicatie gebruikten. Dit is een belangrijke bevinding, hoewel het niet uitsluit dat andere vaccins wel een exacerbatie van JIA zouden kunnen veroorzaken. Het artritogene vermogen van een vaccin is afhankelijk van het antigeen, het adjuvant en het vermogen om een Th1 respons tot stand te brengen. Vaccinatie bij kinderen met auto-immuunziekten kan beschouwd worden als een ‘balancing act’ tussen voldoende beschermende immuniteit en het risico de auto-immuniteit te verergeren. Het is bovenal belangrijk dat vaccinatie de natuurlijke regulatoire netwerken niet verstoort.

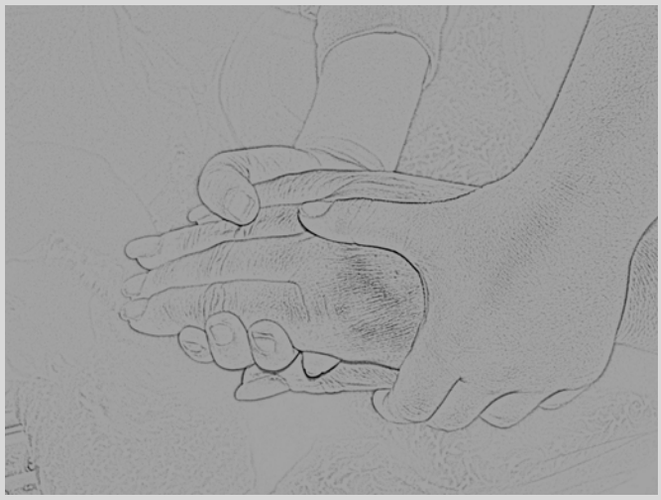
In **hoofdstuk 6** demonstreren we dat patiënten met een ernstige vorm van JIA, de poly-articulaire subgroep, een hogere T cel proliferatie vertoonden op het MenC vaccin. Daarom onderzochten we de regulatoire T cellen van deze groep.

In **hoofdstuk 7** tonen we aan dat de hogere proliferatie van T cellen bij de PA JIA groep correspondeert met een disfunctie van het T regulatoire mechanisme. Hoewel Tregs. Dit suggereert dat vaccinaties, en ook infecties, ervoor kunnen zorgen dat de immuun tolerantie doorbroken wordt bij gepredisponeerden voor auto-immuniteit en zo potentieel kunnen leiden tot een verergering of zelfs inductie van de ziekte. Het is niet ondenkbaar dat subunit vaccinaties geen pro-inflammatoire respons uitlokken die sterk genoeg is om chronische ontsteking te verergeren of induceren. Het risico blijft echter bestaan, vooral bij levende, geattenueerde vaccins, die erom bekend staan een krachtigere immuun respons uit te lokken. Het is daarom belangrijk het effect van ieder nieuw vaccin te evalueren, zowel klinisch als immunologisch. het aantal Tregs hoger is bij de ernstigere PA JIA groep, functioneren zij niet zoals bij de gezonde controlegroep. Dit suggereert dat de hyperproliferatie die gezien wordt bij deze laatste groep een resultaat zou kunnen zijn van een verzwakte functie van de $CD4^{+}CD25^{+}$.

Implicaties voor de toekomst

Reactiviteit van T cellen op lichaamseigen HSP60 hebben aangetoond T regulatoire cellen te induceren die chronische ontsteking kunnen remmen. Deze cellen zijn essentieel voor de instandhouding van een gezond immuunsysteem. In dezelfde lijn zijn therapeutische strategieën ontwikkeld die gebruik maken van HSP60 peptiden om grotere aantallen en betere functie van Tregs te bewerkstelligen. De implicaties van dit soort therapie zijn voor chronische ontstekingsziekten gunstiger dan de huidige therapieën die het hele immuunsysteem onderdrukken en zo het risico op infectie verhogen. In theorie zou ISS de cellulaire immuun respons op HSP peptiden kunnen verhogen en daarmee de anti-artritogene eigenschappen van HSP60-peptide therapie bij mensen kunnen optimaliseren door inductie van Tregs, indien gegeven in de juiste (mucosale) context. Recent werd aangetoond dat activatie door ISS $CD4^+$ $CD25^+$ Tregs kan induceren. Dit verdient nader onderzoek in een experimenteel model van artritis zoals AA.

Dit proefschrift benadrukt het belang van de rol van ISS als adjuvant en het gebruik ervan bij accinatie van zowel gezonde personen als bij JIA patiënten. MenC vaccinatie bleek veilig te zijn bij JIA patiënten, maar voorzichtigheid dient geboden te worden bij patiënten met ernstige PA JIA. Verder onderzoek zal moeten aantonen of een natuurlijke downregulatoire respons op een omgevingstrigger (absoluut of relatief) ontbreekt bij deze patiënten. Dit kan vergaande invloed hebben op toekomstige ontwikkeling van vaccinaties en op immuun therapie bij auto-immuunziekten.



Acknowledgement

Acknowledgements
Were it not for those mentioned here, this thesis would remain a dream and not a reality.

Chance is as important in life as anything
I would love to tell you that my sense of curiosity and eagerness to unlock to secrets of life led me to basic science and that after exposure to the sciences it was immunology that grabbed my heart and mind. I could tell you this, but it would be an outright lie. Two events focused my curiosity towards research in immunology: 1) waking up late for school, and 2) an empty seat on a bus.
As a freshman, I was running late for school one morning at the University of California, San Diego. As I saw the bus 50 meters ahead I started running at full speed and got on just in time. I grabbed the only seat available and tried to catch my breath. The man seated next to me was Dr. Eyal Raz. We started our first conversation and six months later I was in his lab learning immunology. I learned and matured quite a bit in Eyal's lab. That period was stimulating, intensive, and enjoyable.

A special thanks to those without whom this thesis would not have been written
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To the Prakken lab; you all are an enthusiastic, eager group, always willing to help. You all have been so helpful to me throughout this process! Please keep me in mind, and if you ever drop by Boston, or wherever I may be, look me up!

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To those I love
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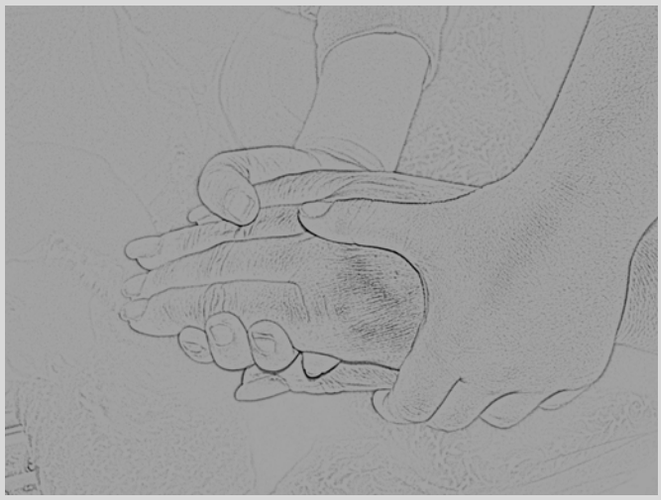
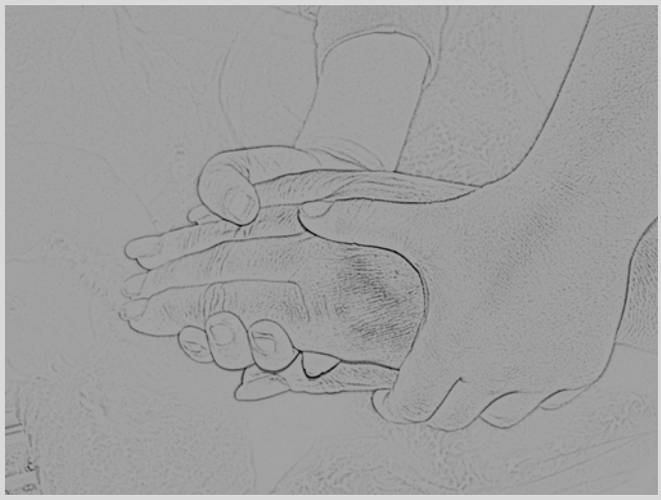


Curriculum Vitae

Arash Ronaghy was born in Shiraz, Iran on the 13th of January, 1973. He and his parents moved to San Diego in 1981. In 1993, he graduated with honors from University City High School. In 1998, he graduated from the University of California, San Diego with a degree in Biochemistry and Cell Biology. He worked as a research assistant for the next three years and then entered a masters degree program at Boston University School of Medicine in 2001. He graduated, in 2002, with a Master of Arts in Graduate Medical Sciences. Soon after, Dr. Berent Prakken asked Mr. Ronaghy to join his lab to begin his formal training as a PhD student. In 2002, he formally entered the doctoral program in immunology at the University of Utrecht, The Netherlands. In 2003, Mr. Ronaghy entered the Boston University School of Medicine as a first year medical student. After one year, he took a leave of absence, to finish his PhD training in Utrecht. On January 4th, 2007, Arash Ronaghy will be awarded his PhD in immunology, after which he will continue his medical education at Boston University. Arash Ronaghy intends to receive his degree of Medical Doctor in June of 2009.



List of publications

List of abbreviations

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^{3}H	thymidine
AA	adjuvant Arthritis
AJ	active joints
AmpR	ampicillin resistance
APC	antigen presenting cells
BALF	bronchoalveolar lavage fluid
Beta-gal	beta-galactosidase
CCR	cc-chemokine receptor
CD	cluster determinant
cDCs	conventional dendritic cells
CFA	complete Freunds' Adjuvant
CFSE	carboxyfluorescin diacetate succinimidyl ester
CHAQ	childhood Health Assessment Questionnaire
CMV	cytomegalovirus
CpG	cytosine-phosphate-guanisine
CPM	counts per minute
CT	cholera toxin
CTL	cytotoxic T lymphocyte
CTLA4	cytotoxic T lymphocyte antigen-4
CY	cychrome
DC	dendritic cells
DMARDs	disease-modifying antirheumatic drugs
DNA	deoxyribonucleic acid
DT	diphtheria-toxoid
E. coli	escherichia coli
ELISA	enzyme-based immunosorbent assay
ESR	erythrocyte sedimentation rates
FITC	fluorescein isothiocyanate
FoxP3	forkhead/winged-helix family of transcriptional regulators
GITR	glucocorticoid induced tumor-necrosis factor receptor
HC	healthy adults/controls
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
HSP	heat shock protein
i.d.	intradermal
i.g.	intragastrically
i.m.	intramuscular
i.n.	intranasal
IFA	incomplete Freund's adjuvant
IFN	Interferon
IL	Interleukin
ILAR	International League of Associations for Rheumatology
ILN	inguinal lymph node
IPEX	immunodysregulation polyendocrinopathy enteropathy X-linked syndrome
ISS	immunostimulatory DNA Sequences
iTregs	induced regulatory T cells
JA	juvenile arthritis
JCA	juvenile chronic arthritis
JIA	Juvenile idiopathic arthritis

JRA	juvenile rheumatoid arthritis
KanR	kanamycin resistance
LOM	limited range of motion
LPS	lipopolysaccharide
LR	low responders
MAMPs	microorganisms-associated molecular pattern
MCP-1	monocyte chemotactic protein-1
MenC	meningococcal serogroup C
MMR	measles-mumps-rubella
Mtb	*mycobacterium tuberculosis*
MTX	methotrexate
MyD88	myeloid differentiation primary response gene 88
NF-kappaB	muclear Factor kappa B
NK	natural killer
NSAID	non-Steroidal Anti-Inflammatory Drugs
nTregs	natural occuring regulatory T cells
OA	oligoarticular
ODN	oligodeoxynucleotides
OVA	ovalbumin
PA	polyarticular
PBMC	peripheral-blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDCs	plasmacytoid dendritic cells
PE	phycoerythrin
PGA	physician Global Assessment
PRRs	pattern-recognition receptors
RA	rheumatoid arthritis
RANKL	receptor activation of NF-kappaB ligand
RANTES	regulated upon Activation, Normal T cell Expressed, and Secreted
RNA	ribonucleic acid
RR	relative risk
SI	stimulation index
SPSS	statistical package for the social sciences
TCR	T cell receptor
TGF	transforming growth factor
Th1	T helper 1
Th3	T helper 3
TLR	Toll-like receptors
TNF	tumor necrosis factor
TR1	regulatory T cells type 1
Tregs	regulatory T cells
TT	tetanus toxoid
VAS	visual analogue scale
WHO	World Health Organization