T cell regulation in Juvenile Idiopathic Arthritis

Controlling chronic inflammation by pulling the right strings

ISBN 90-9018592-5
Cover layout Habari
Layout Habari
Print Print Partners Ipskamp, Enschede

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Controlling chronic inflammation by pulling the right strings

(met een samenvatting in het Nederlands)

Proefschrift ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de Rector Magnificus, Prof Dr. W.H. Gispen, ingevolge het besluit van het College voor Promoties in het openbaar te verdedigen op dinsdag 23 november 2004 des namiddags te 2.30 uur.

door **Ismé Mariëtte de Kleer,** geboren op 24 september 1973 te Oosterbeek

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The printing of this thesis was supported by
Eijkman Graduate School
Amgen B.V., Breda
Aventis Pharma B.V.
Fujisawa Holland B.V.
Novartis Pharma B.V.
Dutch Arthritis Association
Fresenius Kabi

I saw, as in a dream sublime,
The balance in the hand of Time.
O'er East and West its beam impended;
And day, with all its hours of light,
Was slowly sinking out of sight,
While, opposite, the scale of night
Silently with the stars ascended.

Like the astrologers of eld,
In that bright vision I beheld
Greater and deeper mysteries.
I saw, with its celestial keys,
Its chords of air, its frets of fire,
The Samian's great Aeolian lyre,
Rising through all its sevenfold bars,
From earth unto the fixed stars.
And through the dewy atmosphere,
Not only could I see, but hear,
Its wondrous and harmonious strings,
In sweet vibration, sphere by sphere.

Part of “The Occultation of Orion”
by Henry Wadsworth Longfellow

Voor mijn ouders

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1 General Introduction

Introduction

In the healthy person there is a delicately balanced immune system. Antibody concentration or the number of effector T cells progressively increases after antigenic stimulation, but the levels rapidly reach a plateau and eventually wane after elimination of the pathogen. Natural autoimmunity is kept under control and lymphocyte pools are maintained at constant levels through homeostatic processes. More generally: most immune responses are quantitatively and qualitatively adapted to mount an optimal defense against foreign agents and hosts, while at the same time avoiding damage to somatic tissues. For this goal the immune system has developed several checkpoints and regulatory systems. The failing of this system might lead to adversities like autoimmunity or malignancies of the hematopoietic system.

Immune regulatory mechanisms

Multiple mechanisms exist that contribute to the regulation of immune responses. First, repertoire selection in the thymus strongly prevents self-reactive T cells from entering the periphery. This selection is however not 100% efficient, and even autoreactive T cells that have high affinity for auto-antigens can escape the deletion process and migrate to the periphery [1,2]. Several mechanisms have been proposed that deal with those potentially pathogenic lymphocytes that have escaped the deletional mechanism in the thymus [3,4]. They may be rendered anergic or further deleted upon encounter with self-antigens. Self-reactive T cells may fail to be activated because of lack of co-stimulation from antigen-presenting cells (APC), their seclusion from the target self-antigens or because of low avidities of their T cell receptors for self-antigens. In addition to these passive mechanisms of self-tolerance there appears to be a dominant one. This dominant form of self-tolerance is mediated by subsets of T cells functionally distinct from conventional helper or cytotoxic T-lymphocytes, which are now referred to as “regulatory T cells” (Tregs).

Subsets of regulatory T cells

Tregs are heterogeneous in phenotype, function and the way of generation. Some are naturally occurring, others are induced by specific ways of antigenic stimulation. Nowadays Tregs are classified in two major categories: naturally occurring $CD4^{+}CD25^{+}$ Tregs and those induced in the periphery. As is frequently the case these regulatory T cell populations were first discovered in animal models and subsequently identified in human.

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

CD4$^+$CD25$^+$ Tregs are among the best-characterized immune-regulatory subsets shown to prevent the activation of the innate and adaptive immune response and to block the effector function of activated responder cells [5]. The discovery of CD4$^+$CD25$^+$ Tregs was based on a simple in vivo observation: mouse thymectomy on neonatal day 3 leads to the development of multiorgan autoimmune disease [6]. Seminal experiments performed by Sakaguchi and coworkers demonstrated that it was the depletion of CD4$^+$CD25$^+$ T cells that resulted in the onset of systemic autoimmune diseases in these neonatally thymectomized mice [7,8]. Indeed, transfer of large doses of CD25$^-$ T cells into nude mice resulted not only in the production of organ-specific autoantibodies but also in the development of lethal systemic autoimmunity including colitis, gastritis, insulin-dependent autoimmune diabetes, and thyroiditis [9-12], while cotransfer of a small number of CD4$^+$CD25$^+$ T cells with CD25$^-$ T cells completely prevented these autoimmunities.
Besides playing an imminent role in the prevention of autoimmune diseases, experimental animal models also showed that removal of CD4$^+$CD25$^+$ T cells may enhance immune responses to nonself antigens including xenogeneic proteins and allografts.
It even has been shown that a significantly prolonged and even permanent graft tolerance can be established by infusing large doses of CD4$^+$CD25$^+$ Tregs [13]. In contrast, given that many tumor-associated antigens recognized by autologous T cells are antigenically normal self-constituents, CD4$^+$CD25$^+$ Tregs may impede the generation and activation of tumor-effector T cells recognizing autologous tumor cells [14,15].
Since CD4$^+$CD25$^+$ Tregs is a subset present in the periphery of every naïve host these cells can be referred to as naturally occurring Treg cells (nTregs). NTregs develop in the thymus, possibly as a consequence of escape from negative selection [16-18]. They represent a small fraction (1-3%) of CD4$^+$ T cells and constitutively express the α-chain of the IL-2 receptor (CD25) [9], the CTLA-associated antigen-4 (CTLA4) [19], the glucocorticoid induced tumor-necrosis-factor receptor (GITR) [20] and the transcription factor FoxP3 [21]. Although the precise cellular and molecular pathways involved in the suppressive capacity of CD4$^+$CD25$^+$ Tregs remain to be defined, evidence has indicated that in vitro suppression requires activation of Tregs via their T cell-receptor and is mediated through a cell-contact dependent mechanism [22-24]. Although the activation of CD4$^+$CD25$^+$ Tregs is antigen-specific, once activated, these cells inhibit both CD4$^+$ and CD8$^+$ T cell responses in an antigen-nonspecific manner [25,26].
While all the above information is gained from experimental animal models, data on human CD4$^+$CD25$^+$ Tregs, especially under inflammatory conditions, are still scarce. Therefore, the current challenge is to investigate what role CD4$^+$CD25$^+$ Tregs play in human disease and whether these cells can be harnessed to limit and perhaps reverse existing immune pathology.

2a. Induced regulatory T cells: Tr1 and Th3 cells

In addition to the well-established role of natural CD4$^+$CD25$^+$ Tregs in the maintenance of tolerance to self-antigens, there is accumulating evidence for distinct populations of Tregs induced in the periphery after encounter with pathogens and foreign antigens. A variety of in vitro and in vivo protocols have been used to generate these “induced” Tregs (iTregs) [27]; in general they are generated in “tolerogenic” or anti-inflammatory contexts [28,29] and produce high amounts of inhibitory cytokines, IL-10 and/or TGF-ß, and exhibit suppressive activity that depends on these cytokines. These antigen-

specific iTregs include type 1 Tr cells (Tr1) that secrete high levels of IL-10, with or without IL-5, IL-13 or TGF-ß, but little or no IL-2, IL-4 and IFN-ϒ [30], and Th3 cells which secrete high levels of TGF-ß [31,32]. The principal difference between Th1/Th2 cells and Tr1/Th3 cells is that Th1/Th2 cells function only in response to antigen, whereas Tr1/Th3 cells need to be activated by antigen, but once activated, exert their suppressive function independent of the antigen. Th1/Th2 cells are responsible for host defense against bacteria and intracellular infections, while Tr1/Th3 cells act as feedback regulators of Th1/Th2 cells. Indeed, recent published data showed that Tr1 cells, like $CD4^+CD25^+$ nTregs are able to suppress autoimmune diseases after adoptive transfer in vivo [33]. Although all Treg subsets inhibit both Th1 cells and Th2 cells, cytokines produced by the Th1 and Th2 subsets inhibit each other.

$CD4^+CD25^+$ Tregs and Tr1: distinct T cell lineages?

Whether $CD4^+CD25^+$ Tregs and Tr1 cells are derived from distinct T cell lineages is still a matter of debate. In the last few years a considerable amount of progress has been made into the phenotypic characterization of both types of Tregs. However, it was recognized by most workers in the field that CD25 expression, as well as the expression of CTLA4, GITR, CD62L, CD103 and CD45RO, which are all indirect indices of T cell activation, are unsatisfactory markers for Tregs. Furthermore, gene expression analyses have shown that these markers are all constitutively expressed on both Tr1-clones and $CD4^+CD25^+$ Tregs. Thus, no specific surface marker has been identified thus far that is uniquely associated with either one of the regulatory subtypes or is even able to distinguish between $CD4^+CD25^+$ Tregs and conventional T cells that are activated. The differentiation between Tr1 cells and $CD4^+CD25^+$ Tregs is nowadays, as good as it gets, made upon only two distinctions. First, Tr1 cells are recognized by the very defined cytokine profile described earlier and $CD4^+CD25^+$ Tregs by the expression of the transcription factor FoxP3. Second, Tr1/Th3 cells are believed to be generated from naïve T cells in the periphery after encounter with antigen, while naturally occurring $CD4^+CD25^+$ Tregs are believed to develop in the thymus. While until now it was generally believed that these differences confirm a separate lineage origin, very recent data showed that the two subsets have more in common than originally thought (see below). Thus the question how or if the two subsets are related is still open.

2b. Induced regulatory T cells: peripheral acquisition of mRNA FoxP3

Although it is now well established that at least some of the naturally arising Tregs are generated in the thymus as a separate lineage, recent evidence suggest that $CD4^+CD25^+$ Tregs may also be induced in the periphery. First it was shown that retroviral overexpression of FoxP3 into non-regulatory $CD4^+CD25^-$ or $CD8^+$ T cells induces a functional phenotype similar to $CD4^+CD25^+$ nTregs possessing potent suppressive functions in vitro and in vivo [21]. Subsequently, two groups showed

that when peripheral $CD4^+CD25^-$ T cells were stimulated in vitro with plate-bound anti-CD3 and anti-CD28 mAbs in the presence of TGF-ß they acquired FoxP3 expression and regulatory function [34-36]. Together, these data highly suggest that mRNA FoxP3 expression is not restricted to thymic derived Tregs and that $CD4^+CD25^+$ Tregs, like Tr1 cells, can indeed be generated in the periphery. It remains to be determined, however, whether such a process operates in vivo under "normal" physiological conditions, as well.

It's all a matter of balance

A great deal of effort and energy goes on in the immune system to maintain the exquisite balance of activation on the one hand and regulation on the other. Too much activity may result in chronic inflammation or autoimmunity while too much regulation may result in chronic infection or tumor growth. The result of this exquisite balance is a sensitive system of checks and balances that generates an immune response that is fast, appropriate, effective, and –in a properly functioning immune system- self-limiting. It is now widely believed that the regulatory site of the balance is mainly represented by the two types of regulatory cells described above.

Autoimmune disease: an immune deficiency

A major part of the balancing act, orchestrated by regulatory cells, is to prevent potentially hazardous self-reactive T and B-lymphocytes, present in the periphery of every individual, from reacting to self-antigens and cause autoimmune disease. That the reduction in the number of Tregs predisposes to the development of autoimmune disease has extensively been shown in animal models by Sakaguchi and co-workers. As described above these authors showed that reconstitution of nude mice through the infusion of CD4 cells depleted of $CD4^+CD25^+$ T cells results in the development of organ-specific autoimmune disease such as insulitis, gastritis and thyroiditis [9,37], indicating a direct relation between defective number of Tregs and autoimmune attack. Causal or predisposing factors to autoimmune disease may therefore be found in any genetic abnormality or environmental insult that can tip the balance between Tregs and self-reactive T cells toward the dominance of the latter. The specificity and intensity of the developing autoimmune disease may subsequently depend on the degree of deficiency or dysfunction of Tregs (or the balance between Tregs and self-reactive T cells) and on the host genes, including MHC and non-MHC genes [38].
When in autoimmunity either a deficient generation, or reduced effector function of Tregs plays a role in the development of the disease, new therapeutic strategies may be found in either boosting Treg cell activity or stimulating Treg cell induction. Important questions, like how the regulatory T cell pool is maintained under physiological conditions and what mechanisms cause the regulatory T cell pool to shrink, expand or become (de)activated when necessary, than first need to be answered. In other words, what regulates the regulators? And under which circumstances do these mechanisms fail and lead to the development of autoimmune disease? And in case of a breakthrough of tolerance, leading to autoimmunity, how can we restore the regulatory processes?

Juvenile Idiopathic Arthritis

Juvenile Idiopathic Arthritis (JIA) is the most common rheumatic disease of childhood [39]. Local inflammation in the joints results in joint destruction and overall, an estimated 49% of affected children end up with severe functional limitation because of JIA [40]. For two major reasons, JIA lends itself, better than most other human diseases, to basic research in immunoregulation. First, due to the local inflammation in the joints and the accompanying increase in the volume of synovial fluid (SF) it is possible to obtain cells from the inflammatory site. Second, JIA is not a homogenous disease but consists of various well-defined subtypes with striking differences in both severity and outcome [41], allowing us to correlate the findings in vitro with the clinical outcome in vivo. The different subtypes of JIA, based on the type of onset of disease, are currently classified according to The International League Against Rheumatism (ILAR) criteria [41-43]. Three major disease phenotypes are studied in this thesis: (a) oligoarthritis; (b) polyarthritis; (c) systemic-onset disease [41]. Though the histopathological abnormalities found in these three subtypes seem similar there is a striking difference in both severity and outcome of the three subtypes. In oligoarticular JIA the disease has a relative benign course; the disease is restricted to one to four joints, is often self-remitting and sometimes even self-limiting. In polyarticular- and systemic JIA five or more joints are affected and in contrast to oligoarticular JIA, polyarticular and systemic JIA are usually non-remitting and crippling diseases requiring aggressive immunosuppressive therapy.

Regulatory immune components in remitting JIA

Due to the self-limiting and remitting character of the inflammatory process, pers-OA JIA has a unique place among all human autoimmune diseases. The self-limitation of an autoimmune process is often seen in experimental animal models of autoimmune diseases but hardly ever in humans. Why oligoarticular JIA is a more benign, self-remitting disease and which (immune) regulatory mechanisms play a role in controlling the inflammation is still unknown, although several mechanisms with supposedly immune suppressive capacity have been described [44-46].

Th2 counter-regulation in remitting JIA

It is believed that in JIA, T cell activation results in a cascade of events leading to tissue damage in joints and other affected tissues, including B-cell activation, complement consumption, and, in particular, release of interleukin-6 (IL-6) [47], tumor necrosis factor-α (TNF-α), and other pro-inflammatory cytokines [48]. Thus, the synovial lymphocyte infiltrate in JIA exists predominantly of Th1 type cells [49,49] and like Rheumatoid Arthritis (RA) is therefore believed to be the result of a polarization towards a persistent pro-inflammatory Th1 response.
Despite this strong type 1 phenotype in JIA, variable immune components that favor downregulation of inflammation can be present, especially in early oligoarticular disease. In particular, the presence of a Th2 component, in terms of type 2 cytokines (IL-4 and IL-10) and expression of chemokine receptors [44], early in the disease process of JIA is suggested to function in an anti-inflammatory capacity and to correlate to a favorable prognosis [44,50,51].

Regulatory T cells in remitting JIA

In addition to the above-described Th2 components, T cells with specialized regulatory capacity have been suggested to play a role in JIA. Thompson et al. demonstrated equally increased levels of CCR5 and CCR4 bearing synovial fluid lymphocytes, the latter producing greater IL-4 than IFN-ϒ [44]. Increased expression of CCR5 has been associated with a type 1 cytokine profile and is dependent on recent activation events. CCR4 is preferentially expressed on Th2 like cells and is therefore suggested to be a chemokine receptor for regulatory cells. In addition, in previous work by our group, lymphocytes reactive to human heat shock protein 60 (HSP60) have been suggested to play a regulatory role in JIA (see below).

Heat shock proteins (HSPs)

Ubiquitous and abundant proteins, essential for cellular viability [52] and present in cells of almost all living organisms. They are induced when a cell undergoes various types of environmental stresses like heat, cold and oxygen deprivation. HSPs are highly conserved during evolution, which has resulted in extensive amino acid sequence identities between mammalian and microbial HSPs.

Heat shock protein 60 and immune regulation

Nowadays evidence is accumulating that T cells with specificity for self HSPs may serve an immunoregulatory function and play a role in the down regulation of pathogenic, inflammatory processes. Most evidence for a protective role for HSP60-specific T cells came from the experimental animal model Adjuvant Arthritis (AA). In this model, preimmunization with mycobacterial HSP60 protected animals from the development of arthritis. This regulation has shown to be mediated by regulatory T cells cross-reactive with the self-60 kDa heat shock protein (HSP60) and capable of downregulating inflammation [53,54].

JIA shares many features with the experimental animal model AA. Besides a clinical and histopathological resemblance evidence is growing that HSPs play a central role in the immuneregulation of JIA as well. Synovial lining cells of patients with JIA show an increased expression of endogenously produced HSP60 [55]. This endogenous HSP60 is a target for the immune system as clearly shown by the detection of IgG antibodies and T cell reactivity to human as well as mycobacterial HSP60 in both serum and synovial fluid from patients with JIA [56-58]. Also T cell reactivity to other HSPs, in particular to E. Coli derived HSP65 (GroEL) [59], and to DnaJ [60] is seen. Since most of the T cell responses to human HSP60 can be found in HLA B27 negative oligoarticular JIA, the subtype of JIA with the best prognosis, it was suggested that self-HSP60 specific T cells contribute to regulatory mechanisms that down-regulate inflammation [61]. Further characterization of phenotype, cytokine profile and mRNA FoxP3 expression of HSP60 specific T cells is now needed to evaluate whether these cells are a new category of regulatory T cells or can be classified as part of the earlier described subsets (see above).

Current therapies for JIA

The treatment of JIA is to a large extent dependent on the disease subtype, and combines drug use with physical and occupational therapy [62,63]. In general the initial treatment of JIA consists of non-steroidal anti-inflammatory drugs (NSAIDs). NSAIDs, with or without the treatment with intraarticular corticosteroid injections, especially used in oligoarticular JIA, may induce disease remission in as many as 60% of patients [64,65]. These modalities are usually not adequate for treating patients with polyarticular or systemic-onset JIA. Patients in whom polyarthritis develops are usually treated with methotrexate [66,67]. However, only two-thirds of the patients with polyarticular JIA and even a smaller proportion of patients with systemic onset JIA respond to this therapy [66,67]. Furthermore, methotrexate is not always well tolerated and has only a moderate effect on the prevention of disability [68]. Other disease-modifying or immunosuppressive drugs often are less effective or are not tolerated. The use of systemic corticosteroids must be restricted because of their adverse effects, particularly on growth.
For the treatment resistant polyarticular and systemic JIA patients, not responding to either of the now described therapies, two new advances in the treatment of arthritis emerged recently: anti-TNFα therapy and autologous stem cell transplantation.

Anti-TNFα therapy

TNFα is a proinflammatory cytokine and induces many effects in the inflammatory process that eventually cause cartilage damage and joint destruction. Two therapeutic approaches were adopted to reduce TNF activity in arthritis: anti-TNF monoclonal antibodies (infliximab, Remicade) [69] and a genetically engineered soluble TNF receptor (etanercept, Enbrel) [70]. Interestingly, anti-TNFα therapy does not only bind and inactivate TNFα and lymphotoxin-a, it has also shown to restore peripheral blood $CD4^+CD25^+$ Treg frequencies and the capacity of Tregs to inhibit cytokine production in RA [71].
The first clinical results of anti-TNFα therapy were promising [70,72]. In RA, infliximab as well as etanercept have shown to be effective and well tolerated. In JIA, most experience exists with the use of etanercept. Results of a multicenter pediatric trial performed in the US demonstrated that etanercept was effective and well tolerated in 74% of children with JIA and polyarthritis, regardless of the type of disease onset [70]. However, to date, having 5 years experience with the use of anti-TNFα therapy in JIA and RA, serious concerns are evolving regarding the long-term safety of the therapy. An increasing number of studies now report that the long-term treatment of arthritis with etanercept and infliximab may be associated with a wide spectrum of severe side effects, including pancytopenia, severe infection, neurologic or psychiatric disorders and systemic lupus like symptoms [73,74].

Autologous stem cell transplantation

Altogether, 5-10% of the children with the systemic and polyarticular onset forms of JIA are treatment-resistant or non-tolerant to conventional therapy, including anti-TNFα therapy. In such cases, patients suffer considerable morbidity from the disease itself but also from serious adverse effects caused by the long-term treatment with the immunosuppressive drugs. For this group of children autologous stem cell transplantation (ASCT) has emerged as an alternative treatment option. The use of intensive

immunosuppressive treatment coupled with (ASCT) to treat human autoimmune diseases (AID) follows anecdotal observations of responses of AID in survivors of hematopoietic stem cell transplants for malignancy [75-77] and extensive animal work describing the treatment of AID with ASCT [78-80]. The first results of a pilot study conducted in 1997 and 1998 to the clinical efficacy of ASCT for JIA were promising [81] and since than more than 40-50 children have been transplanted worldwide. More extensive and long-term studies should now be performed to give better insight in the cost-benefit ratio, especially in terms of survival and the sustainability of the clinical effect. Furthermore, while clinical experience with this relatively new treatment is rapidly accumulating, still very little is known about which changes in the immune system induced by ASCT are responsible for the favorable effect. Combining careful clinical follow-up studies of JIA patients treated with ASCT and detailed immunological investigations may, in fact, offer a unique opportunity to improve our understanding both of ASCT and of the pathogenesis of the disease.

Aim of the study

The present way to control the unwanted autoreactive immune response in JIA -and other autoimmune diseases- is with immunosuppressive drugs, that operate by penalising the whole immune system even though only a small number of autoreactive T cells (and B-cells and macrophages) are actually responsible for the tissue damage. Furthermore, though autologous stem cell transplantation may hypothetically offer a cure, all other available drugs mostly have to be given for a prolonged time, since stopping the drug will result in the full recurrence of the disease. This means that the physician has to carefully balance the dose of drug against the risk of infection, cancer and other unwanted side effects.
It would be preferable to control the inflammation more specifically either by controlling just those cohorts of lymphocytes that are responsible, leaving others free to handle infectious agents normally, or by enforcing the regulatory mechanisms that are supposed to keep them silenced. Indeed, in several animal models it has now been shown that the induction of regulatory T cells by autoantigens can suppress disease, even if the primary, initiating autoantigens are unknown and if inflammation is progressive. That the same mechanisms may apply in human is highly suggested by the results of a Phase I clinical trial in which patients with early rheumatoid arthritis were treated orally with a peptide of the heat shock protein dnaJ (dnaJP1): This treatment induced an intriguing change from proinflammatory to regulatory T cell function [82]. Together, these data suggest that boosting the (intrinsic) activity and/or induction of regulatory T cells may offer a promising treatment strategy for autoimmune related illnesses and hopefully substituting for conventional Ag-nonspecific immunosuppression with all of its hazards. Thus far, studies on human regulatory T cells, especially in pathological situations, have been limited. Therefore the current challenge is to investigate what role the different types of regulatory cells play in the prevention of human autoimmune diseases and whether these cells can limit and perhaps reverse existing immune pathology. New and promising therapeutic strategies might than be found in inducing these regulatory cells in order to re-educate the immunesystem.

Outline of the thesis

In this study we hoped to acquire more insight on the involvement of regulatory T cells in JIA. Knowledge on these cells may be pivotal in designing the next generation of immune regulatory approaches for JIA. The study itself can be subdivided in two parts. The first part identifies what types of regulatory T cells are involved in the inflammatory process in JIA (chapter 2 and 3), how these cells can be induced and/or modulated and whether HSP60 can play a role in this (chapter 4). The second part involves an evaluation of the effectiveness of ASCT as the only curative treatment nowadays available for JIA (chapter 5) and identifies two mechanisms, including regulatory T cells that play a major role in the induction of tolerance by this treatment.

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2 Human HSP60 induces CD30 expressing T cells capable of producing the regulatory cytokine IL-10 in the remitting form of juvenile idiopathic arthritis

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Arthritis and Rheumatism 2003 July; 48(7):2001-2010

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Summary

Objective: To test the hypothesis that T cell reactivity to self heat-shock protein 60 (HSP60) in patients with remitting Juvenile Idiopathic Arthritis (JIA) is part of an anti-inflammatory, regulatory mechanism.
Methods: Using peripheral blood-derived mononuclear cells (PBMCs) and synovial fluid-derived mononuclear cells (SFMCs) obtained from patients with JIA, we analysed the expression of CD30 and the induction of regulatory cytokines in response to human and mycobacterial HSP60.
Results: In oligoarticular JIA patients, in vitro activation of PBMCs and SFMCs with HSP60 induced a high expression of CD30 on $CD4^+$, activated (HLA-DR^+), memory ($CD45RO^+$) T cells. The expression of CD30 induced by human HSP60 was much higher than that induced by mycobacterial HSP60. In oligoarticular JIA patients with active disease, the expression of CD30 in response to human HSP60 was paralleled by a high interleukin-10 (IL-10):interferon-ϒ (IFN-ϒ) ratio. In addition, restimulated human HSP60-specific T cell lines of oligoarticular JIA patients showed a high production of IL-10 and a low production of IFN-ϒ. In contrast, PBMCs and SFMCs from polyarticular JIA patients responded to human HSP60 with virtually no expression of CD30 and a low IL-10:IFN-ϒ ratio.
Conclusion: The results show that T cells responding to human HSP60 in oligoarticular JIA patients express CD30, and during active phases of the disease, these T cells have a cytokine profile with a high IL-10:IFN-ϒ ratio. These findings suggest that in oligo-articular JIA patients, human HSP60-specific $CD4^+$ cells have a regulatory function and contribute to disease remission.

Introduction

Juvenile Idiopathic Arthritis (JIA) is one of the most common rheumatic diseases of children and a major cause of chronic disability. Different subtypes of JIA can be distinguished on the basis of clinical parameters at the onset of disease [1]. Although the histopathological abnormalities found in the different subtypes seem to be similar, there is a striking difference in severity and clinical course. Oligoarticular-onset JIA has a relative benign clinical course, whereas polyarticular and systemic-onset types often are nonremitting and disabling diseases requiring aggressive immunosuppressive treatment [2,3].
It is still not known why oligoarticular JIA is a more benign, self-remitting disease and which (immune) regulatory mechanisms play a role in controlling the inflammation, although some evidence suggests that regulatory $CD4^+$ T cells play a key role [4,5].
In recent years, it has become evident that peripheral or postthymic tolerance is mediated, at least in part, by various types of regulatory $CD4^+$ T cells [6]. The regulatory T cells are now classified as naturally occurring regulatory T cells, which are enriched in the $CD4^+CD25^+$ population [7] and as several induced populations, including Th2, Th3 [8], Tr1 [9], and anergic cells [10]. All regulatory cells can actively inhibit immunopathological responses, either by direct cell-cell contact or by the production of inhibitory cytokines [6]. In particular, interleukin 10 (IL-10) and transforming growth factor ß have been reported to play a role in mediating the suppressive function of T regulatory cells [9,11,12]. Although much progress has been made, many key aspects of regulatory T cells in human diseases still remain to be resolved, particularly concerning their antigen specificity, mechanisms of action and interrelationship.
In several experimental autoimmune models, T cells responding to heat-shock proteins (HSPs) play an important role in the regulation of peripheral tolerance and suppressing pathogenic immune responses [13]. HSPs are evolutionary highly conserved proteins that are present in the cells of virtually all living organisms and which play an important role in various cell processes [14,15]. The production of HSPs is subjected to various forms of cellular stress [16,17]. Most evidence for a regulatory role of HSP-specific T cells has been gathered in experimental models of arthritis. In almost all models of experimentally induced arthritis preimmunization with HSPs protected animals from arthritis [18-20]. In the adjuvant-induced arthritis (AA) model, it was shown that the protection resulted from the induction of self HSP60-cross-reactive T cells capable of downregulating inflammation [21].
The findings in AA raised the question of whether similar regulatory mechanisms could play a role in oligoarticular JIA. In previous studies, we showed an increased expression of endogenously produced HSP60 in the membranes of synovial lining cells of patients with JIA [22]. Subsequently, T cell reactivity to mycobacterial and human HSP60 was documented in patients with JIA [23,24]. In a prospective follow-up study in newly diagnosed patients with JIA, we showed that this T cell reactivity occurred very early in the course of oligoarticular JIA, and that patients lacking early responsiveness to HSP60 later developed a polyarticular course of the disease [25]. Altogether these

data suggested that in patients with oligoarticular JIA, T cells responding to human HSP60 exert a regulatory function and subsequently contribute to disease remission. To better understand the mechanisms of this apparent T cell control we studied in a group of oligoarticular JIA patients and a control group of patients with polyarticular JIA the phenotypic characteristics and the production of cytokines by $CD4^+$ T cells responding to human HSP60. We found that human HSP60 induced the expression of the Th2 marker CD30 on $CD4^+$ T cells and a high IL-10/IFN-ϒ cytokine ratio in oligoarticular JIA patients but not in polyarticular patients. These findings strongly support the hypothesis that in oligoarticular patients human HSP60 specific $CD4^+$ cells are regulatory cells, contributing to disease remission.

Patients and methods

Patients

Forty patients with JIA with a persistent oligoarticular course and 36 control patients with polyarticular JIA were evaluated for this study. Patients were included from both the Netherlands (University Medical Center Utrecht, Wilhelmina Children's Hospital, Utrecht, the Netherlands) and Germany (Kinder und Rheumaklinik, Garmisch Partenkirchen, Germany). The diagnosis of the patients was defined according to the revised criteria for JIA [1]. Active disease was defined by the presence of joint swelling or limitation of movement with either pain on movement or tenderness. Non-active disease was defined by the absence of joint swelling or limitation of movement with either pain on movement or tenderness. The general characteristics of the patients and the controls are shown in **Table 1.**

Blood samples were obtained by venepuncture. From 15 oligoarticular JIA patients and 2 polyarticular JIA patients synovial fluid (SF) was obtained at the time of a diagnostic or therapeutic joint aspiration. Due to the small size of the samples available per patient, not all experiments could be performed on samples from all patients. Blood samples and SF from the German patients were both immediately sent to Utrecht by mail courier and analysed within 24 hours. Lymphocytes from both peripheral blood (PB) and SF were isolated directly after being obtained from the patients. Informed consent was obtained either from parents/guardians or from the children directly when they were >12 years old.

Tabel 1

Clinical characteristics of the patient population*

	Oligoarticular JIA patients (n = 40)	Polyarticular JIA patients (n = 36)
No. male/no. female	16/24	9/27
Age, median years	10.1	9.5
Disease duration, median years	3.0	5.0
No. ANA positive†	20	19
No. RF positive‡	0	6

* JIA = Juvenile Idiopathic Arthritis; ANA = antinuclear antibody; RF= Rheumatoid Factor

† Determined by immunofluorescence on Hep-2 cells

‡ Determined by Rose-Waaler test and latex agglutination

SF and PB direct culture assays

From heparinized PB and SF of patients with oligoarticular and polyarticular JIA, mononuclear cells were isolated using Ficoll Isopaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden). The cells (2 x 10^5 cells/well) were cultured in 96-well round-bottom plates in RPMI 1640, containing 10% heat-inactivated (for 30 minutes at 56ºC) human AB serum, 100 µg/ml penicillin, 100 U/ml streptomycin, 200 mg/ml glutamine (Gibco BRL, Gaithersburg, MD USA) and 2 x 10^{-5} M 2-mercaptoethanol (2-ME). The cultures were stimulated with medium only, 10 µg/ml purified recombinant mycobacterial HSP60 (Faculty of Veterinarian Medicine, Utrecht University, Utrecht, The Netherlands), 10 µg/ml human HSP60 [22] (Stressgen, Victoria, BC Canada), 1.5 µg/ml tetanus toxoid or 1.5 µg/ml diphtheria toxoid. Concanavalline A (Con A), (2.5 µg/ml, Calbiochem, La Jolla, CA) was used as a mitogen. The cells were cultured for 7 days at 37ºC in 5% CO_2 with 100% relative humidity. After 96 hours of culture, supernatants were collected and replaced with fresh medium.

Short-term T cell lines

From PB- and SF-derived lymphocytes, short-term cell lines were set up as described before [26]. Brief-ly, PB-derived mononuclear cells (PBMCs) and SF-derived mononuclear cells (SFMCs) were cultured at 2 x 10^5 cells/well in 96-well round-bottom plates in RPMI 1640, containing 10% heat-inactivated autologous serum, 100 µg/ml penicillin, 100 U/ml streptomycin, 200 µg/ml glutamine and 2 x 10^{-5} M 2-ME. The cultures were stimulated with 10 µg/ml human HSP60 for 3 days. On days 3, 6 and 9, half of the used medium was replaced with fresh medium containing 10% autologous serum, 10 units/ml of recombinant IL-2 and 10 units/ml of recombinant IL-4. On day 13, the cells were counted and tested for expression of CD30 and other cell surface markers as described below. In addition, aliquots of 2 x 10^4 cells were incubated for 24 hours with human HSP60 or medium only and 2 x 10^4 irradiated (4,000 rads) autologous PBMCs as antigen-presenting cells. Following this restimulation, the cells were harvested and evaluated for surface staining and the intracellular production of cytokines.

Lymphocyte cell surface markers

At different time points following culture, PBMCs and SFMCs were washed twice in fluorescence-activated cell sorting (FACS) buffer (phosphate buffered saline [PBS] containing 2% fetal calf serum [FCS] and 0.1% NaN_3). Subsequently, PBMCs or SFMCs (0,5-1 x 10^6) were incubated for 30 min at 4ºC in 100 ml PBS containing 10% FCS in order to reduce aspecific binding. The cells were then incubated in 50 ml FACS buffer containing appropriately diluted phycoerythrin (PE)-, fluorescencein isothiocyanate (FITC)- or Cy-Chrome (CY)-labeled monoclonal antibodies (mAb) against human CD4, CD25, HLA-DR, CD45RA, CD45RO and CD30. CD30 mAb was obtained from DAKO (Glostrup, Denmark). All other mAbs were obtained from Becton Dickinson (San Jose, CA, USA). Stained mononuclear cells were diluted in sheath fluid and run on a FACStar Plus flow cytometer (Becton Dickinson). CellQuest software (BD Biosciences, San Jose, CA) was used for analysis.

Cytokine analysis by lymphocyte intracellular staining and flowcytometry

T cell lines were generated as described above and restimulated for 24 hours with human HSP60 or medium only. During the last 4 hrs of culture, Golgistop (BD

Biosciences) was added (2uM final concentration). The cells were harvested, washed in cold PBS with 2% FCS, blocked in PBS with 10% FCS for 20 minutes at 4ºC, washed twice, and stained with CY-conjugated anti-CD4 for 20 minutes at 4ºC. Subsequently, the cells were fixed in Cytofix/Cytoperm solution (BD Biosciences) for 20 minutes at 4ºC and washed twice in Perm/Wash solution (BD Biosciences). After the second wash the cells were resuspended in 50 ml Perm/Wash solution containing a predetermined optimal concentration of PE-conjugated anti-IL-10, PE-conjugated anti-IL-4 and FITC-conjugated anti-IFN-ϒ. After incubation for 30 minutes at 4ºC, cells were washed twice. Cells were analysed as described above.

Analysis of cytokines and soluble CD30 (sCD30) by enzyme-linked immunosorbent assay (ELISA)

PB and SF levels of sCD30 were assayed with a commercially available ELISA kit (Ki-1 antigen ELISA; Bender MedSystems, Vienna, Austria) according to the manufacturer's instructions. Cytokines were measured after activation of the lymphocytes in a lymphocyte activation assay as described above. In preliminary experiments, optimal timing for activation was determined. After 96 hours, the supernatants of the cell cultures were collected and stored at -80°C.

Levels of IL-4, IL-10, and IFN-ϒ were determined using sandwich ELISAs. IL-4 was measured using a solid-phase sandwich ELISA (BioSource Europe, Nivelles, Belgium) according to the manufacturer's instructions. IL-10 and IFN-ϒ ELISA kits were purchased from Sanguin (Amsterdam, The Netherlands). Briefly, 96-well polystyrene microtiter plates were coated with a specific mAb. Standards and samples were added after the plates were blocked and incubated overnight at 4°C. After washing, a specific biotinylated antibody was added to all wells and incubated for 1 hour at room temperature. Thereafter, the plates were washed and incubated for 30 minutes with horseradish peroxidase (HRP)-conjugated streptavidin. After removal of nonbound HRP conjugate by washing, 3,3′ ,5,5′ -tetramethylbenzidine substrate reagent solution (ICN Biomedicals, Aurora, OH) was added to the wells. The reaction was terminated by addition of 1.8M sulfuric acid.

Absorbencies of all ELISAs were read at 450 nm against air using a Milenia microtiter plate reader (Diagnostic Products Corporation Nederland, Breda, The Netherlands). From the absorbencies of samples and those of the standard curve, the concentrations of the cytokines and of sCD30 were determined by interpolation with the standard curve using Elisa Plus software version 3.01 (Meddata, New York, NY).

Statistics

Basic descriptive statistics were used to describe the patient population. A Mann-Whitney U test was used to compare CD30 expression and cytokine production between oligoarticular and polyarticular JIA patients and between patients with active disease and those with nonactive disease and to compare the difference in CD30 expression between SFMCs and PBMCs. A paired t-test was used to compare CD30 expression in response to mycobacterial and human HSP60. Correlations between the expression of CD30 in response to human HSP60 and age, sex, the presence of antinuclear antibody and rheumatoid factor, and medication were calculated using Spearman's rank correlation coefficient.

Expression of CD30 on CD4+ T lymphocytes and levels of sCD30 in PB and SF from patients with oligoarticular- or polyarticular-onset JIA

The expression of CD30 was determined on freshly isolated PBMCs from 12 oligoarticular and 7 polyarticular JIA patients and on SFMCs from 12 oligoarticular and 2 polyarticular JIA patients with active disease. Levels of sCD30 were measured in the serum of 12 oligoarticular and 14 polyarticular JIA patients (both with disease in remission and with active disease) and in the SF from 8 oligoarticular JIA patients with active disease. The results are shown in **Table 2**. The expression of CD30 on $CD4^+$ T cells was low on PBMCs from both oligoarticular and polyarticular JIA patients and did not differ between the two groups. In oligoarticular JIA patients, the expression of CD30 on $CD4^+$ T cells was significantly higher on SFMCs than on PBMCs. SF was available from only 2 polyarticular JIA patients. In both of these patients, CD30 expression on $CD4^+$ T cells was <0.5%.
No significant difference in mean ± SEM PB sCD30 levels was found between polyarticular JIA patients (167 ± 45 units/ml) and oligoarticular JIA patients (119 ± 34 units/ml), between oligoarticular JIA patients with disease in remission (106 ± 13.0 units/ml) and those with active disease (128 ± 11 units/ml), or between polyarticular JIA patients with disease in remission (171 ± 28.8 units/ml) and those with active disease (163 ± 30.1 units/ml). In oligoarticular JIA patients with active disease, SF sCD30 levels were significantly higher than PB sCD30 levels ($P < 0.05$). No SF from polyarticular JIA patients was available to measure levels of sCD30.

Table 2

Percentages of $CD4^+$ cells coexpressing CD30 and levels of soluble CD30 (sCD30) in peripheral blood (PB) and synovial fluid (SF) from patients with polyarticular and oligoarticular juvenile idiopathic arthritis (JIA)*

	Polyarticular JIA patients		**Oligoarticular JIA patients**	
	PB	SF	PB	SF
CD30+CD4+ (%)	0.1 ± 0.02	0.04 ± 0.05†	0.2 ± 0.04	1.4 ± 0.57‡
SCD30 (units/ml)	167 ± 45	-	119 ± 34	218 ± 76.2ξ

* Values are the mean ± SEM.

† Only 2 patients

‡ $P < 0.05$ versus PB mononuclear cells from oligoarticular JIA patients.

ξ $P < 0.05$ versus PB from oligoarticular JIA patients.

Expression of CD30 on CD4+ T lymphocytes in PB from patients with oligoarticular or polyarticular JIA and in SF from patients with oligoarticular JIA after in vitro activation with human HSP60 in a short-term cell line

PBMCs from 14 oligoarticular JIA patients and 7 polyarticular JIA patients were activated in vitro with human HSP60 (10 µg/ml) in a short-term cell line, as described in Patients and Methods. CD30 expression on $CD4^+$ T cells was determined before (on day 0) and after 2 rounds of activation in vitro with HSP60 in the presence of IL-2 and IL-4 (on day 13). The results are shown in **Fig. 1a.** After PBMCs were activated with human HSP60, a mean ± SEM 10.5 ± 1.8% increase in $CD4^+CD30^+$ cells compared with unactivated PBMCs ($P < 0.05$) was found in patients with oligoarticular JIA. In polyarticular JIA patients, activation with human HSP60 led to only a slight increase (mean ± SEM 1.2 ± 0.5%) in CD30 expression on PBMCs ($CD4^+CD30^+$ cells). The difference in CD30 expression on $CD4^+$ lymphocytes after in vitro activation with human HSP60 between oligoarticular and polyarticular JIA patients was significant ($P < 0.05$). In addition, SFMCs from 11 patients with active oligoarticular JIA were activated in vitro with human HSP60 (10 µg/ml) in a short-term cell line as described in Patients and Methods. No SF from polyarticular JIA patients was available for this experiment. CD30 expression on $CD4^+$ lymphocytes was determined before and after activation in vitro with human HSP60. The results are shown in **Fig. 1b.** After activation with human HSP60, a mean ± SEM 16.8 ± 6.6% increase in $CD4^+CD30^+$ cells compared with unactivated SFMCs ($P < 0.05$) was found in patients with oligoarticular JIA.

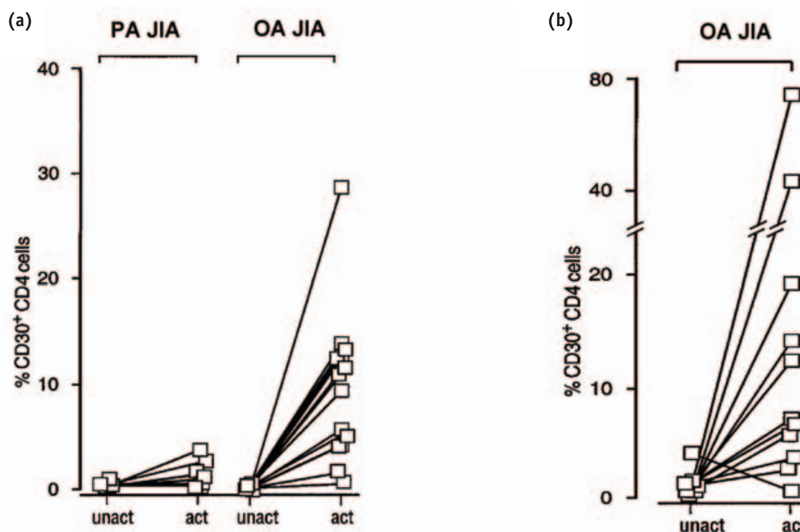

Figure 1. (a) Expression of CD30 on $CD4^+$ T cells of unactivated (unact) and activated (act) peripheral blood mononuclear cells (PBMCs) from patients with oligoarticular juvenile idiopathic arthritis (OA JIA) and patients with polyarticular JIA (PA JIA). **(b)** Expression of CD30 on $CD4^+$ T cells of unactivated and activated synovial fluid mononuclear cells (SFMCs) from patients with oligoarticular JIA. PBMCs and SFMCs were activated twice in vitro with human HSP60 (10 µg/ml) in a short-term cell line.

Difference in CD30 expression on $CD4^+$ T cells from oligoarticular JIA patients after in vitro activation with either mycobacterial or human HSP60.

PBMCs from 26 oligoarticular JIA patients (14 patients with active disease and 12 patients with disease in remission) were cultured in a direct assay (no restimulation) for 7 days with medium only, human HSP60, mycobacterial HSP60, or tetanus toxoid. Remarkably, in these direct culture assays, human HSP60 induced a significantly higher expression of CD30 on $CD4^+$ T lymphocytes than did mycobacterial HSP60 (mean ± SEM 2.5 ± 0.7% versus 0.9 ± 0.1%; $P < 0.05$) (**Fig. 2**). As could be expected, tetanus toxoid also led to up-regulation of CD30 on CD4+ T lymphocytes (6.3 ± 1.2%). However, the response to tetanus toxoid was not disease specific, since polyarticular JIA patients also responded to tetanus toxoid with up-regulation of CD30 (4.4 ± 1.0%).

The highest expression of CD30 in response to human HSP60 was found in oligoarticular JIA patients whose disease was in remission at the time their blood was obtained. These patients had a significantly greater percentage of CD4+CD30+ cells than did patients with active disease (3.9 ± 1.1% versus 1.2 ± 0.9%; $P < 0.05$).

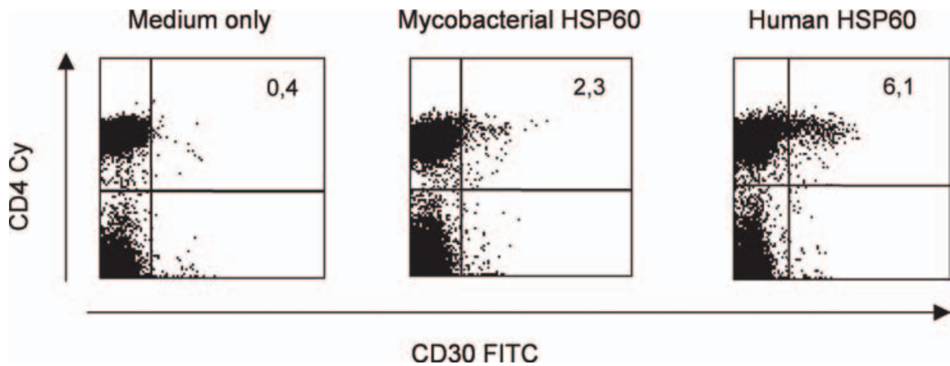

Figure 2. Difference in CD30 expression on $CD4^+$ lymphocytes in response to mycobacterial heat-shock protein 60 (HSP60) and human HSP60 in an oligoarticular JIA patient with remission of disease. PBMCs were cultured in vitro in the presence of mycobacterial HSP60 (10 µg/ml), or medium only. After 7 days, $CD4^+$ T cells were tested for the expression of CD30. In oligoarticular JIA patients, $CD4^+$ lymphocytes showed a significantly higher expression of CD30 in response to human HSP60 than they did in response to mycobacterial HSP60 ($P < 0.05$). CY = Cy-Chrome; FITC = fluorescein isothiocyanate (see Figure 1 for other definitions).

Phenotypic characteristics of $CD30^+$ lymphocytes from oligoarticular JIA patients

CD30 expression on PBMCs and SFMCs of 12 patients with oligoarticular JIA after in vitro activation was predominantly found on $CD4^{bright}$ lymphocytes (i.e., lymphocytes with a high expression of CD4). $CD4^{bright}$ lymphocytes are uniformly positive for CD45RO

(a marker for memory cells) and negative for CD45RA (a marker for naive cells), and they express the activation marker HLA-DR (**Fig. 3**). No difference in phenotype was found between SF and PB CD30-expressing lymphocytes.

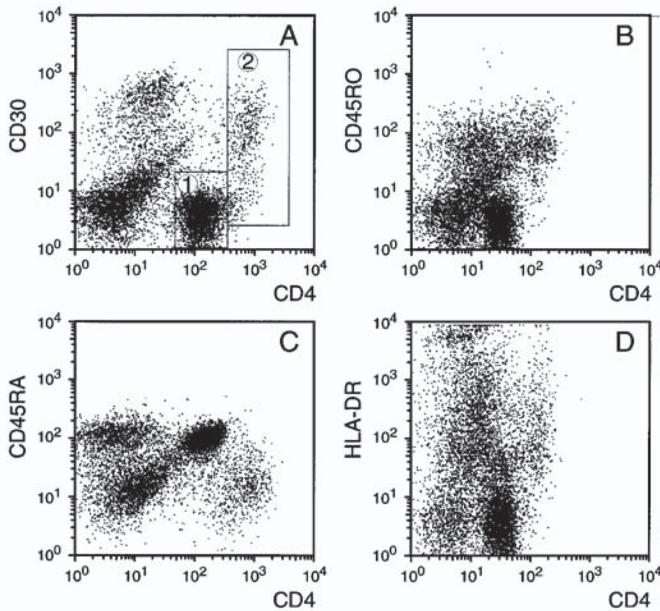

Figure 3. Phenotypic characteristics of CD30-expressing CD4+ T lymphocytes. Peripheral blood and synovial fluid lymphocytes from a patient with oligoarticular juvenile idiopathic arthritis were cultured for 13 days in a short-term cell line and activated in 2 rounds with human HSP60. Cells were subsequently stained with phycoerythrin (PE)-conjugated CD4 (A and C) or FITC-conjugated CD4 (B and D) combined with FITC-conjugated CD30 (A), PE-conjugated CD45RO (B), FITC-conjugated CD45RA (C), or PE-conjugated HLA-DR (D). For ease of comparison, CD4 fluorescence is always plotted on the x-axis. Within the CD4+ cells, a distinction between intermediate cells (gate 1) and bright cells (gate 2) can be made (A). See Figure 2 for other definitions.

Cytokine production induced by activation of PBMCs with human and mycobacterial HSP60 as measured by ELISA

From 20 patients with oligoarticular JIA (10 with active disease and 10 with disease in remission) and 23 patients with polyarticular JIA (13 with active disease and 10 with disease in remission), PBMCs were isolated and cultured in a direct assay as described in Patients and Methods. After culturing the cells for 96 hours with ConA, tetanus toxoid, mycobacterial HSP60, or human HSP60, supernatants of the cell cultures were collected, and production of IL-4, IL-10, and IFN-ϒ was measured by ELISA. IL-4 could be detected in none of the conditions (concentration <0.5 pg/ml). Mean ± SEM concentrations of IFN-ϒ and IL-10 induced by human HSP60 in oligoarticular and polyarticular JIA patients are shown in **Fig. 4a and 4b**, respectively. Both human and mycobacterial HSP60 induced the production of IL-10 as well as IFN-ϒ.

During remission of disease, the production of IFN-ϒ exceeded the production of IL-10 in both patient groups. However, in oligoarticular JIA patients with active disease, but not in polyarticular JIA patients with active disease, the IL-10:IFN-ϒ ratio was reversed in favor of IL-10. The difference in IFN-ϒ production between oligoarticular JIA patients with disease in remission and oligoarticular JIA patients with active disease approached statistical significance ($P < 0.06$). Tetanus toxoid, used as a positive control antigen, induced higher mean concentrations of IFN-ϒ and lower mean concentrations of IL-10 than did HSP60 (oligoarticular JIA patients: IL-10 23 pg/ml, IFN-ϒ 181 pg/ml; polyarticular JIA patients: IL-10 24 pg/ml, IFN-ϒ 217 pg/ml). Differences between the different patient groups in response to tetanus toxoid were not significant.

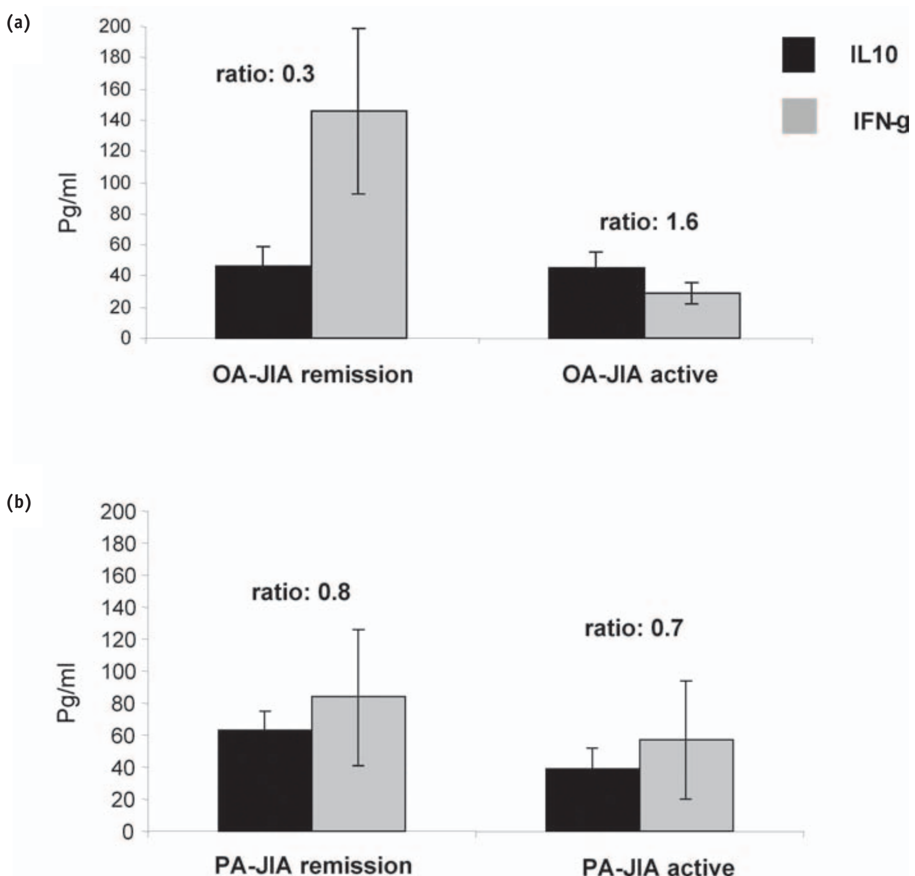

Figure 4. Interferon-ϒ (IFNϒ) and interleukin-10 (IL-10) production by mononuclear cells from patients with oligoarticular juvenile idiopathic arthritis (OA-JIA) and polyarticular JIA (PA-JIA) during active disease or disease remission. Mean and SEM cytokine levels were determined in supernatants of 96-hour cultures of cells stimulated with human heat-shock protein 60.

Cytokine production in short-term HSP60 T cell lines from patients with oligoarticular JIA as measured by intracellular staining and flowcytometry

From 4 patients with oligoarticular JIA in remission, a short-term T cell line from PB was generated. After restimulating the cells for 24 hours with human HSP60, intracellular cytokine staining for IL-10 and IFN-ϒ was performed. Restimulation of the cell lines with human HSP60 (10 mg/ml) in the presence of irradiated APCs induced in all 4 cell lines a high production of IL-10 (mean ± SEM 7.8 ± 2.2% increase in IL-10+CD4+ cells) and a low production of IFN-ϒ (mean ± SEM 1.7 ± 0.6% increase in IFN-ϒ+CD4+ cells). No IL-4 could be detected. An example is shown in **Fig. 5**.

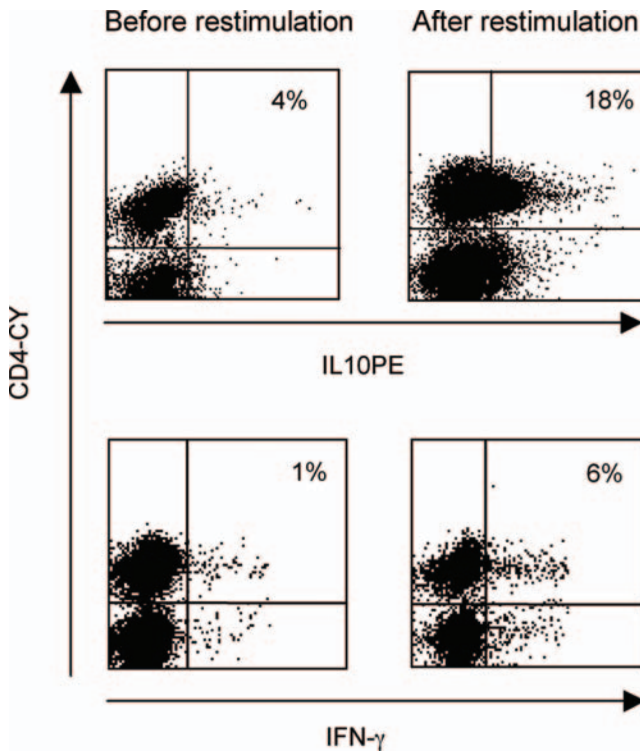

Figure 5. Intracellular IL-10 (upper panels) and IFN-ϒ (lower panels) in a representative T cell line from an oligoarticular JIA patient with remission of disease. Intracellular levels of IL-10 and IFN-ϒ were measured after restimulation for 24 hours with 10 μg/ml human HSP60. PE = phycoerythrin (see Figures 2 and 4 for other definitions).

Discussion

Previous studies suggested that T cell reactivity against self HSP60 in oligoarticular JIA patients was a part of the regulatory processes leading to down-regulation of inflammation and thus to suppression of disease. In the present study, we analysed the relationship between T cell responsiveness to HSP60 and both T cell surface expression of CD30 and the production of regulatory cytokines in a group of oligoarticular JIA patients and in a group of polyarticular JIA patients.

Although CD30 expression can also be found in Th1 and Th0 clones, CD30 surface expression has mainly been described as an in vitro feature of Th2 cells [27-29], and there is in vivo evidence that CD30 has a role in both function and development of Th2-like human $CD4^{+}$ cells [30,31]. As in rheumatoid arthritis (RA), the synovial lymphocyte infiltrate in JIA consists predominantly of Th1 cells [32,33], and both diseases are therefore believed to be the result of a polarization toward a persistent proinflammatory Th1 response. The presence of a Th2 component, in terms of type 2 cytokines and expression of chemokine receptors [4,5], early in the disease process of JIA has been suggested to function in an anti-inflammatory capacity and may play a role in determining disease phenotype. Gerli et al. recently proposed the hypothesis that $CD30^{+}$ T cells play a local counter-regulatory role at sites of inflammation in RA as well as in other Th1-polarized diseases [34,35]. Until now, no data were available on the presence of $CD30^{+}$ T cells in PBMCs or SFMCs from patients with JIA.

We found virtually no expression of CD30 on unstimulated PBMCs from patients with JIA. In addition, we found a low expression of CD30 on unstimulated SFMCs of patients with oligoarticular JIA. Previous reports describe a higher expression of CD30 on SF-derived lymphocytes from RA patients [34]. This difference between JIA and RA seems to be due to shedding of the molecule, since SF levels of sCD30 are much higher in JIA than in RA. It is not clear whether this shedding is the result of differences in disease characteristics.

After in vitro activation with human HSP60, PB- and SF-derived lymphocytes from patients with oligoarticular JIA showed a high expression of CD30 in short-term cell lines as well as in direct cultures. In contrast, in vitro activation with HSP60 failed to induce a similar increased expression of CD30 on PB-derived lymphocytes from patients with polyarticular JIA. Since the response in terms of CD30 expression to human HSP60 was much higher than the response to mycobacterial HSP60, it is unlikely that CD30 merely functions as an activation marker.

In addition to a high expression of CD30, in vitro activation of PBMCs with mycobacterial or human HSP60 resulted in a high production of IL-10 and a low production of IFN-ϒ in oligoarticular JIA patients with active disease, while in polyarticular JIA patients with active disease, this ratio was reversed in favor of IFN-ϒ. In addition, restimulation of the cell lines with human HSP60 induced a high intracellular level of IL-10 and a low intracellular level of IFN-ϒ. Similar observations have been made for AA: in the animal model, protective autoproliferative T cells specific for HSP60

produced IFN-ϒ as well as IL-4/IL-10 [36]. IL-10 is a very potent immunoregulatory cytokine with an overall suppressive effect on the generation of Th1 responses. Several studies on IL-10 and arthritis suggest that insufficient IL-10 in the presence of inflammation can result in a persistent proinflammatory milieu and joint destruction [37-40]. We could not detect IL-4 in any of our experiments. Since CD30+ cells correlated with the synthesis of IL-4 in RA, and since HSP60-specific T cells produced IL-4 in the AA model as well, this might have been due to a technical problem.

The high IL-10:IFN-ϒ ratio and the up-regulation of CD30 in response to HSP60 in oligoarticular JIA patients are consistent with the benign clinical course of this subgroup. It seems likely that the presence of HSP60-specific T cells in oligoarticular JIA patients constitutes an example of human Tr1 cells (simultaneously producing IFN-ϒ and IL-10) [6]. Tr1 clones can suppress the immune responses of other T cells in vitro and in vivo, including inhibiting the development of autoimmunity [9]. Interestingly, also in human clinical infections, there are first reports of the presence of Tr1-like IL-10 and IFN-ϒ double-producing T cells functioning as regulators of the antiparasite response while preventing hyperinflammation [41]. The stimuli that induce these double-producing T cells are still largely unknown. The data collected from the present study suggest that in oligoarticular JIA patients, IL-10 and IFN-ϒ double-producing T cells may develop from T cells recognizing the autoantigen HSP60.

We now suggest the following mechanistic principle in oligoarticular JIA. During periods of active arthritis, inflammation in the joint leads to local cellular stress and therefore to the up-regulation of self HSPs in the synovial tissue. T cells recognizing these self HSPs are activated in the inflamed joint, express CD30, and are triggered to produce high levels of IL-10 and low levels of IFN-ϒ. These autoreactive T cells may then down-modulate the pathogenic T cells, either directly through recognition of self HSP60 on the pathogenic T cells or indirectly through bystander suppression in a suppressive, IL-10-containing environment. In polyarticular JIA patients, T cell responses against human HSP60 are not completely absent but are qualitatively different; they appear later in the course of the disease, probably through bystander activation, and show no regulatory features.

Although the above-described principle is still speculative and is definitely not the only mechanism that leads to the benign clinical course of oligoarticular JIA, the above findings might have important implications. As a possible consequence of the identification of an antigen triggering regulatory T cells, new ways for immunotherapy in chronic arthritis may be found in strategies aimed at restoring the natural regulatory responses in patients with polyarticular JIA, through vaccination with HSP60 or peptides containing defined HSP epitopes. Taking into account the present observations, such an immunotherapy approach should then trigger self HSP60-specific regulatory T cells that can down-modulate inflammation in either a Th2-counterbalancing or Tr1-regulatory manner.

Acknowledgements

We wish to thank Erica Roks and Jos Keyzer for administrative support throughout the study.

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3

CD4^{+}CD25^{bright} regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of Juvenile Idiopathic Arthritis

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Journal of Immunology 2004 May 15;172(10):6435-43.

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Summary

This study investigates the role of $CD4^{+}CD25^{+}$ regulatory T cells during the clinical course of Juvenile Idiopathic Arthritis (JIA). Persistent oligoarticular JIA (pers-OA JIA) is a subtype of JIA with a relatively benign, self-remitting course while extended oligoarticular JIA (ext-OA JIA) is a subtype with a much less favorable prognosis. Our data show that patients with pers-OA JIA display a significant higher frequency of $CD4^{+}CD25^{bright}$ T cells with concomitant higher levels of mRNA FoxP3 in the peripheral blood than ext-OA JIA patients. Furthermore, while numbers of synovial fluid (SF) $CD4^{+}CD25^{bright}$ T cells were equal in both patient groups, pers-OA JIA patients displayed a higher frequency of $CD4^{+}CD25^{int}$ T cells and therefore of $CD4^{+}CD25^{total}$ in the SF than ext-OA JIA patients. Analysis of FoxP3 mRNA levels revealed a high expression in SF $CD4^{+}CD25^{bright}$ T cells of both patient groups and also significant expression of FoxP3 mRNA in the $CD4^{+}CD25^{int}$ T cell population. The $CD4+CD25^{bright}$ cells of both patient groups and the $CD4^{+}CD25^{int}$ cells of pers-OA JIA patients were able to suppress responses of $CD25^{negative}$ cells in vitro. A markedly higher expression of CTLA-4, glucocorticoid-induced TNFR, and HLA-DR on SF $CD4^{+}CD25^{bright}$ T regulatory cells (Tregs) compared to their peripheral counterparts, suggests that the $CD4^{+}CD25^{+}$ Tregs may undergo maturation in the joint. In correlation with this mature phenotype, the SF $CD4^{+}CD25^{bright}$ T cells showed an increased regulatory capacity in vitro compared with peripheral blood $CD4^{+}CD25^{bright}$ T cells. These data suggest that $CD4^{+}CD25^{bright}$ Tregs play a role in determining the patient's fate toward either a favorable or unfavorable clinical course of disease.

Introduction

The adaptive immune system represents a highly effective and dynamic system that can protect the host from a wide array of pathogens. However, the establishment of a full repertoire of pathogen specific lymphocytes is coincident with the formation of T cells able to recognize self-antigen. Some of these potentially autoreactive T cells escape negative selection in the thymus and are released into the periphery. To maintain peripheral tolerance the immune system has evolved a number of regulatory mechanisms. Failure of this regulatory network can result in autoimmune disease.
One of the key players of immune-regulation is the $CD4^{+}CD25^{+}$ regulatory T cell (Treg). These spontaneously occurring T cells can actively and dominantly prevent both the activation and the effector function of autoreactive T cells that escape other mechanisms of tolerance. $CD4^{+}CD25^{+}$ Tregs were initially identified in mice and rats by their ability to inhibit the development of autoimmune gastritis following neonatal thymectomy [1] and since then to inhibit autoimmune diabetes [2,3], prevent inflammatory bowel disease [4], impede antitumor immunity [5], prevent the expansion of other T cells in vivo [6] and inhibit T cell activation in vitro [7,8]. It is now clear that $CD4^{+}CD25^{+}$ Tregs are also part of the normal human immune repertoire, and it seems likely that the constitutive presence of this regulatory T cell subset also controls autoaggressive T and B cells in humans [9-13]. There is a remarkable similarity between $CD4^{+}CD25^{+}$ Tregs in mice and men. Therefore, the current challenge is to investigate what role human $CD4^{+}CD25^{+}$ Tregs play in the prevention of human autoimmune diseases and whether these cells can limit and perhaps reverse existing immune pathology.
We report here data on $CD4^{+}CD25^{+}$ Tregs in Juvenile Idiopathic Arthritis (JIA) patients and healthy children. JIA is the most frequent rheumatic disease of childhood and is defined as the development, in children under the age of 16 years, of arthritis in one or more joints lasting for >6 wk [14,15]. Two major reasons make JIA an ideal model for the study of immune regulatory processes in human diseases. First, due to the local inflammation in the joint and the accompanying increase in the volume of synovial fluid (SF) it is possible to obtain inflammatory cells from the inflammatory site. Second, JIA is not a homogenous disease but consists of various subtypes with striking differences in both severity and outcome [16], allowing us to correlate the findings in vitro with the clinical outcome in vivo. One subtype is oligoarticular JIA, in which a child has arthritis affecting one to four joints during the first 6 months of disease. If the condition extends to involve more than four joints after the first 6 months the patient is considered to have extended oligoarticular JIA (ext-OA JIA). When the disease persists as oligoarticular (pers-OA JIA), the disease has a relatively benign course, which is frequently self-remitting and sometimes even self-limiting. The progression towards ext-OA JIA leads to a much less favorable prognosis. In these children, the disease frequently involves a non-remitting destructive and disabling arthritis, requiring aggressive immunosuppressive therapy. It is still not known which mechanisms determine this wide difference in clinical appearance between the two

subtypes, although evidence is accumulating that T cells, such as CCR4 expressing T cells and T cells specific for heat shock protein 60, play a key role [17-19].
As a result of the self-limiting and remitting character of the inflammatory process, pers-OA JIA has a unique place among all human autoimmune diseases. The self-limitation of an autoimmune process is often seen in experimental animal models of autoimmune diseases but hardly ever in humans: it is therefore intriguing to propose that immune regulatory mechanisms may play a major role in this process. In this study we analysed whether $CD4^+CD25^+$ Tregs play a role in the reversal of the auto-immune process in pers-OA JIA patients and whether differences in this regulatory cell population between the different subsets of JIA can explain the difference in clinical course.

Methods

Patients

Sixty patients with JIA with a pers-OA course, 34 patients with an ext-OA course, and 34 healthy children were evaluated for this study. Patients were included from both The Netherlands (University Medical Center, Utrecht) and Great Britain (Great Ormond Street Hospital, London). The diagnosis of the patients was defined according to the revised criteria for JIA [16]. Blood samples were obtained by venepuncture. SF was obtained at the time of a diagnostic or therapeutic joint aspiration. Informed consent was obtained either from parents/guardians or from the children directly when they were older than 12 years (The Netherlands) or 16 years (Great Britain). **Table 1** shows the clinical characteristics of the included children at the time of blood draw and/or SF aspiration.

Table1

Clinical characteristics

	Pers-OA JIA (n = 60)	Ext-OA JIA (n = 34)	Healthy controls (n = 34)
Male	20 (33%)	9 (26%)	24 (62%)
Age (mean, range)	11y3m (2y7m-25y4m)	10y3m (2y7m-19y9m)	7y3m (2y9m-17y3m)
Duration of disease	5y6m 92m-17y8m)	6y6m (1m-14y)	NA [a]
Joint count (mean, range) at time of sampling	1.1 (1-4)	2.7 (0-10)	NA
Medication			
Prednison, low dose, orally	0	1 (4%)	
MTX low dose [b]	8 (13%)	9 (26%)	
MTX high dose [c]	0	2 (6%)	
Anti-TNFα and/or other immunosuppressives	0	0	NA
Salazopyrine	1 (2%)	1 (4%)	
Nonsteroidal anti-inflammatory drugs	29 (48%)	21 (62%)	

[a] NA, not applicable
[b] Two and one-half to 12.5 mg/week
[c] More than 12.5 mg/week

Proliferation assays and co-culture experiments

PBMC and SF mononuclear cells (SFMC) were isolated using Ficoll-Isopaque density centrifugation (Ficoll-Paque, Pharmacia, Sweden). For the analysis of the proliferative response of peripheral blood (PB) and SF, CD4CD25- and $CD4^{+}CD25^{bright}$ T cells were sorted by FACS (EPICS ALTRA, Beckman-Coulter, Fullerton, California, USA) and subsequently incubated with 1 μM CFSE (Molecular Probes, Oregon, USA) for 5 minutes at 37°C. The cells were washed and cultured for 5 days in the presence or absence of 1 μg/ml phytohaemaglutinin (PHA, Sigma, UK) and/or 10 ng/ml IL-2 (Eurocetus, Amsterdam, The Netherlands). Cells were then stained and analysed by FACS.
For the co-culture experiments $CD4^{+}$ T cells from PB and SF were obtained using magnetic goat anti-mouse IgG beads and VarioMACS-positive selection columns (Miltenyi Biotec, Bisley, Surrey, UK). The resulting $CD4^{+}$ T-cell fraction was subsequently sorted into $CD4^{+}CD25^{-}$, $CD4^{+}CD25^{intermediate\ (int)}$ and $CD4^{+}CD25^{bright}$ T cells by FACS (FACS Vantage, Becton-Dickinson, San Jose, CA, USA). For functional assays $CD4^{+}CD25^{-}$, $CD4^{+}CD25^{int}$ and $CD4^{+}CD25^{bright}$ T cells were directly sorted into a 96-well plate. The purity of the sorted populations, determined by FACS reanalysis of an aliquot of sorted cells, was 95% on average. $5x10^{3}$ FACS sorted $CD4^{+}CD25^{-}$ T cells were cultured in the absence or presence of $5x10^{3}$ FACS sorted $CD4^{+}CD25^{bright}$ or $CD4^{+}CD25^{int}$ T cells in plate-bound anti-CD3-coated wells (OKT-3, 1 μg/ml). As controls, $CD4^{+}CD25^{-}$ T cells were co-cultured with $CD4^{+}CD25^{-}$ T cells at the same ratio. The negative fraction of cells obtained after MACS sorting were used as antigen-presenting cells (APC) after depletion of T cells by a second round of MACS sorting using anti-CD3 beads followed by irradiation (3500 rad). $3x10^{4}$ APC were added to each well. The cells were incubated at 37°C for 6 days, the last 18 h in the presence of [^{3}H]thymidine (1 μCi/well).
The suppressive activity was determined by calculating the relative difference in proliferative response (mean [3H]thymidine incorporation (cpm) of triplicate wells) between $CD4^{+}CD25^{-}$ T cells cultured alone and $CD4^{+}CD25^{-}$ T cells cultured in the presence of $CD4^{+}CD25^{bright}$ or $CD4^{+}CD25^{int}$ T cells.

Flow cytometry

PBMC and SFMC were washed twice in PBS containing 2% FCS (PBS-FCS) and adjusted to 0.5-1 x 10^{6} cells/ml in staining buffer (PBS-FCS containing 0.1% sodium azide) and blocked with the appropriate normal serum (30 min at 4ºC). Subsequently, the cells were incubated in 50 μl FACS buffer containing three or four appropriately diluted PE, FITC, CY or APC labeled mAb against human CD4 (clone RPA-T4), CD25 (clone 2A3), HLA-DR (clone L243), CD40L (clone ACT35), CD45RA (clone L48), CD45RO (clone 4CHL-1), CD69 (clone L78), CCR4 (clone 1G1) or GITR (clone 110416). For cytoplasmic staining of CTLA-4 (clone BN13) the cells were first surface stained, then fixed in Cytofix/Cytoperm solution (20 min, 4ºC) and washed twice in Perm/Wash solution (Cytofix/perm kit, BD, San Jose, CA), followed by incubation with anti-CTLA-4 mAb. GITR specific mAb was obtained from R&D (Germany). All other mAbs were obtained from Becton Dickinson (San Jose, CA, USA). Stained mononuclear cells were diluted in sheath fluid and run on a FACSCalibur (Becton Dickinson). CellQuest software (BD Biosciences) was used for analysis.

mRNA analysis by quantitative PCR

From three ext-OA and nine pers-OA JIA patients $CD4+CD25^{bright}$ and $CD4^{+}CD25^{-}$ T cells were isolated from PB and SF by FACS sorting. Total RNA was isolated using

Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 500 ng/ml. First-strand cDNA was synthesized from total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV, Promega, Madison WI, USA) with 1 µg/µl Oligo (dT) and 10 mM dNTP (both Amersham Pharmacia Biotech AB, Uppsala, Sweden). The reaction mixture was incubated at 40°C for 90 minutes followed by incubation at 70°C for 15 minutes.
To ensure the fidelity of mRNA extraction and reverse transcription to first-strand cDNA all samples were subjected to real time PCR amplification with primers specific for the constitutively expressed gene GAPDH or beta-2 microglobulin (ß2m). Messenger RNA expression levels of the cytokines IL-10, IFN-ϒ and TGF-ß and of the transcription factors GATA3 and T-bet were determined by real-time quantitative PCR on a Taqman instrument (ABI PRISM® 7700 thermal cycler, Perkin Elmer). GAPDH was analysed as a housekeeping gene. The following combination of primers and probes were used: IFN-ϒ Forward 5′ CCA ACG CAA AGC AAT ACA TGA 3', IFN-ϒ Reverse 5′ TTT TCG CTT CCC TGT TTT AGC T3′, IFN-ϒ Probe-JOE 5′ TCC AAG TGA TGG CTG AAC TGT CGC C3′, IL-10 Forward 5′ TGA GAA CAG CTG CAC CCA CTT 3, IL-10 Reverse 5′ GCT GAA GGC ATC TCG GAG AT 3′, IL-10 Probe-FAM 5′ CAG GCA ACC TGC CTA ACA TGC TTC GA 3′, T-bet Forward 5′ GTT TGT GGA CGT GGT CTT GGT 3′, T-bet Reverse 5′ CTT TCC ACA CTG CAC CCA CTT 3′, T-bet Probe-JOE 5' ACC AGC ACC ACT GGC GGT ACC AGA G 3', GAPDH Forward 5′ CCA CCC ATG GCA AAT TCC 3′, GAPDH Reverse 5′ TGG GAT TTC CAT TGA TGA CAA G 3′, GAPDH Probe-TET 5′ TGG CAC CGT CAA GGC TGA GAA CG 3′. To quantify mRNA amounts of IL-10, IFN-ϒ, T-bet and GATA-3 induction indices were calculated using the comparative Ct method. Differences in the Ct for the target gene and Ct for the housekeeping gene (GAPDH), indicated as ∆Ct, were calculated to normalize differences in the mRNA extractions and the efficiency of reverse transcription. The relative mRNA amount for each target gene in $CD4^+CD25^{bright}$ and $CD4^+CD25^{int}$ Treg cells is calculated as ∆∆Ct and expressed as n-fold difference relative to $CD4^+CD25^-$ T cells in the same compartment (induction index).
Next, from PB and SF of 4 pers-OA and 4 ext-OA JIA patients and from PB of 4 healthy controls $CD4^+CD25^-$, $CD4^+CD25^{int}$ and $CD4^+CD25^{bright}$ T cells were isolated by FACS sorting and mRNA was isolated as described above. For FoxP3 and ß2m transcripts real-time quantitative PCR was performed with a LightCycler (Roche Diagnostics, Mannheim, Germany) based on specific primers and general fluorescence detection with SYBR Green. The following primer combinations were used; FoxP3 Forward 5′ TCA AGC ACT GCC AGG CG 3′, FoxP3 Reverse 5′ CAG GAG CCC TTG TCG GAT 3′ and ß2m Forward 5′ CCA GCA GAG AAT GGA AAG TC 3′, ß2m Reverse 5′ GAT GCT GCT TAC ATGTCT CG 3′. All PCR reactions were performed using LightCycler-FarstStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). A pool of cDNA from tetanus stimulated human peripheral blood mononuclear cells was used as a standard and normalization to ß2m was performed for each sample. Semi-quantitative levels of FoxP3 are expressed as percentage of the FoxP3 expression of the cDNA pool.

Statistical analysis

Basic descriptive statistics were used to describe the patient population. A Mann-Whitney U test and a paired t-test were used to compare numbers of $CD4^+CD25^+$ T cells in the PB and the SF of the different patient groups. A paired t-test was used to compare the expression of molecules on PB and SF $CD4^+CD25^+$ T cells.

Results

Numbers of $CD4^+CD25^{bright}$ T cells in PB and SF of JIA patients and in PB of healthy controls

Sixty PB and 35 SF samples from 60 pers-OA JIA patients, 34 PB and 26 SF samples from 34 ext-OA JIA patients and 34 PB samples from healthy children were evaluated on frequencies of $CD4^+CD25^+$ T cells. Since the regulatory $CD4^+$ T cells preferentially reside within the $CD4^+CD25^{bright}$ population [21] we analysed PB and SF samples for the relative numbers of both $CD4^+CD25^{bright}$ T cells and $CD4^+CD25^{total}$ T cells (**Fig. 1a and b**). The range of the PBL values of the patients whose synovial fluid values are shown fall in the overall distribution of PBL values. The number of $CD4^+CD25^{bright}$ and $CD4^+CD25^{total}$ T cells is expressed as a percentage of all $CD4^+$ T cells.

Our results reveal a significantly lower number of $CD4^+CD25^{bright}$ T cells in the PB of pers-OA JIA patients (mean ± SEM: 1.2% ± 0.2%) as well as in the PB of ext-OA JIA patients (mean ± SEM; 0.5% ± 0.2%) when compared to healthy controls (mean ± SEM; 1.6% ± 0.1%) ($p < 0.001$, $p < 0.0001$ respectively, **Fig. 1a**). The difference in PB $CD4^+CD25^{bright}$ T cell frequency between pers-OA and ext-OA JIA patients was also significant ($p < 0.05$). In the SF of both pers-OA and ext-OA JIA patients the number of $CD4+CD25^{bright}$ T cells was highly increased (mean ± SEM; 6.2% ± 0.7%, mean ± SEM; 5.2%, 0.9% respectively, **Fig. 1b**) when compared to PB ($p < 0.0001$, $p < 0.0001$ respectively). Also, the CD25 density on the synovial fluid cells was brighter than on cells from peripheral blood as reflected by a higher mean fluorescence intensity (**Fig. 1d**). There was no significant difference in number of SF $CD4^+CD25^{bright}$ T cells between pers-OA and ext-OA JIA patients. However, when $CD4^+CD25^{total}$ cells were analysed as a whole, patients with pers-OA JIA had significantly higher numbers of CD25 positive cells within the joints (mean ± SEM: 30.5% ± 2.3%) than patients with ext-OA disease (mean ± SD: 20.3% ± 1.7%) ($p < 0.01$) and this was explained by a higher frequency of $CD4^+CD25^{int}$ T cells. The difference in relative numbers of PB $CD4^+CD25^{bright}$ Treg cells between pers-OA and ext-OA JIA patients seems not to be the result of a difference in number of aggressor cells, since the absolute $CD4^+$ T cell counts were equal in both patient groups (**Fig. 1c**). Data on absolute blood counts in the healthy controls and absolute cell counts in the SF were not available.

Furthermore, more ext-OA JIA patients are treated with the immunosuppressive drug Methotrexate (MTX). We therefore analysed whether this difference in treatment can explain the differences in $CD4^+CD25^+$ T cell frequencies. However, no significant differences in absolute and relative numbers of PB or SF $CD4^+CD25^{bright}$ and $CD4^+CD25^{total}$ T cells could be found between patients treated with or without MTX.

High expression of the regulatory cell marker FoxP3 in PB and SF $CD4^+CD25^{bright}$ T cells but also significant expression in $CD4^+CD25^{int}$ T cells

Recently FoxP3 has been identified as a specific marker of $CD4^+CD25^+$ Treg cells, distinguishing them from recently activated, non-regulatory $CD4^+CD25^+$ T cells [22,23].

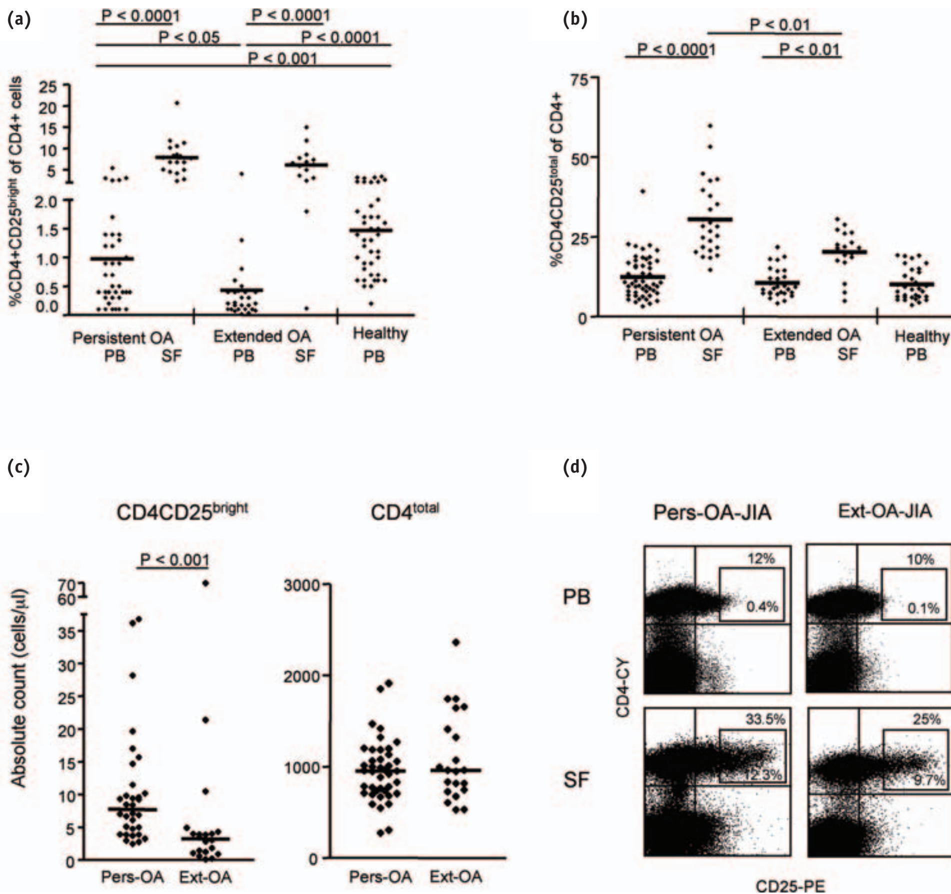
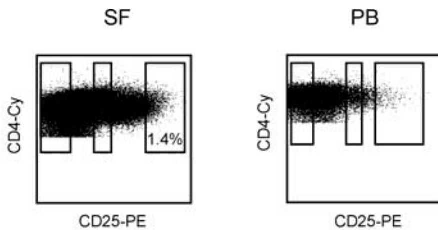


Figure 1. Number of CD4CD25^{bright} and CD4CD25^{total} T cells in JIA and healthy children. Sixty PB and 35 SF samples from pers-OA JIA patients, 34 PB, and 26 SF samples from ext-OA JIA patients and 34 PB samples from healthy children were screened by flow cytometry for the presence of CD4^{+}CD25^{bright} T cells (a) and CD4^{+}CD25^{total} T cells (b). (c) Absolute counts of PB CD4^{+}CD25^{bright} and CD4^{+}CD25^{total} T cells. (d) Dotplots indicate the gates used for the analysis of number of CD4^{+}CD25^{bright} and CD4^{+}CD25^{total} T cells in PB and SF.

We therefore sorted CD4^{+}CD25^{-}, CD4^{+}CD25^{int} and CD4^{+}CD25^{bright} T cells from the PB and SF of four pers-OA and four ext-OA JIA patients and from the PB of four healthy controls by FACS and evaluated the sorted populations for the expression of FoxP3 mRNA by quantitative PCR. To obtain clear populations we defined CD4^{+}CD25^{bright} T cells as the 1.4% CD25 brightest of the CD4^{+} T cells.
Healthy controls and pers-OA JIA patients displayed higher amounts of mRNA FoxP3 in PB CD4^{+}CD25^{int} and CD4^{+}CD25^{bright} T cells than ext-OA JIA patients (**Fig. 2**). In the SF of all pers-OA as well as ext-OA JIA patients each cell population analysed displayed higher levels of mRNA FoxP3 than the same PB population. Especially the SF CD4^{+}CD25^{bright}

T cells showed a high expression of the FoxP3 gene, but also the SF CD4^{+}CD25^{int} T cells expressed much higher levels of mRNA FoxP3 than their PB counterparts. Comparing the two patient groups pers-OA JIA patients displayed higher amounts of mRNA FoxP3 in both, SF CD4^{+}CD25^{int} and SF CD4^{+}CD25^{bright} T cells. These differences in mRNA FoxP3 expression between the two patient groups were not statistically significant due to the low number of patients analysed. However, when analyzing more closely at clinical outcome of the affected joint the correlation of mRNA FoxP3 levels with clinical outcome seemed to be further confirmed. Each SF aspiration was followed by an intra-articular injection of corticosteroids. Interestingly, the two pers-OA JIA patients with the lowest numbers of CD4^{+}CD25^{int} and CD4^{+}CD25^{bright} T cells in the SF relapsed very soon (within 3 months) after the local immune suppressive treatment. Likewise, the ext-OA JIA patient with the highest number of CD4^{+}CD25^{int} and CD4^{+}CD25^{bright} T cells in the joint showed a long-term remission (> 6 months; data not shown).

Altogether these findings indicate that the accumulation of CD4^{+}CD25^{bright} T cells in the SF of JIA patients is due to the accumulation of professional CD4+CD25+ Treg cells. The Tregs do not only reside within the CD4^{+}CD25^{bright} T-cell population but can also be found in the CD4^{+}CD25^{int} T-cell population. A good correlation was found between the measured amounts of mRNA FoxP3 and the relative numbers of CD4^{+}CD25^{int} and CD4^{+}CD25^{bright} T cells in the PB and the SF (**Fig. 1**). Also, the measured amounts of mRNA FoxP3 seem to correlate well with clinical subtype and clinical course after local treatment.



	Pt-group	CD25-	CD25^{int}	CD25^{bright}
PB	Healthy controls	26.3 ± 13.4	230.3 ± 51.6	608.8 ± 159.4
	Pers-OA JIA	7.5 ± 3.05	161.2 ± 58.8	644.5 ± 196.5
	Ext-OA JIA	23.2 ± 9.4	96.4 ± 21.5	326.7 ± 45.0
SF	Pers-OA JIA	78.4 ± 27.4	1102.9 ± 447.4	3245.9 ± 1071.5
	Ext-OA JIA	92.8 ± 28.5	733.2 ± 397	1691.6 ± 751.9

Figure 2. CD4^{+}CD25^{bright} and CD4^{+}CD25^{int} T cells in the SF of JIA patients express the regulatory T cell marker FoxP3. CD4^{+}CD25^{-} T cells, CD4^{+}CD25^{int} T cells and CD4^{+}CD25^{bright} T cells were isolated from the SF of pers-OA and ext-OA JIA patients. (a) Sort-gates used during FACS isolation of PB and SF CD4^{+}CD25^{+}, CD4^{+}CD25^{int}, and CD4^{+}CD25^{bright} T cells. The dot plots were obtained from a pers-OA-JIA patient. (b) The mean expression of mRNA (as percentage of expression in standardized control cDNA) FoxP3 in the different cell populations.

SF $CD4^+CD25^{bright}$ Treg-cells show a state of functional maturation when compared to PB

We investigated the expression of a number of molecules on $CD4^+CD25^-$ and $CD4^+CD25^{total}$ T cells in the PB and on $CD4^+CD25^-$, $CD4^+CD25^{int}$ and $CD4^+CD25^{bright}$ T cells in the SF (**Fig. 3**). In all children evaluated the phenotype of PB $CD4^+CD25^+$ T cells differed from that of $CD4^+CD25^-$ T cells in a manner consistent with published literature, i.e., by the constitutive expression of CTLA4 and GITR4 [24-26].

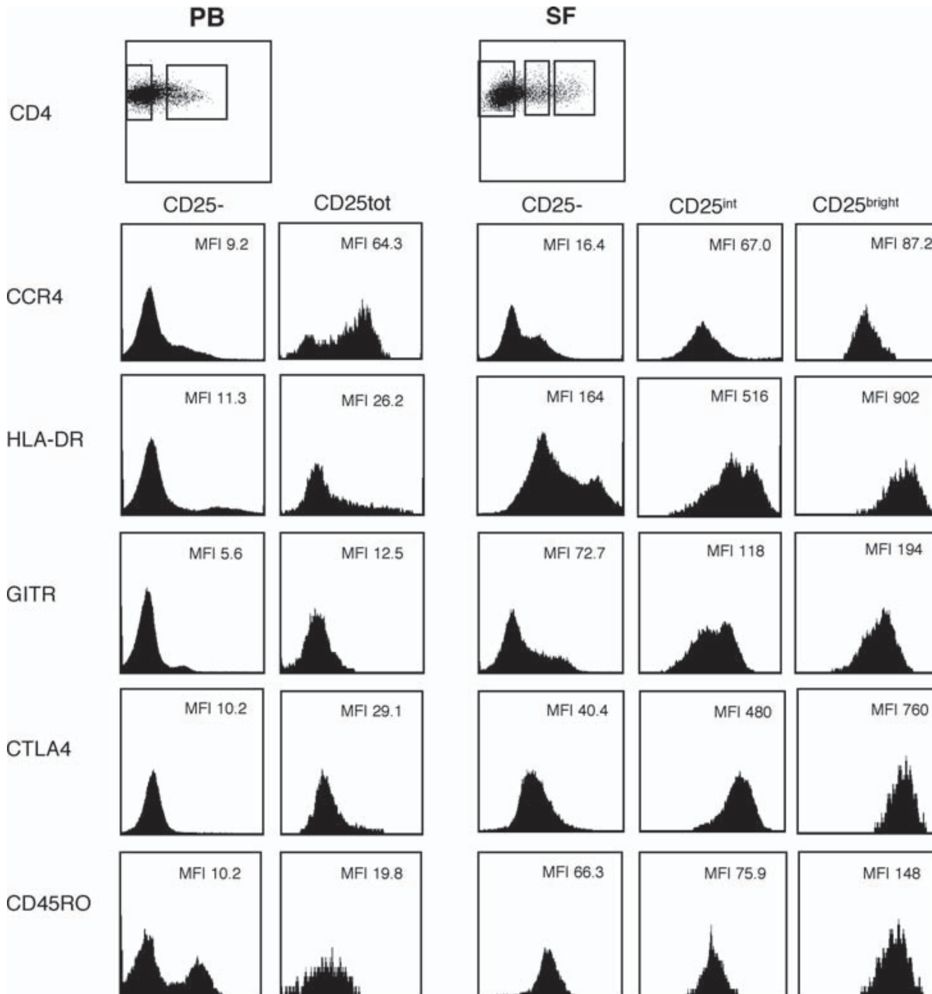


Figure 3. Phenotype of PB and SF $CD4CD25^-$ and $CD4CD25^+$ T cells. PB $CD4^+CD25^-$ and $CD4^+CD25^{total}$ T cells and SF $CD4^+CD25^-$, $CD4^+CD25^{int}$, and $CD4^+CD25^{bright}$ T cells were evaluated on the expression of molecules associated with regulatory T cells. The dot plots indicate the sorting gates used for the different subpopulations. There were no differences in the phenotype of the $CD4^+CD25^{bright}$ and $CD4^+CD25^{int}$ T cells of the two patient groups. The depicted FACS plots were obtained from a pers-OA JIA patient. Mean Fluorescence Intensity (MFI) values of the whole population are shown in each plot.

Comparing PB CD4$^+$CD25^{bright} Treg cells with their synovial counterparts, the SF populations showed a significantly higher expression of the activation marker HLA-DR ($p < 0.01$) and a significantly higher expression of CTLA-4 ($p < 0.0001$) and GITR ($p < 0.0001$). CTLA-4 density on SF CD4$^+$CD25^{bright} T cells was 10-15 times higher when compared to their peripheral counterparts and two times higher when compared to SF CD4$^+$CD25$^-$CD69$^+$ T cells. We observed a gradual increase in the expression of HLA-DR, CTLA-4 and GITR with the increase in the expression of CD25 (**Fig. 3**), which was consistent for all samples measured. PB as well as SF CD4$^+$CD25^{bright} T cells were highly positive for the chemokine receptor CCR4. No statistically significant difference was found in the expression of CCR4 between PB and SF CD4$^+$CD25^{bright} T cells.
Beside the markers shown in **Figure 3** we evaluated the expression of CD69, CD45RB, CD40L and CD134. PB CD4$^+$CD25$^+$ T cells showed no expression of CD69 while the SF CD4$^+$CD25$^+$ T cells showed a high expression of CD69 (mean ± SEM; 53% ± 5.2%).
The expression of CD40L and CD134 did not differ significantly between the different subsets. In both PB and SF all CD4$^+$CD25^{bright} T cells were CD45RB^{low}. There were no differences in phenotype of the CD4$^+$CD25^{bright} and CD4$^+$CD25^{int} T cells between pers-OA and ext-OA JIA patients.

Hyporesponsiveness of SFMC is due to active suppression by CD4$^+$CD25^{bright} T cells and can be overcome by IL-2

In rheumatoid arthritis (RA) as well as in JIA, SF-derived T cells show a state of hyporesponsiveness [27,28] and few cells in active cell division [29]. Thus, although these cells have a high activation status they appear relatively inactive in situ and respond only weakly to diverse stimuli ex vivo. We hypothesized that this relative hyporesponsiveness could be due to the presence of CD4$^+$CD25$^+$ Treg cells in the SF. We therefore compared the proliferative response of PB and SF T cells from children with pers-OA and ext-OA JIA to the T cell mitogen PHA. A representative example of three experiments is shown in **Fig. 4**. In all three experiments, SFMC showed very poor proliferation to PHA compared with paired PBMC (**Fig. 4A, middle panel**). We hypothesized that this hyporesponsive state could be due to suppression by the CD4$^+$CD25$^+$ Treg cells, which are abundantly present in the SF. Since high doses of IL-2 can abrogate the suppressive activity and the anergic phenotype of CD4$^+$CD25$^+$ Treg cells [8,10,29], we evaluated whether the addition of IL-2 to the cultures could overcome the hyporesponsiveness of SF T cells. The addition of 10 ng/ml IL-2 was found to overcome the hyporesponsive state of the SF T cells (**Fig. 4a, right panel**). Depletion of the CD4$^+$CD25^{bright} T cells from SF by FACS sorting resulted in an increased response of the remaining SF CD4$^+$ T cells (**Fig. 4b, right panel**). The CD4$^+$CD25^{bright} T cells alone were profoundly anergic (**Fig. 4b, middle panel**) compared with unsorted SFMC (**Fig. 4b, left panel**).
To demonstrate active suppression by CD4$^+$CD25^{bright} T cells we mixed CD4$^+$CD25$^-$ and CD4$^+$CD25^{bright} T cells (ratio 2:1) and evaluated the proliferative response (**Fig. 4c**). The mixed cells proliferated poorly (**Fig. 4c, right panel**) compared to CD4$^+$CD25$^-$ cells alone (**middle panel**), indicating an active suppressive role for the SF CD4$^+$CD25^{bright} T cells.

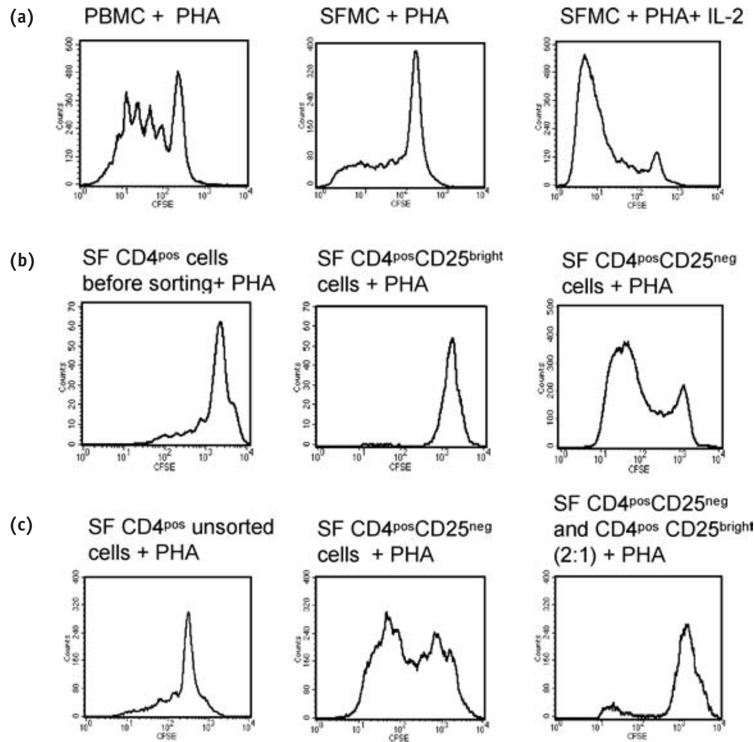


Figure 4. Hyporesponsiveness of SFMC is due to active suppression by CD4+CD25bright Treg cells and can be overcome by IL-2. Proliferation of PBMC or SFMC from children with oligoarticular JIA followed by CFSE labeling. Plots in (a) are gated on CD3, while (b) and (c) are gated on CD4 and CD3. (a) SFMC show very poor proliferation to the T cell mitogen PHA (middle panel) compared to paired PBMC (left panel); this hyporesponsive state is overcome by addition of IL-2 (right panel). (b) After FACS sorting of SFMC cells, CD4posCD25bright T cells alone are profoundly anergic to PHA (middle panel) compared to unsorted SF CD4 cells (left panel), while CD4posCD25neg T cells, from which CD25bright have been depleted, respond well (right panel). (c) Suppression by CD25bright T cells is active, as shown by mixing of CD4posCD25neg and CD4posCD25bright T cells at a ratio of 2:1, (right panel), which proliferate poorly compared to CD4posCD25neg T cells alone (middle panel). For data in C, middle and right panels, only the CD4+CD25- cells are CFSE labeled and are gated on CFSE+ cells to illustrate active suppression.

Suppressive function of SF CD4+CD25bright T cells of both persistent oligoarticular and extended oligoarticular patients in vitro.

We next investigated the suppressive capacity in vitro of CD4+CD25bright T cells by a direct functional comparison of CD4+CD25bright T cells from PB and SF of JIA patients and from PB of healthy children. We sorted CD4+CD25- and CD4+CD25bright T cells from PB and SF. To obtain enough cells the gate for each sort of SF as well as PB CD4+CD25bright T cells was set to include the brightest 1.4% of CD4+CD25+ T cells. The regulatory capacity of the CD4+CD25bright T cells was measured in co-cultures. The suppressive activity was determined by calculating the relative difference in proliferative response to plate bound anti-CD3 between CD4+CD25- T cells cultured

alone and $CD4^+CD25^-$ T cells cultured in the presence of $CD4^+CD25^{bright}$ T cells (ratio 1:1). We tested the PB of 10 healthy children, 13 pers-OA and 6 ext-OA JIA patients and the SF of 6 pers-OA and 3 ext-OA JIA patients.
In 2 healthy children (20%), 3 pers-OA (23%) and 3 ext-OA JIA patients (50%) the PB $CD4^+CD25^{bright}$ T cells were not suppressive when tested in the above-described assays. The PB $CD4^+CD25^{bright}$ T cells of the remaining 8 healthy controls, 10 pers-OA JIA and 3 ext-OA JIA patients showed a suppressive activity of 44% ± 7%, 68% ± 7% and 62% ± 14% (mean ± SEM), respectively. The SF $CD4^+CD25^{bright}$ T cells of the 6 pers-OA and the 3 ext-OA JIA patients all showed good suppressive effect (mean ± SEM: 82% ± 0.1%, 80% ± 0.01% respectively). There was no significant difference in percentage of inhibition between the different study groups.

SF $CD4^+CD25^{int}$ T cells of pers-OA JIA patients are able to suppress

Pers-OA JIA patients harbor significantly higher amounts of (mRNA FoxP3 expressing) $CD4^+CD25^{int}$ T cells in the SF than ext-OA JIA patients. To test whether the SF $CD4^+CD25^{int}$ T cell compartment of pers-OA-JIA patients contain enough regulatory T cells to suppress we performed a co-culture experiment with SF $CD4^+CD25^-$ and $CD4^+CD25^{int}$ T cells of 3 pers-OA JIA patients. In all three patients the SF $CD4^+CD25^{int}$ T cells were able to suppress CD4CD25- responder T cells, though to a lesser extent than SF $CD4^+CD25^{bright}$ T cells (**Fig. 5**).

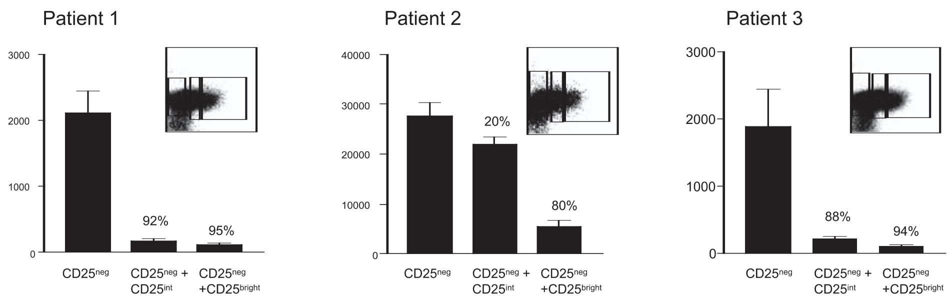


Figure 5. SF $CD4^+CD25^{int}$ T cells of pers-OA JIA patients are able to suppress proliferation of synovial responder cells. Coculture experiments in three pers-OA JIA patients comparing the suppressive capacity of SF $CD4+CD25^{int}$ T cells and $CD4^+CD25^{bright}$ T cells. The FACS plots indicate the sorting gates used for the different SF subpopulations. The percentages indicate the percentage of suppression by $CD4^+CD25^{int}$ and $CD4^+CD25^{bright}$ T cells respectively. In each patient SF $CD4^+CD25^{int}$ T cells were able to suppress the proliferative responses of SF $CD4^+CD25^-$ T cells, although to a lesser extent than SF $CD4^+CD25^{bright}$ T cells.

SF $CD4^+CD25^{bright}$ T cells express higher IL-10, lower IFN-ϒ and similar TGF-ß mRNA levels when compared to SF $CD4^+CD25^-$ T cells.

The role of immunosuppressive cytokines such as TGF-ß and IL-10 in relationship to $CD4^+CD25^+$ Treg cells is still unclear, but has been implicated in suppressive function in some reports. We therefore evaluated the expression of mRNA TGF-ß, IL-10 and IFN-ϒ in FACS sorted SF $CD4^+CD25^{bright}$ T cells and $CD4^+CD25^-$ T cells by quantitative PCR.
The expression of mRNA in $CD4^+CD25^{bright}$ T cells was compared with the expression in

CD4+CD25- T cells and expressed as the mean induction index relative to CD4+CD25- T cells. The SF CD4+CD25bright T cells expressed similar amounts of mRNA TGF-ß (mean ± SEM; 0.91 ±0.22), lower amounts of mRNA IFN-ϒ (mean ± SEM; 0.59 ± 0.23) and significantly higher amounts of mRNA IL-10 (mean ± SEM; 1.42 ± 0.36) than SF CD4+CD25- T cells.
To evaluate whether the CD4+CD25bright T cells in the SF show a polarization towards either a Th1 or Th2 phenotype we evaluated mRNA expression of GATA-3 and T-bet in FACS sorted SF CD4+CD25bright T cells. Both transcription factors were hardly detectable in the sorted populations and neither population showed an increased expression, either when compared to the other, or to CD4+CD25- T cells (data not shown).

SF CD4+CD25bright T cells have an increased regulatory capacity when compared to PB CD4+CD25bright T cells.

The phenotype and in vitro suppressive activity of the CD4+CD25bright T cells in the SF suggests an increased regulatory capacity compared to PB CD4+CD25bright T cells. To test this hypothesis we isolated CD4+CD25- and CD4+CD25bright T cells from two JIA patients. We co-cultured a fixed number of SF CD4+CD25- T cells (5,000/well) with increasing numbers of SF or PB CD4+CD25bright T cells as described above. Proliferative responses of SF CD4+CD25- T cells in the presence of SF CD4+CD25bright T cells were compared with proliferative responses of SF CD4+CD25- T cells in the presence of PB CD4+CD25bright T cells (**Fig. 6**). In each patient a clear enhanced suppression was observed when SF CD4+CD25- cells were co-cultured with SF CD4+CD25bright T cells when compared to co-culture with PB CD4+CD25bright T cells. Furthermore, while in both patients the PB CD4+CD25bright T cells were not able to suppress SF CD4+CD25- responder T cells, good suppression was observed when PB CD4+CD25bright T cells were co-cultured with PB CD4+CD25- responder T cells (data not shown). This may indicate that the SF CD4+CD25- responder T cells are harder to suppress than the PB CD4+CD25- responder T cells, presumably due to their activation status.

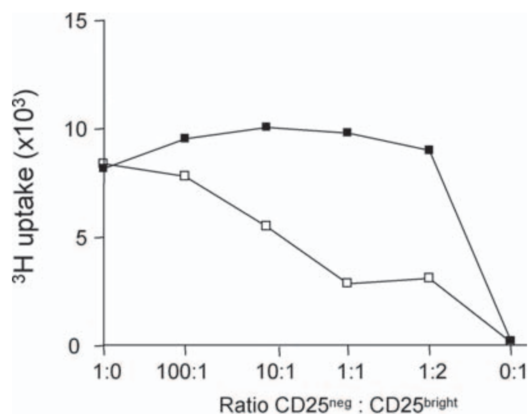


Figure 6. SF CD4CD25bright T cells have an increased suppressive capacity compared to PB CD4CD25bright T cells. Fixed numbers of SF CD4+CD25- T cells of two JIA patients were co-cultured with either SF CD4+CD25bright or PB CD4+CD25bright T cells at different ratios. In both patients the addition of SF CD4+CD25bright T cells led to a higher suppression of the CD4+CD25- responder T cells than the addition of PB CD4+CD25bright T cells. The effector/suppressor ratio curve of only one patient is shown.

Discussion

Many questions on whether and how $CD4^+CD25^+$ Treg cells can actively regulate autoimmunity in human still need to be answered. We here provide data on $CD4^+CD25^{bright}$ Treg cells in Juvenile Idiopathic Arthritis, comparing patients with a mild self-limiting and remitting course (pers-OA JIA) with patients displaying a less favorable, non-self-remitting course (ext-OA JIA).
While in previous studies variable immune components have been identified that seem to favor downregulation of inflammation in pers-OA JIA patients [17,30] it has not previously been clear which T cell subsets are involved in either sustaining or controlling the inflammation in the different patient groups. In this study we analysed whether $CD4^+CD25^{bright}$ Treg cells contribute to the different clinical outcome of the two disease subtypes.
In both groups of JIA patients we found a significantly lower frequency of $CD4^+CD25^{bright}$ Treg cells in the PB when compared to the blood of healthy controls. Since the PB $CD4^+CD25^{bright}$ Treg cells were functional in most patients we can only speculate whether or not this reduced number contributed to the development of the autoimmune disease. Despite this low frequency of $CD4^+CD25^{bright}$ Treg cells in the PB of JIA patients, the number of $CD4^+CD25^{bright}$ Treg cells in the SF was highly enriched. This enrichment at the site of inflammation is consistent with a recent study that showed an increased number of $CD4^+CD25^{bright}$ T cells in the SF of rheumatoid arthritis (RA) patients [31].
Interestingly, a comparison of the different patient groups of JIA showed that patients with pers-OA JIA display significant higher frequencies of PB $CD4^+CD25^{bright}$ T cells with concomitant higher levels of mRNA FoxP3 than patients with ext-OA JIA. Furthermore, while numbers of synovial fluid (SF) $CD4^+CD25^{bright}$ T cells were equal in both patient groups, pers-OA JIA patients displayed a higher frequency of $CD4^+CD25^{int}$ T cells, and thus a higher number of $CD4^+CD25^+$ cells in total in the synovial fluid (SF) than ext-OA JIA patients. Analysis of mRNA FoxP3 levels in SF $CD4^+CD25^{bright}$ and $CD4^+CD25^{int}$ T cells separately, revealed besides a high expression of the gene in SF $CD4^+CD25^{bright}$ T cells of both patient groups, also significant levels of expression in the SF $CD4^+CD25^{int}$ T cell population. Again, pers-OA JIA patients displayed higher levels of mRNA FoxP3 than ext-OA JIA patients. Though it is expected that the SF $CD4^+CD25^{int}$ T cell population consist of a mixture of activated and regulatory T cells, suppression assays with SF $CD4^+CD25^{int}$ T cells of pers-OA JIA patients showed that even this mixture is able to suppress $CD4^+CD25^-$ responder T cells in vitro. This finding is especially interesting, since it shows that though, consistent with reports by Baecher-Allan et al. most $CD4^+CD25^+$ Treg cells reside in the $CD4^+CD25^{bright}$ T cell population [21], during inflammation, $CD4^+CD25^+$ Treg cells can also be found among $CD4^+CD25^{int}$ T cells. Even more, low levels of mRNA FoxP3 were found in the SF $CD4^+CD25^-$ T cell population. Taking our results together, we propose that the differences in mRNA FoxP3 levels, number of PB $CD4^+CD25^{bright}$ T cells and number of SF

$CD4^+CD25^{int}$ T cells between pers-OA and ext-OA JIA patients contribute to the differences in clinical course. It needs to be noted however that, despite highly statistically significant differences in $CD4^+CD25^+$ T cell frequencies between the two patient groups, it will not be possible to use the analysis of PB and/or SF $CD4^+CD25^+$ T cell frequencies as a test to predict whether a patient with OA JIA will progress to ext-OA stages or persist as oligoarticular, as $CD4^+CD25^+$ T cell frequencies in the two patient groups are too much overlapping.

As in RA, the isolated SF $CD4^+CD25^{bright}$ T cells as well as the SF $CD4^+CD25^{int}$ T cells of pers-OA JIA patients exhibited an extremely potent suppressive capacity in vitro. Different groups previously reported a profound state of hyporesponsiveness of SFMC in RA [27,28] as well as in JIA and that synovial T cells in JIA show little evidence of proliferation [29]. Our results now show that this cellular hyporesponsiveness is the result of active suppression by highly activated $CD4^+CD25^+$ Treg cells present in the SF. Interestingly, besides depletion of the $CD4^+CD25^{bright}$ T cells from the SF also the addition of IL-2 could overcome this relative hyporesponsiveness. It has been shown that IL-2 can overcome the suppressive capacity of $CD4^+CD25^+$ Treg cells [8,10,32]. It is therefore conceivable that the addition of IL-2 to our cultures caused a breakdown in the hyporesponsive state by abrogating the suppressive capacity of the SF $CD4^+CD25^+$ Treg cells.

The expression of FoxP3, suggests that the SF $CD4^+CD25^{bright}$ T cells are so called professional Treg cells. Recently it has been shown, in both mice as well as in human, that activation of $CD4^+CD25^-$ T cells can lead to expression of FoxP3 and the acquisition of T regulatory activity [33]. Thus clear evidence exists now that there are two pathways for the generation of $CD4^+CD25^+$ Tregs: one as a result of thymic selection, and a distinct pathway as a consequence of immune responses in the periphery [33,34]. Our data do not provide information on the origin of the $CD4^+CD25^+$ Treg cells in the SF. It has been suggested that thymus-derived Treg cells, which are autoreactive and have self-renewing capabilities allow for regulation of autoreactivity [35]. Part of the SF $CD4^+CD25^+$ Tregs, presumably thymus-derived, may therefore have entered the joint in the initial phase of the autoimmune response. Since all SF $CD4^+CD25^{bright}$ T cells express the chemokine receptor CCR4, CCR4 may have a role in the homing of $CD4^+CD25^+$ Treg cells from the periphery to the joint. The expression of CCR4 is an intrinsic feature of $CD4^+CD25^+$ Treg cells [36], and it has been suggested before that the presence of CCR4 expressing T cells in the SF of JIA patients may function in an anti-inflammatory capacity [17]. In addition, it is possible that part of the $CD4^+CD25^+$ Tregs in the SF were generated during the excessive (bystander) activation of $CD4^+CD25^-$ T cells at the site of inflammation. The presence of functional Tregs in the $CD4^+CD25^{int}$ T cell population may than mirror an intermediate stage of development.

Our data on the phenotype of the SF $CD4^+CD25^{bright}$ T cells suggest that, independent of the origin the $CD4^+CD25^+$ Treg cells undergo maturation at the site of inflammation. The SF $CD4^+CD25^{bright}$ Treg cells show a marked increase in the expression of CD25, CTLA-4 and GITR compared to their peripheral counterparts. Furthermore, we observed a gradual increase in the expression of CTLA-4 and GITR on SF $CD4^+CD25^+$ T cells with the increase in the expression of CD25. It has been shown in human $CD4^+CD25^+$ T cell clones that the expression level of CTLA-4 and GITR clearly correlate with their suppressive capacity [37]. Herewith consistent is our finding that the striking expression of CTLA-4 and GITR on the SF $CD4^+CD25^{bright}$ T cells in JIA correlates with an increased

regulatory capacity in vitro. Thus, both the local enlargement of the CD4$^+$CD25$^+$ Treg cell population, either due to homing or due to induction at the site of inflammation, and the change in phenotype leading to enhanced suppressive capacity seem to be mechanisms by which CD4$^+$CD25^{bright} Treg cells try to regulate local inflammation.

As well as the marked increase in the expression of CTLA4 and GITR we found that the SF CD4$^+$CD25^{bright} T cells of oligoarticular JIA patients contain an increased amount of mRNA IL-10 when compared to PB CD4$^+$CD25^{bright} T cells or to SF CD4$^+$CD25$^-$ responder T cells. This finding suggests an active role for IL-10 during the suppression of inflammation by CD4$^+$CD25$^+$ Treg cells in the joints of these patients. Previous studies on IL-10 and arthritis have suggested that insufficient IL-10 in the presence of inflammation could be one of the mechanisms allowing joint inflammation to continue [38-40]. Though data on the secretion of the immunosuppressive cytokines IL-10 and TGF-ß and their involvement in suppression by CD4$^+$CD25$^+$ T cells have been very controversial in literature [8,13,41], our findings are in line with data from Fontenot et al. showing that expression of FoxP3 correlates with increased amounts of IL-10 mRNA in CD4$^+$CD25$^+$ Treg cells [23]. The controversy surrounding the data concerning the function of IL-10 in suppression by CD4$^+$CD25$^+$ Treg cells in general may be partly due to differences in isolation techniques or the use of different in vitro assays. In the study of Fontenot et al and in our study, mRNA IL-10 was measured in CD4$^+$CD25$^+$ Treg cells that were taken directly ex vivo.

Altogether our data lead to the hypothesis that the outcome of disease may in part be a matter of balance. Although the CD4$^+$CD25^{bright} Treg cells could not prevent the development of the disease in pers-OA JIA patients, the remitting clinical course in these patients suggests that they contribute to reversing ongoing inflammation. In the ext-OA JIA patients, the disease is neither prevented nor reversed presumably because the number of CD4$^+$CD25^{bright} T cells in the SF is not sufficient to suppress the inflammation.

In conclusion, pers-OA JIA is an example of a human disease in which CD4$^+$CD25$^+$ Treg cells, in addition to other regulatory mechanisms, seem to play an active role in the limiting and even reversal of established autoimmune pathology. The progression to ext-OA JIA in some patients seems not the result of an intrinsic defect of the CD4$^+$CD25$^+$ Treg cells but rather a failure to either home to or expand at the site of inflammation. Therefore, future studies need to be focused on finding ways to enhance local proliferation of CD4$^+$CD25$^+$ Treg cells without abrogating their regulatory capacity.

Acknowledgements

The authors wish to thank Ger Arkesteijn for his excellent help and advice on FACS sorting, Wilco de Jager and Brenda Hendriks for their help on the molecular work and Erica Roks for excellent editorial assistance.

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4

Human heat shock protein 60 regulates immune responses by the induction of FoxP3⁺CD4⁺CD25⁺ regulatory T cells

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Summary

Recent evidence has indicated that, besides being a separate lineage derived from the thymus, $CD4^+CD25^+$ Tregs can be induced in the periphery. This study shows that the activation of human CD4+CD25- T cells with autologous heat shock protein 60 (HSP60), induces the differentiation of $CD4^+CD25^-$ T cells into highly suppressive, $FoxP3^+CD4^+CD25^+$ Tregs, coexpressing CD30. In contrast, tetanus induced $CD4^+CD25^+$ T cells expressed much lower levels of mRNA FoxP3 and failed to suppress. The induction of $CD4^+CD25^+$ Tregs was dependent on ligation of HSP60 with TLR4 on APC.
Using a newly developed technique to capture peptide specific $CD4^+$ T cells, we were able to sort human HSP60-epitope specific $CD4^+$ T cells ex vivo from arthritis patients. mRNA analysis of these cells revealed a high expression of mRNA FoxP3. Together these data indicate that the peripheral induction of $CD4^+CD25^+$ Tregs by HSP60 may involve two mechanisms: an innate effect through ligation with TLR4 on APC and TCR recognition of HSP60 epitopes. This study provides the first evidence that $CD4^+CD25^+$ Tregs respond to autoantigens and demonstrate that Tregs play an important role in the detection and prevention of deleterious tissue damage.

Introduction

To date the naturally occurring CD4+CD25+ Treg subset (nTregs) is the best described regulatory T cell population [1-3]. The CD4+CD25+ Tregs are characterized by the expression of mRNA FoxP3 and have been shown to inhibit the activation of autoreactive T cells [4] and to prevent autoimmunity [5;6], and allograft rejection [7]. Importantly, besides the natural occurrence of CD4+CD25+ Tregs, it is now well established that FoxP3 expressing CD4+CD25+ Tregs can be induced in the periphery upon differentiation of conventional CD4+CD25- T cells (iTregs) [8-11]. These peripherally generated CD4+CD25+ Tregs are indistinguishable in cell surface phenotype, lifespan and functional aspects from intrathymically generated FoxP3+CD4+CD25+ cells (nTregs). The exact mechanism and circumstances by which this peripheral differentiation of naïve T cells into professional CD4+CD25+ Tregs takes place remains to be defined, especially in human and under inflammatory conditions.

Recently, we reported a highly increased frequency of CD4+CD25+ Tregs in the joints of patients with Juvenile Idiopathic Arthritis (JIA) [12]. This local accumulation of CD4+CD25+ Tregs raised the question what factors may be involved in the homing, proliferation or induction of Tregs at the inflammatory site. In mice Tregs can become activated by LPS, increasing their survival/proliferation and suppressor efficiency, probably through ligation with TLR4 [13]. It has recently been shown that endogenous heat-shock protein 60 (HSP60) can also functionally ligate TLR4 on macrophages and DCs [14;15]. Given the fact that the membranes of synovial lining cells of patients with JIA show an increased expression of endogenously produced HSP60 [16] and evidence is accumulating that HSPs serve a role in T cell mediated immunoregulation, we questioned whether HSP60 could in part be responsible for the accumulation of CD4+CD25+ Tregs at inflammatory sites, either through the activation and proliferation of migrated CD4+CD25+ nTregs or through the local induction of CD4+CD25+ iTregs.

Human HSPs share a unique degree of homology with bacterial HSPs and are expressed both constitutively and under stressful conditions. HSPs can leak into the extracellular compartment upon necrotic cell death [17], or be released in response to a number of stressful conditions [18-20]. Once released, HSPs have shown to be potent activators of the innate immune system [21-23], what prompted the suggestion that HSPs act as potentially harmful autoantigens, providing a link between bacterial infection and autoimmunity [24-28]. However, more recent findings have led to the suggestion that T cell reactivity against human HSPs, especially HSP60, might serve a regulatory role, the best-studied conditions in this field being adjuvant arthritis (AA) and the nonobese diabetic mouse model. In almost all models of experimental arthritis, including AA, preimmunization with HSP65 protects animals from arthritis [29;30]. In addition, in children with JIA, T cell responses to human HSP60 have been associated with an improved prognosis [31;32]. Together these data form a strong body of evidence supporting the concept that HSPs are “negotiators between danger and control mechanisms of autoimmunity” (Van Eden et al., 2003) [33]. However, the exact mechanism through

which HSPs may control T cell mediated immunoregulation in humans is still largely unknown. In the present study, we further analysed the regulatory component of human HSP60. Our data show that, the stimulation of human $CD4^{+}CD25^{-}$ T cells with HSP60 results in the peripheral induction of highly suppressive, mRNA FoxP3 expressing, $CD4^{+}CD25^{+}$ iTregs. In addition, part of the established $CD4^{+}CD25^{+}$ Tregs, isolated ex vivo from arthritis patients, recognizes HSP60 epitopes. Taken together, these data indicate that human HSP60 functions as a signalling molecule that, at inflammatory conditions or at sites of tissue damage, can augment T cell mediated immunoregulation through the induction of highly suppressive $CD4^{+}CD25^{+}$ Tregs.

Patients and controls

For most experiments mononuclear cells were isolated from the human buffy coat of healthy volunteers (n = 13) using Ficoll Isopaque density gradient centrifugation (Amersham Biosciences, New Jersey, USA). Where indicated, synovial fluid mononuclear cells (SFMC) were used obtained from oligoarticular JIA patients (n = 3).

Culture medium and reagents

Very low endotoxin RPMI 1640 (Seromed, Berlin, Germany) containing 10mM HEPES (Seromed), 2mM L-glutamine (Seromed), 20 µg/ml gentamicin, 60 µg/ml penicillin, 50 mM 2-ME, and 10% human AB-serum was used as a culture medium. For treatment with polymyxin B (PmB, Sigma-Aldrich, Dorset, UK), 1 µg/ml LPS (Sigma-Aldrich, Dorset, UK) and/or 10 µg/ml HSP60 (Stressgen, Victoria, Canada) were incubated with 10 µg/ml PmB for 1 hour at room temperature before addition to the cell cultures. For heat denaturation, LPS or HSP60 was incubated at 95ºC for 20 min. In some experiments TLR4 blocking antibody was used (clone HTA152, Serotec, Oxford, UK). For these experiments, APC were incubated (45 minutes, 37 ºC) with TLR4 blocking antibody (20 µg/ml) before addition to the cultures.

Flow cytometry

PBMC and SFMC were washed twice in FACS buffer (PBS containing 2% FCS) and adjusted to 0.5-1 x 10^6 cells/ml in staining buffer (FASC buffer containing 0.1% sodium azide) and blocked with AB serum (30 min at 4ºC). Subsequently, the cells were incubated in 50 ml FACS buffer containing three or four appropriately diluted PE, FITC, CY or APC labeled mAb's against human CD4 (clone RPA-T4), CD25 (clone 2A3), CD30 (clone Ber-H83), CCR4 (clone 1G1), GITR (clone 110416) and TLR-4 (HTA125). For cytoplasmic staining of CTLA-4 (clone BN13) the cells were first surface stained, then fixed in Cytofix/Cytoperm solution (20 min, 4ºC) and washed twice in Perm/Wash solution (Cytofix/perm kit, BD, San Jose, CA), followed by incubation with anti-CTLA-4 mAb. GITR specific mAb was obtained from R&D (Germany). All other mAbs were obtained from Becton Dickinson (San Jose, CA, USA). Stained mononuclear cells were diluted in sheath fluid and run on a FACSCalibur (Becton Dickinson). CellQuest software (BD Biosciences) was used for analysis.

Cell-culture

$CD4^+CD25^-$ T cells from healthy donors were obtained using magnetic goat anti-mouse IgG beads and VarioMACS-positive selection columns (Miltenyi Biotec, Bisley, Surrey, UK). The irradiated (3500 Rad) negative fraction of cells, obtained after MACS sorting, were used as antigen-presenting cells (APC). $CD4^+CD25^-$ T cells and APC (1:3) were cultured in the presence of human HSP60 (10 µg/ml), tetanus toxoid (1.5 µg/ml) or LPS (1 µg/ml) for 7 days. After 7 days the cells were evaluated on the expression of the

above-described markers. Furthermore, $CD4^+CD25^-$ and induced $CD4^+CD25^+$ T cells were sorted by FACS and analysed on the expression of mRNA FoxP3. The purity of the sorted populations, determined by FACS reanalysis of an aliquot of sorted cells, was 95% on average.

The 3% $CD4^+CD25^+$ T cells with the brightest expression of CD25 were tested in co-culture experiments on suppressive function. Fresh $CD4^+CD25^-$ responder T cells ($5x10^3$) from the same donor and induced $CD4^+CD25^{bright}$ T cells were directly sorted by FACS (FACS Vantage, Becton-Dickinson, San Jose, CA, USA) into plate-bound anti-CD3-coated wells (OKT-3, 1 µg/ml), in different ratios. T cell depleted, irradiated PBMC (3500 Rad) from the same donor were used as APC (1:3). The cells were incubated at 37ºC for 6 days, the last 18 h in the presence of [^{3}H]thymidine (1 µCi/well). The suppressive activity was determined by calculating the relative difference in proliferative response (mean [^{3}H]thymidine incorporation (cpm) of triplicate wells) between $CD4^+CD25^-$ T cells cultured alone and $CD4^+CD25^-$ T cells cultured in the presence of $CD4^+CD25^{bright}$ T cells.

To evaluate the proliferative capacity of established $CD4^+CD25^+$ Tregs, FACS sorted $CD4^+CD25^{bright}$ T cells ($5x10^3$/well) were cultured with medium alone, human HSP60, LPS or IL-2 (10 ng/ml, Eurocetus, Amsterdam, The Netherlands), in the presence of APC and with or without TCR stimulation with anti-CD3. After 4 days the proliferative response was measured (mean [^{3}H]thymidine incorporation (cpm) of triplicate wells).

mRNA analysis by quantitative PCR

Total RNA from FACS sorted cells was isolated using Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 500 ng/ml. First-strand cDNA was synthesized from total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV, Promega, Madison WI, USA) with 1 µg/µl Oligo (dT) and 10 mM dNTPs (both Amersham Pharmacia Biotech AB, Uppsala, Sweden). The reaction mixture was incubated at 40°C for 90 minutes followed by incubation at 70°C for 15 minutes. To ensure the fidelity of mRNA extraction and reverse transcription to first-strand cDNA all samples were subjected to real time PCR amplification with primers specific for the constitutively expressed gene beta-2 microglobulin (ß2m). For FoxP3 and ß2m transcripts real-time quantitative PCR was performed with a LightCycler (Roche Diagnostics, Mannheim, Germany) based on specific primers and general fluorescence detection with SYBR Green. The following primer combinations were used; FoxP3 Forward 5′ TCA AGC ACT GCC AGG CG 3′, FoxP3 Reverse 5′ CAG GAG CCC TTG TCG GAT 3′ and ß2m Forward 5′ CCA GCA GAG AAT GGA AAG TC 3′, ß2m Reverse 5′ GAT GCT GCT TAC ATGTCT CG 3′. All PCR reactions were performed using LightCycler-FarstStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany).

A pool of cDNA from tetanus toxoid stimulated human peripheral blood mononuclear cells was used as a standard and normalization to ß2m was performed for each sample. Semi-quantitative levels of FoxP3 are expressed as percentage of the FoxP3 expression of the cDNA pool.

T cell capture and artificial APC

This technique is extensively described earlier [34]. Compared to the previously described protocol a few improvements were made. In short, PBMC of one DR4 homozygotic JIA

patient, one DR4 heterozygotic JIA patient and one DR4 heterozygotic RA patient obtained before and after transplantation were cultured with or without a peptide of human heat shock protein 60, HSP60 280-294 (GEALSTLVLNRLKVG). After 4 days the cells were prestained with anti-CD4-CY and subsequently incubated with Artificial Antigen-Presenting Cells (aAPCs) for 2 hours at 37°C. Before sorting the cells on the FACSvantage (Becton Dickinson), cells and aAPCs were washed twice and resuspended in FACS buffer. The aAPCs were prepared as follows. Phosphatidylcholine and cholesterol (Sigma) were combined in a glass tube at a molar ratio of 7:2. The solvent was evaporated under an Argon stream for 30 min and dispersed at a final concentration of 10 mg/ml in 140 mM NaCl and 10 mM Tris·HCl, pH 8 (buffer A) containing 0.5% sodium deoxycholate. Monosialoganglioside-GM1 (Sigma G-7641) was added at a final concentration of 0.28 mM. The solution was sonicated until clear and was stored at -20°C. Liposomes were formed through dialysis at 4°C against PBS in a 10-kDa Slide-A-Lyzer (Pierce) for 48 h. Biotinylated recombinant MHC was incubated with the peptide (6h, room temperature). The resulting MHC-peptide complexes were incorporated in rafts, engineered on the aAPC surface. The rafts were constructed by mixing biotinylated HLA-DR4 molecules, biotinylated antibodies to CD28 and anti-LFA-1, and biotinylated Cholera toxin subunit B-FITC conjugated (CTB-FITC; Sigma) in the appropriate (equal) molar ratio. Next, neutravidin (NA; Pierce) was added in a molar ratio of four biotinylated moieties per molecule of NA. CTB-FITC was used to visualize T cells bound by the aAPCs. After incubation (1.5h at room temperature), the Raft-NA mixture was added to the liposomes for 2 h, again at room temperature and washed 3 times in PBS. Finally, once the aAPCs were generated, they were incubated with the stained cells. CD4-CY and CTB-FITC double positive cells were sorted by FACS (FACS Vantage, Becton-Dickinson, San Jose, CA, USA) and analysed on the expression of mRNA FoxP3.

Results

Human HSP60 does not enhance the proliferation of $CD4^+CD25^{bright}$ Tregs

$CD4^+CD25^+$ Tregs are unable to proliferate in vitro when stimulated by most types of APC although proliferation does occur if the TCR is ligated in the presence of high doses of IL-2 [35;36]. A recent report suggested that LPS can also induce $CD4^+CD25^+$ Tregs survival/proliferation in mice even in the absence of TCR triggering [13], presumably via direct TLR4 ligation, which was found to be expressed in low levels on $CD4^+CD25^+$ T cells. Consistent with previous reports showing that human $CD4^+$ T cells do not express TLR4 [37], we found no expression of TLR4 on sorted $CD4^+CD25^{bright}$ T cells obtained from the peripheral blood of healthy controls nor on highly activated $CD4^+CD25^{bright}$ T cells obtained from the synovial fluid of JIA patients (data not shown).
To determine whether autologous HSP60 is able to induce the proliferation of established $CD4^+CD25^+$ Tregs via other undetermined receptors, we sorted $CD4^+CD25^{bright}$ T cells (1.0% brightest) of a healthy control and stimulated them with either medium alone, human HSP60, LPS or IL-2, in the presence of APC and with or without TCR stimulation with anti-CD3. In our experiments IL-2 plus anti-CD3 indeed resulted in a vigorous proliferation of the sorted $CD4^+CD25^{bright}$ T cells. However, the $CD4^+CD25^{bright}$ T cells did not proliferate with IL-2 alone or with HSP60 or LPS with or without anti-CD3 (**Fig. 1**). Furthermore, neither HSP60, nor LPS induced an upregulation of GITR, CTLA4, HLA-DR or CD69, or an increased expression of mRNA FoxP3 in sorted $CD4^+CD25^{bright}$ T cells. Taken together, these results demonstrate that, in human, autologous HSP60 and LPS do not enhance the proliferation and/or maturation of established $CD4^+CD25^{bright}$ Tregs (data not shown).

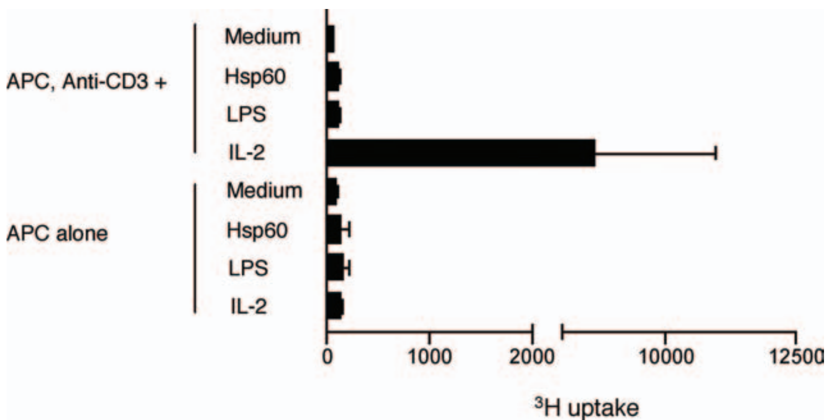


Figure 1: HSP60 does not enhance the proliferation of sorted $CD4^+CD25^{bright}$ T cells. FACS sorted $CD4^+CD25^{bright}$ T cells ($5x10^3$/well) were cultured with either medium alone, human HSP60, LPS or IL-2, in the presence of APC and with or without TCR stimulation with anti-CD3.

Activation of CD4$^+$CD25$^-$ T cells with human HSP60 induces a clear population of CD4$^+$CD25^{bright} Tregs that express the transcription factor FoxP3.

CD4$^+$CD25$^-$ T cells were isolated by MACS from the peripheral blood of healthy donors and cultured for 7 days in the presence of medium only, human HSP60, tetanus toxoid or LPS and in the presence of APC. After 7 days we evaluated the cultured cells on the expression of CD25 and the expression of mRNA FoxP3. Human HSP60 (**Fig. 2a**), as well as tetanus toxoid and LPS (data not shown) induced a large population of CD25 expressing cells. The CD25 intensity on the induced CD4$^+$CD25$^+$ T cells varied between intermediate and high expression, suggestive of two populations. The by HSP60 induced CD4$^+$CD25^{total} T cells varied between 1.5% and 36.4% (mean ± SEM: 17.1 ± 6.4), the induced CD4$^+$CD25^{bright} T cells varied between 0 and 10.1% (mean ± SEM: 3.8 ± 2.0) of total CD4$^+$ cells. Controls using polymyxin B or heat treatment excluded that an endotoxin contamination accounted for the bioactivity of the human HSP60.
We determined mRNA FoxP3 expression in MACS sorted CD4$^+$CD25$^-$ T cells before culturing and FACS sorted CD4$^+$CD25$^-$, CD4$^+$CD25^{int} and CD4$^+$CD25^{bright} T cells after culturing of CD4$^+$CD25$^-$ T cells with human HSP60, tetanus and LPS.

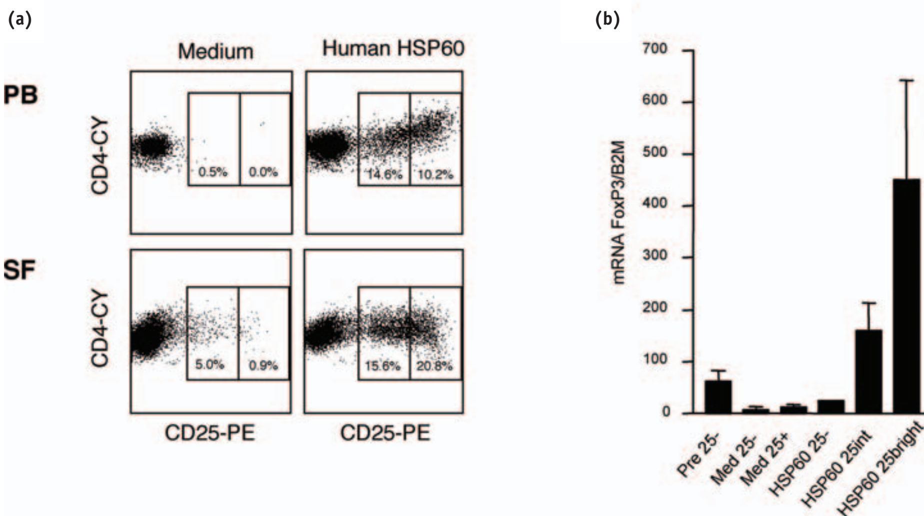


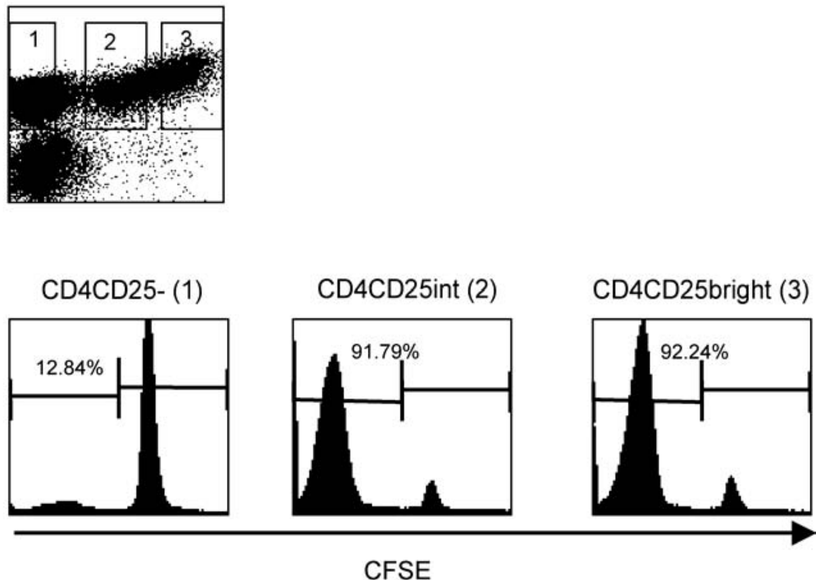
Figure 2. Stimulation of human CD4$^+$CD25$^-$ T cells with human HSP60 results in the induction of FoxP3$^+$CD4$^+$CD25$^+$ T cells. (a) MACS sorted CD4$^+$CD25- T cells from the PB of healthy controls or the SF of JIA patients were stimulated with human HSP60 in the presence of APC. After 7 days the expression of CD25 was evaluated by FACS. Shown are representative examples. **(b)** After 7 days CD4$^+$CD25$^-$ T cells and induced CD4$^+$CD25$^+$ T cells were sorted and evaluated on the expression of mRNA FoxP3 by quantitative PCR. Pre 25$^-$: CD4$^+$CD25$^-$ T cells before stimulation, Med 25$^-$: CD4$^+$CD25$^-$ T cells after 7 days of culture in medium, Med 25$^+$: CD4+CD25+ T cells induced after 7 days of culture in medium only, HSP60 CD25$^-$: CD4$^+$CD25$^-$ T cells after 7 days of culture with human HSP60, HSP60 25int: CD4$^+$CD25intermediate expressing T cells induced after 7 days of culture with human HSP60, HSP60 25bright: CD4$^+$CD25^{bright} expressing T cells induced after 7 days of culture with human HSP60.

Interestingly, human HSP60 (**Fig. 2b**) and LPS induced $CD4^{+}CD25^{bright}$ T cells, expressed significant amounts of mRNA FoxP3. Though the sorted $CD4^{+}CD25^{bright}$ T cells that were induced upon activation of $CD4^{+}CD25^{-}$ T cells with tetanus expressed some mRNA FoxP3 (mean ± SEM: 106.69 ± 38.14), the amounts were significantly lower than induced by HSP60 (**Fig. 2b**) and LPS (mean ± SEM: 385.2 ± 148.0). Thus, the quantity of Tregs induced upon activation (iTregs) seems to depend on the type of antigen. Interestingly, stimulating $CD4^{+}CD25^{-}$ T cells obtained from the synovial fluid of JIA patients resulted in similar CD25 and mRNA FoxP3 expression levels (**Fig. 2a**). This indicates that the inflammatory status in the joint has not exhausted the SF $CD4^{+}CD25^{-}$ T cell population and further induction of $CD4^{+}CD25^{+}$ Tregs is still possible.

Human HSP60 induced $CD4^{+}CD25^{bright}$ T cells have proliferated vigorously and have a highly activated phenotype.

To determine whether the induced $CD4^{+}CD25^{+}$ T cells had proliferated we stained the $CD4^{+}CD25^{-}$ T cells with CFSE before culture. After 7 days the CFSE intensity of $CD4^{+}CD25^{-}$, $CD4^{+}CD25^{int}$ and $CD4^{+}CD25^{bright}$ T cells was determined. All $CD4^{+}CD25^{+}$ T cells appeared to have proliferated vigorously in response to human HSP60 (**Fig. 3a**), LPS and tetanus (data not shown). Furthermore, we evaluated the induced $CD4^{+}CD25^{+}$ T cells on the expression of markers associated with $CD4^{+}CD25^{+}$ Tregs. The induced $CD4^{+}CD25^{+}$ T cells appeared to express extremely high levels of CTLA, GITR, CCR4 and CD30 (**Fig. 3b**). The expression of each of these 4 markers was a 10-fold higher than on conventional peripheral blood $CD4^{+}CD25^{bright}$ T cells [12].

(a)



(b)

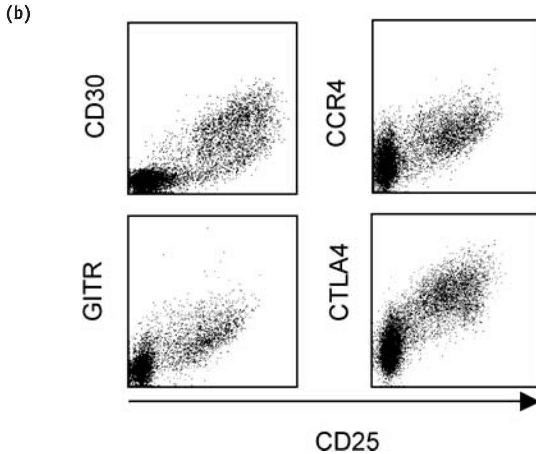


Figure 3: HSP60 induced CD4⁺CD25⁺ T cells have proliferated vigorously and gained a highly activated phenotype. (a) Representative example of MACS sorted $CD4^+CD25^-$ T cells obtained from a healthy volunteer stained with CFSE and cultured with human HSP60. After 7 days the percentage of divided cells was calculated. The induced $CD4^+CD25^+$ T cells had proliferated vigorously. **(b)** After 7 days of culture the expression of CD30, GITR, CCR4 and CTLA4 was evaluated. The expression of each of these markers clearly correlated with the expression of CD25.

The induction of $FoxP3^+CD4^+CD25^+$ Tregs by HSP60 is in part mediated via TLR4 on APC

The HSP60 induced expression of CD25 required the addition of APC (**Fig. 4a**). To assess whether HSP60 induced the expression of CD25 on cultured $CD4^+CD25^-$ T cells through the engagement of the TLR4 receptor on APC; we used a TLR4 blocking antibody in the above-described assays. Blocking the TLR4 receptor on APC before addition to the cultures resulted in a significantly decreased expression of CD25, as well as in a reduced expression of mRNA FoxP3 in response to human HSP60 (**Fig. 4b**) as well as to LPS (data not shown). These results indicate that LPS and HSP60 do not induce the differentiation to $CD4^+CD25^+$ Tregs by a direct effect on $CD4^+CD25^-$ T cells, but through the interference of APC and at least in part through ligation of TLR4 on APC.

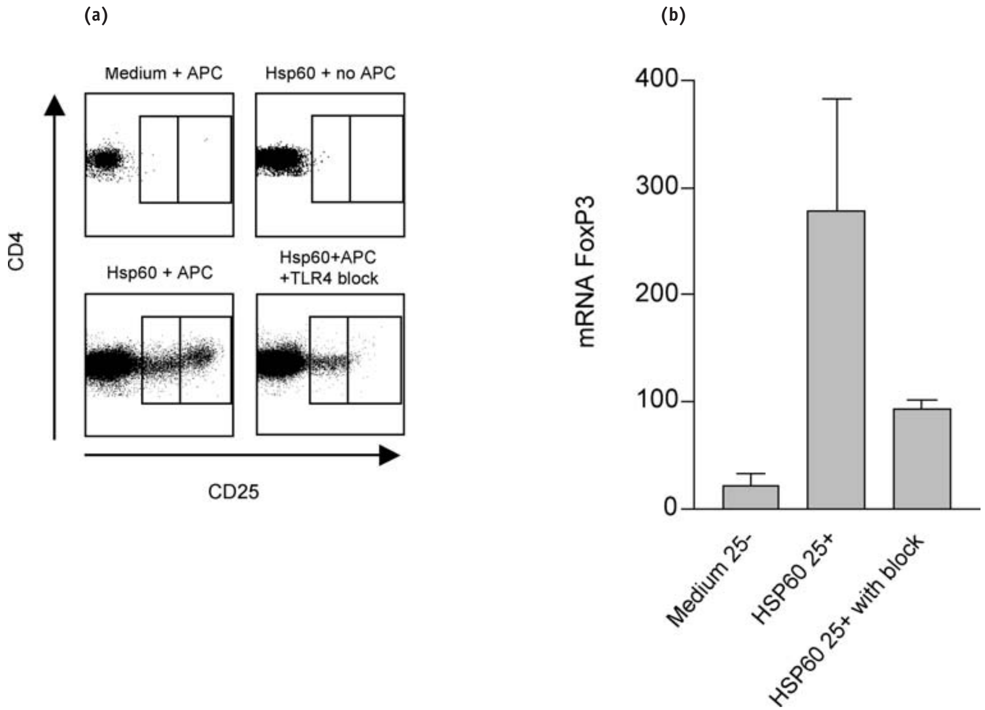


Figure 4: TLR4-block reduces the induction of CD4⁺CD25⁺ T cells upon stimulation of CD4⁺CD25⁻ T cells with human HSP60 (a) and the expression of mRNA FoxP3 in these cells (b). Macs sorted CD4⁺CD25⁻ T cells and APC were either blocked with anti-TLR4 blocking antibody or not and cultured with human HSP60. (a) Blocking TLR4 on APC resulted in a reduced expression of CD25, (b) as well as in a reduced expression of mRNA FoxP3.

Human HSP60 and LPS, but not tetanus, induce $CD4^{+}CD25^{bright}$ T cells that are highly suppressive in vitro.

To evaluate the suppressive capacity of the induced $CD4^{+}CD25^{bright}$ T cells we performed a suppression assay as described before. Fresh MACS sorted $CD4^{+}CD25^{-}$ T cells, stimulated with anti-CD3, in the presence of APC, were used as responder cells. FACS sorted $CD4^{+}CD25^{bright}$ T cells induced by a 7 day culture of $CD4^{+}CD25^{-}$ T cells in the presence of human HSP60, LPS or tetanus were added in different ratios (**Fig. 5**). The $CD4^{+}CD25^{bright}$ T cells induced by human HSP60 and LPS appeared to be highly suppressive, while the tetanus induced $CD4^{+}CD25^{bright}$ Tregs were not. These findings indicate that, in line with mRNA FoxP3 levels, the $CD4^{+}CD25^{bright}$ Treg cell population induced by human HSP60 or LPS, quantitatively and/or qualitatively differs from the tetanus induced population.

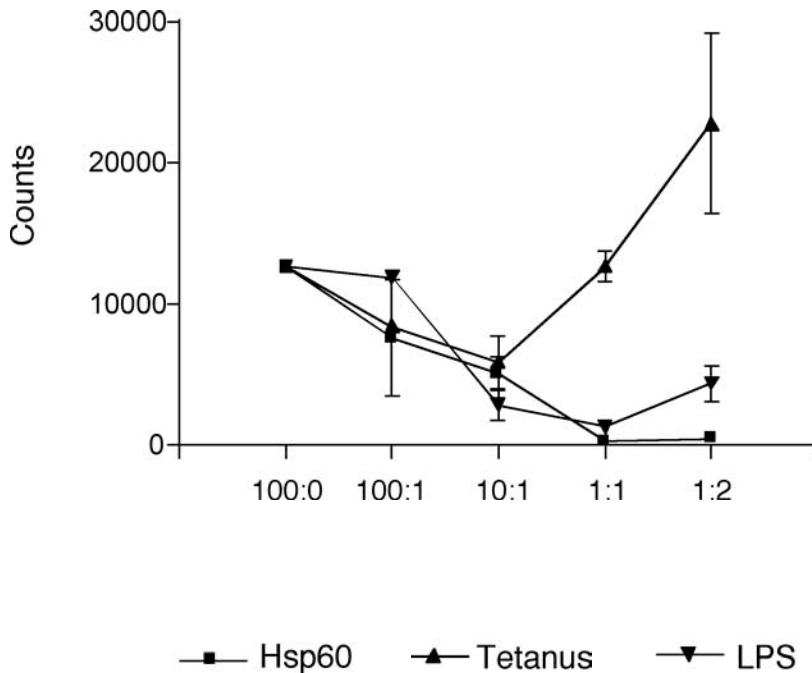


Figure 5: HSP60 and LPS but not tetanus induced CD4+CD25bright T cells are able to suppress CD4+CD25- responder cells. The induced CD4+CD25bright T cells were tested in suppression assays as previously described [12]. HSP60 as well as LPS induced CD4+CD25bright T cells were able to suppress CD4+CD25- T cells in the presence of APC and anti-CD3, while tetanus induced CD4+CD25bright Tregs were not.

Sorted human HSP60 epitope specific T cells express high levels of mRNA FoxP3

To investigate whether established CD4+CD25+ Tregs recognize epitopes of human HSP60, we used a newly developed technique, that uses artificial APC (aAPC), and is designed to capture peptide specific CD4+ T cells [34]. Artificial APC containing HLA-DR4 molecules were loaded with an epitope of human HSP60. Using the aAPC loaded with this self-HSP60 epitope, it was possible to sort HSP60 specific CD4+ T cells from one DR4 homozygotic JIA patient, one DR4 heterozygotic JIA patient and one DR4 heterozygotic RA patient. After sorting, mRNA FoxP3 expression levels of the human HSP60 specific T cells were analysed (**Fig. 6**). In each of the three patients, the sorted cells specific for the human HSP60 epitope, expressed high levels of mRNA FoxP3.

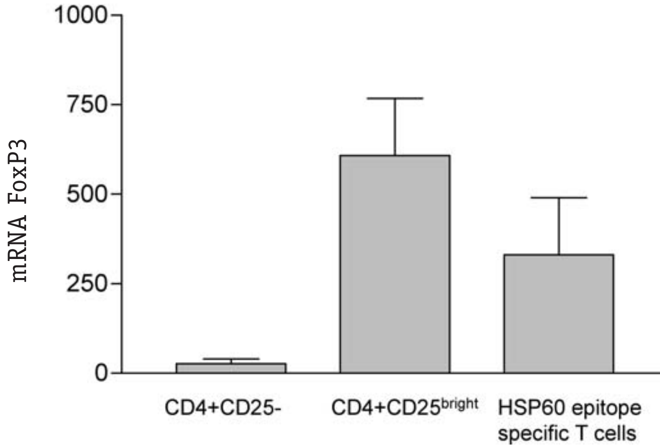


Figure 6: HSP60 specific T cells, isolated ex vivo, express mRNA FoxP3. HSP60 specific $CD4^+$ T cells were isolated from the peripheral blood of two JIA patients and one rheumatoid arthritis patient using aAPC. The sorted peptide specific $CD4^+$ T cells were lysed and analysed on the mRNA expression of the FoxP3 by quantitative PCR. MRNA levels are expressed as the mean percentage of expression compared to a standardized control cDNA.

Human HSP60 induced $CD4^+CD25^+$ Tregs coexpress CD30

Confirming previous observations in JIA [38], the cells that responded to human HSP60 showed a high expression of the surface marker CD30 (**Fig. 3b**). CD30, a member of the TNF receptor (TNFR) superfamily, is expressed on activated T and B-lymphocytes. Although earlier in vitro studies have shown that the engagement of CD30 by its ligand provides costimulatory signals to activated T cells and enhances cytokine production [39], the in vivo role of CD30 remains unclear. Recent studies suggest that $CD30^+$ T cells, present at sites of inflammation in autoimmune diseases such as rheumatoid arthritis and JIA may serve a regulatory role [38;40]. In addition, the expression of CD30 on antigen-induced Tregs has been suggested to be critical for their ability to suppress allograft rejection [41].

To investigate whether the expression of CD30 is more related to regulatory T cells or to activated T cells we analysed the relationship between the expression of CD30 and mRNA FoxP3 in sorted SF $CD4^+CD25^-$, $CD4^+CD25^{int}$ and $CD4+CD25^{bright}$ T cells (**Fig. 7a and b**). In each population, significantly higher levels of mRNA FoxP3 were found when CD30 was coexpressed on the membrane, suggesting a specific role of CD30 in either the development or function of $CD4^+CD25^+$ Tregs. Interestingly, the expression of CD30 also distinguished mRNA FoxP3 expressing cells present in the $CD4^+CD25^-$ population.

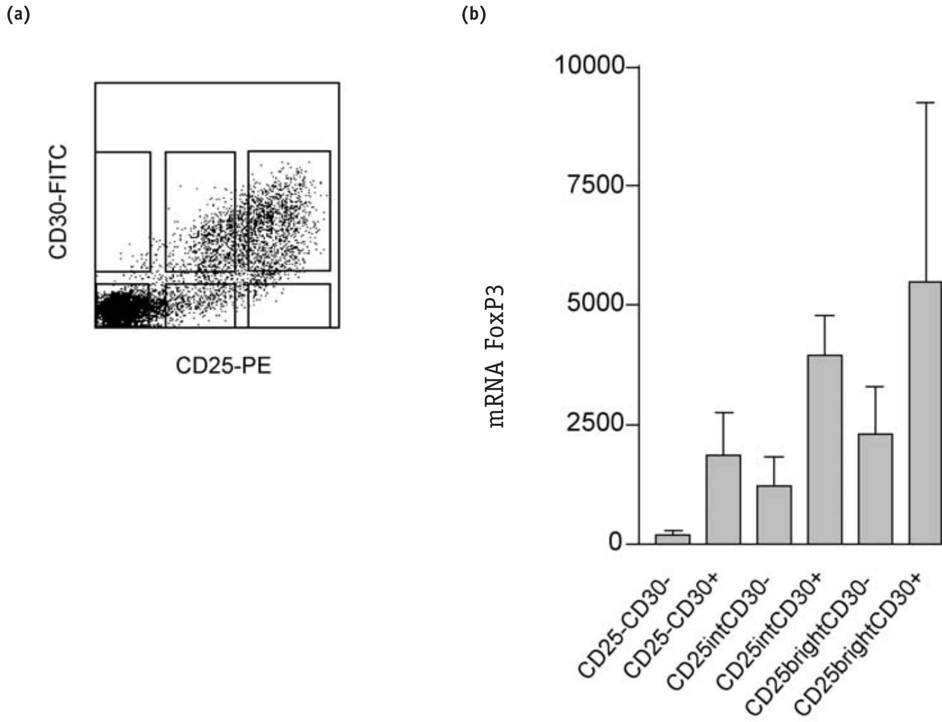


Figure 7. CD30 is preferentially expressed on FoxP3 expressing CD4⁺ T cells. CD4+CD25- T cells were cultured with HSP60. (a) After 7 days the indicated cell populations were FACS sorted and analysed on the expression of MRNA FoxP3. **(b)** $CD4^{+}CD25^{-}$ and $CD4^{+}CD25^{+}$ T cells co-expressing CD30, expressed the highest levels of mRNA FoxP3.

Discussion

It has only recently been shown, that besides the natural occurrence of $CD4^+CD25^+$ Tregs, $FoxP3^+CD4^+CD25^+$ T cells can also be generated in peripheral lymphoid organs [8;9]. Factors influencing the peripheral generation of $CD4^+CD25^+$ T cells are of considerable interest but so far poorly understood, especially in human and under inflammatory conditions. This study now shows that human HSP60 is able to drive the differentiation of human $CD4^+CD25^-$ T cells into highly suppressive, mRNA FoxP3 expressing $CD4^+CD25^+$ iTregs. In contrast, tetanus toxoid induced a similar expression of CD25 on $CD4^+CD25^-$ T cells, but much lower levels of mRNA FoxP3, and consequently, no in vitro suppressive effect.

Since HSPs are stress proteins, released in response to stressfull conditions, the above-described mechanism may drive T cell mediated immunoregulation specifically at sites of tissue damage. We previously described increased frequencies of $CD4^+CD25^+$ Tregs in the joints of patients with Juvenile Idiopathic Arthritis (JIA) and showed that this frequency inversely correlates with the clinical course of the disease. Interestingly, besides highly increased levels of mRNA FoxP3 in the synovial fluid $CD4^+CD25^{bright}$ T cells, significant expression levels of mRNA FoxP3 were found in the synovial fluid $CD4^+CD25^{int}$ T cells and even in the $CD4^+CD25^-$ T cell population. Taking this observation into account, the present data suggest that, besides being recruited from the periphery, part of the Tregs in the joints of JIA patients might have been differentiated from a FoxP3-negative T cell at the site of inflammation, perhaps via the presentation of HSP60 epitopes. Though not conclusive, we further substantiated this hypothesis by isolating human HSP60-epitope specific T cells from the peripheral blood of arthritis patients with the use of a newly developed technique to capture $CD4^+$ T cells [34]. These ex vivo isolated HSP60 specific T cells expressed high levels of mRNA FoxP3, indicating that part of the established $CD4^+CD25^+$ Tregs in arthritis patients are able to recognize epitopes of human HSP60. Though we could not determine whether the ex vivo isolated HSP60 specific T cells gained FoxP3 in the periphery or whether they were as such derived from the thymus, we herewith identified human HSP60 as the first known autoantigen, recognized by human naturally occurring $CD4^+CD25^+$ Tregs.

The induction of $FoxP3^+CD4^+CD25^+$ Tregs by human HSP60 was partly suppressed by blocking the TLR4 receptor on APC. In mice it has been shown that signalling through TLR4 on $CD4^+CD25^+$ Tregs elicits their proliferation, prolongs their survival, and augments their in vitro suppressive activity, even in the absence of APC [13]. Thus in mice, besides driving the local induction of $CD4^+CD25^+$ Tregs, HSP60, may play a role in the activation and/or proliferation of naturally occurring $CD4^+CD25^+$ Tregs that homed to the site of inflammation. Importantly, our data reveal an essential difference between mice and human $CD4^+CD25^+$ Tregs: In contrast to what was found in mice, human $CD4^+CD25^+$ Tregs do not express TLR4. Consequently, neither HSP60, nor LPS, exerted any effect on the maturation/differentiation status of established $CD4^+CD25^+$ nTregs, in terms of expression levels of activation markers and/or mRNA FoxP3. In addition, sor-

ted nTregs showed no proliferation in response to human HSP60 or LPS. Thus, while in mice TLR4 signalling effects T cell mediated immunoregulation through a direct effect on $CD4^{+}CD25^{+}$ Tregs, in human the interference of APC is needed.
Recently, Pasare and co-authors showed that ligation of TLRs on DCs overcomes suppression by $CD4^{+}CD25^{+}$ T cells [42]. This block of suppressor activity was dependent on the production of IL-6, which renders responder T cells refractory to suppressors. The data in this study show now that, besides blocking suppressor activity, ligation of TLR4 on APC results in the induction of newly generated $CD4^{+}CD25^{+}$ Tregs. Taking into account that TLRs are primary sensors of microbial infection, these two observations make sense. At the start of an infection ligation of TLRs should be able to overcome the function of Tregs so that pathogen specific T cell responses can be initiated. However, when the pathogen has been cleared and the production of IL-6 decreased, inflammatory cells will regain susceptibility to suppression by naturally as well as induced Tregs, enabling the downregulation of inflammation. Thus, depending on the local milieu, HSPs confer signals that either augment or attenuate T cell mediated immunoregulation.
Besides the expression of CD25 and mRNA FoxP3, human HSP60 induced the expression of CD30 on part of the $CD4^{+}CD25^{-}$ T cells. CD30, a member of the TNF receptor (TNFR) superfamily, is expressed on activated T and B lymphocytes [39]. Although, in vitro studies have shown that CD30-CD30L interaction has effect on both co-stimulation and cytokine production [43;44], the in vivo role of CD30 remains unclear. Recent studies implicated a role for CD30 in the regulation of inflammatory processes [38;40;45;46] and in the suppression of allograft rejection [41]. However, direct evidence that CD30 is required for or contributes to immune regulation is still lacking. Interestingly, the present data clearly show a positive correlation between the expression of CD30 on $CD4^{+}CD25^{+}$ T cells as well as $CD4^{+}CD25^{-}$ T cells and the expression levels of mRNA FoxP3. Since the expression levels of mRNA FoxP3 are an indication of the regulatory capacity of the cell [12;47], this finding confirms a role for CD30 in regulating immune responses. CD30 may therefore function as an extra marker for the identification of $CD4^{+}CD25^{+}$ Tregs.
$CD4^{+}CD25^{+}$ Tregs require recognition of their antigen through the TCR to exert their suppressive activity. However, due to linked suppression, $CD4^{+}CD25^{+}$ Tregs need only recognize a limited number of antigens from the inflammatory tissue to be able to regulate responses to other antigens in that tissue [48;49]. The identification of HSP60 as an antigen that can be recognized by part of the established $CD4^{+}CD25^{+}$ Tregs and that is presented at sites of inflammation, like in the gut of patients with Crohn's disease [50] and in the joints of patients with JIA and RA [16;24;51], now opens the way towards new antigen-specific and site-specific therapies for autoimmune diseases. Since the proinflammatory effects of HSPs seem to be the result of the activation of innate immunity and the regulatory effects are mediated through TCR recognition, it may be possible to specifically induce HSP60 specific $FoxP3^{+}CD4^{+}CD25^{+}$ Tregs, while preventing innate activating side effects of HSP60.

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5

Autologous stem cell transplantation for refractory juvenile idiopathic arthritis: analysis of clinical efficacy, mortality and transplant related morbidity

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Annals of Rheumatic Diseases 2004 October; 63(10): 1318-26

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Summary

Objective: To evaluate safety and efficacy of Autologous Stem Cell Transplantation for refractory Juvenile Idiopathic Arthritis.
Design: Retrospective analysis of follow up data on 34 children with JIA who were treated with ASCT in nine different European transplant centers. Rheumatological evaluation employed a modified set of core criteria. Immunological reconstitution and infectious complications were monitored at three months interval after transplantation.
Results: Clinical follow up ranged from 12 to 60 months. Eighteen of the 34 patients (53%) with a follow up of 12 to 60 months achieved a drug-free remission. Seven of these patients had previously failed treatment with anti-TNF. Six of the 34 patients (18%) showed a partial response (ranging from 30-70% improvement) and seven (21%) were resistant to ASCT. Infectious complications were common. There were three cases of transplant related mortality (9%) and two cases of disease related mortality (6%).
Conclusions: ASCT in severely ill JIA patients induces a drug-free remission of the disease and a profound increase in general wellbeing in a substantial proportion of patients, but the procedure carries a significant mortality risk. The following adjustments are proposed for future protocols: (1) elimination of total body irradiation from the conditioning regimen, (2) prophylactic administration of antiviral drugs and intravenous immunoglobulines until there is a normal $CD4^+$ T cell count.

Introduction

Juvenile Idiopathic Arthritis (JIA) is the most common rheumatic disease in childhood and a major cause of disability. Although the overall prognosis for most children with chronic arthritis is good, in 5-10% of children with the systemic and polyarticular onset forms, the disease is refractory to conventional therapies, which comprises combinations of non-steroidal anti-inflammatory drugs (NSAID) and immunosuppressive drugs such as methotrexate (MTX) and corticosteroids [1-5]. In such cases, patients have considerable morbidity from severe joint destruction, growth retardation, psychosocial morbidity and various adverse effects from long-term treatment with immunosuppressive drugs. Mortality in the total group of systemic onset children is estimated on 2-4% [6]. The major causes of mortality relate to drug toxicity, intercurrent infection, amyloidosis [7] or the evolution of disease to conditions overlapping with other rheumatic diseases, such as systemic lupus erythematosis or scleroderma [8].
Recently, the introduction of anti-tumor necrosis factor (TNF) therapy appeared to have a major impact on outcome of children with polyarticular JIA who were unresponsive to methotrexate, with a persistent response of up to 80% [9]. However, in active systemic onset JIA this treatment seems less effective [10-12]. Another new treatment approach for patients with severe autoimmune disease involves intense immunosuppression followed by autologous hematopoietic stem cell transplantation (ASCT) [13-17]. The rationale of this treatment is based on the concept of intense immunosuppression, with subsequent regeneration of naïve T lymphocytes derived from reïnfused hematopoietic progenitor cells. The European League Against Rheumatism (EULAR) and the European Group for Blood and Marrow Transplantation (EBMT) have published guidelines on inclusion criteria, conditioning regimen and manipulation of the graft [18,19]. Since 1997, ASCT has been applied as an experimental procedure in a substantial group of children with refractory polyarticular and systemic JIA [20-23]. The majority of these patients (n = 41) can be identified in the registry of the EBMT. Recently we published a short report on the general outcome of 31 of these children [24]. The reported mortality of ASCT in JIA is a major concern. We here report a more detailed survey of the follow up of 34 of the registered JIA patients who were treated with ASCT in 9 different European centers. Safety and efficacy of ASCT in this severely ill patient group are evaluated with specific attention to infectious complications and transplant related mortality. New adjustments in the transplantation protocol are proposed.

Patients and methods

Patient selection

We present data on 34 of the 41 JIA patients who received ASCT, identified in the registry of the EBMT. The patients were transplanted in nine different pediatric transplantation centers in Europe (**Table 1**). Of seven patients, registered at the EBMT, data were not detailed enough to allow follow up evaluation.

Assessment of efficacy

For rheumatological follow up the core set criteria described by Giannini and adopted by the Pediatric Rheumatology International Trials Organization group (PRINTO) were used [25-29]. The core set consists of:
1) physician's global assessment of overall well-being (visual analogue scale, VAS);
2) parent/patient assessment of pain (VAS);
3) functional disability (Child Health Assessment Questionnaire, CHAQ);
4) number of joints with active arthritis;
5) number of joints with limited range of motion (pediatric EPM-ROM);
6) erythrocyte sedimentation rate (ESR) [27-29].

The evolution of the disease in the patients was analysed by collecting the outcome of the core set of variables as recorded before stem cell collection and at three months intervals after transplantation.
Owing to the retrospective nature of this study, not all core set criteria were available from every time point for every child; therefore the Giannini's criteria for improvement were adapted. To meet the definition of improvement at a certain time point, patients had to have a persistent 30% or more improvement from baseline in at least three of the available variables (instead of three out of six variables according to Giannini). Partial responders were thus defined as patients with a persistent 30%, 50% or 70% improvement from baseline in at least three response variables. They could also have worsening of 30% in no more than one of the available variables (instead of no more than one out of 6 variables according to Giannini).
As no criteria for complete clinical remission in JIA have been defined, the American College of Rheumatology (ACR) criteria for complete remission in rheumatoid arthritis (RA) were used [30]. Full responders were defined as patients in complete drug-free remission, fulfilling at least 5 or more of the following requirements for the last two consecutive months; morning stiffness less than 30 minutes, no fatigue, no joint pain, no joint tenderness or pain on motion, no soft tissue swelling in joints or tendon sheaths and ESR <20 mm/h.

A relapse was defined as a persistent worsening of 30% or more in three of the available core set criteria variables and a minimum of two active joints. There could also be improvement of 30% or more in no more than one of the six response variables. Flare up of disease was defined as a transient worsening of 30% or more in three of the available core set criteria variables and a minimum of two active joints.

Table 1

Clinical characteristics

Pt	Prot.	Gender	Type	Onset	Medication history	Toxicity antirheumatica	ASCT	f.u.
1	A	f	Syst	11m	St, MTX, NSAID, CsA, Aza, iv Ig	GD, O	6y, 7m	5y
2	A	m	Syst	4y, 7m	St, MTX, NSAID, CsA, Sulfa, Aza, Plaq, Cy, Aza, Plaq, Cy	GD, O	10y, 6m	4y
3	A	m	Syst	2y, 6m	St, MTX, CsA, NSAID	GD, O	11y, 5m	4y, 6m
4	A	m	Syst	1y, 2m	St, MTX, CsA, NSAID, Cy, anti-TNFα	GD, O, vertebral fractures, hypertension, hypertonia	4y, 11m	†
5	A	m	Syst	4y, 10m	St, MTX, CsA, NSAID, Aza, iv Ig	GD, O	9y, 5m	3y, 6m
6	A	m	Poly	1y, 6m	St, MTX, CsA, Sulfa, Gold im, hydroxy	GD, O, severe Cushing, hypertensive convulsions, proteinuria	9y, 3m	3y
7	A	f	Syst	5y, 2m	St, MTX, CsA, NSAID, Sulfa, Aza, iv Ig	GD, O	11y, 3m	4y
8	A	f	Syst	3y, 8m	St, MTX, CsA, NSAID, Aza, hydroxy	GD, O, severe relapsing pneumonitis	14y, 11m	†
9	A	f	Poly	3y, 11m	St, MTX, CsA, NSAID.	GD, O	7y, 10m	5y
10	A	f	Syst	7y, 7m	St, MTX, CsA, NSAID	GD, O, liverfailure	10y, 7m	2y, 6m
11	A	m	Syst	1y, 8m	St, MTX, CsA, NSAID, anti-TNFα	GD, O	5y, 2m	1y
12	A	m	Poly	2y, 2m	St, MTX, CsA, NSAID, Sulfa	GD, O, severe GI toxicity	8y, 5m	2y
13	A	m	Syst	4y, 1m	St, MTX, NSAID, Sulfa, auro, Aza	GD, O, severe myelosuppression	13y, 10m	4y, 6m
14	A	f	Syst	3y, 1m	St, MTX, CsA, NSAID	GD, O	5y, 5m	2y
15	A	m	Syst	3y, 2m	St, MTX, CsA, NSAID	GD, O, Cushing	4y, 3m	†
16	A	m	Syst	5y, 5m	St, MTX, CsA, NSAID, anti-TNFα	GD, O, severe GI toxicity	7y, 9m	1y, 6m
17	A	m	Syst	2y, 10m	St, MTX, CsA, NSAID, anti-TNFα	GD, O	11y, 7m	1y
18	A	m	Syst	1y, 6m	St, CsA, MTX, NSAID	GD, O, hypertension	8y, 2m	1y, 6m
19	A	m	Poly	3y, 1m	St, MTX, NSAID, sala	GD, O	5y, 8m	4y
20	A	m	Syst	8y, 11m	St, MTX, CsA, NSAID, Cy,	GD, severe myelosuppression anti-TNFα, iv Ig	12y, 4m	1y

21	B	f	Syst	2y, 11m	St, MTX, NSAID, iv Ig	GD, cataracts, avascular necrosis of femoral head	11y, 1m	2y, 6m
22	B	m	Syst	8m	St, MTX, CsA, NSAID	Growt delay, cataracts, avascular necrosis of femoral head	8y, 9m	2y
23	B	f	Syst	4y, 3m	St, MTX, CsA, NSAID, Cy, iv Ig	GD, Cushing, GI hemorrhage, avascular necrosis of femoral head	17y	3y, 6m
24	C	f	Syst	2y, 9m	St, MTX, CsA, NSAID, Cy, Chlor, iv Ig, anti-TNF-α	GD, severe hypertension	5y	2y
25	C	f	Syst	9y, 2m	St, MTX, CsA, NSAID, Cy, Chlor, iv Ig, anti-TNFα	GD, severe GI toxicity	14y, 9m	2y
26	C	f	Syst	2y, 8m	St, MTX, CsA, NSAID, Chlor, iv Ig, anti-TNFα	GD, severe myelosuppression, hypertension, photosensitivity	9y, 9m	1y
27	B	m	Syst	10m	St, MTX, CsA, NSAID, Cy, anti-TNFα	GD, O, compression vertebrae	5y, 6m	1y, 6m
28	B	m	Syst	8y, 4m	St, MTX, CsA, NSAID	GD, O, avascular necrosis femoral head, compression vertebrae	11y	1y
29	B	f	Syst	7y, 1m	St, MTX, CsA, NSAID	GD, O, intestinal perforation, compression vertebrae	9y,1m	†
30	A	f	Syst	2y, 1m	St, MTX, CsA, NSAID	GD, O, compression fractures	4y, 10m	2y, 6m
31	A	f	Syst	2y, 7m	St, MTX, CsA, NSAID, anti-TNFα	GD, O	8y, 5m	†
32	A	m	Syst	10y, 2m	St, MTX, CsA, NSAID, Cy, iv Ig	GD, O, hepatic dysfunction	18y, 3m	3y
33	A	f	Syst	4y, 8m	St, CsA, MTX, NSAID	GD, O, severe hypertension	6y, 9m	1y, 6m
34	B	m	poly	3y, 3m	St, MTX, NSAID, iv Ig	GD, O, vertebral fractures, cataracts	9y, 11m	1y, 6m
Total		**19/15 (m/f)**	**29/5**	**3y, 11m**			**9y, 5m**	**2y, 4m**

Table 1: Clinical characteristics before ASCT

anti-TNFα = anti-Tumor Necrosis Factor receptor therapy, Auro = Auromyosine, Aza = Azathioprine, Chlor = Chlorambucil, CsA = Ciclosporine A, Cy = Cyclophosphamide, f.u. = follow up in years and months, GD = growth delay, Hydroxy = hydroxychloroquine, iv Ig = intravenous Immunoglobulins, m/f = male/female, MTX = Methotrexate, NSAID = Non Steroidal Anti-Inflammatory Drugs, O = osteoporosis, Plaq = Plaquenil, poly = polyarticular JIA, prot. = conditioning protocol, Pt = patient, St = steroids, Sulfa = Sulfasalazine, syst = systemic JIA. Age of onset, age of ASCT and follow up are expressed in years (y) plus months (m), the totals (Tot) are given as the mean. † = mortality case.

Statistical methods

Treatment efficacy was evaluated by testing whether there was a difference in the core set criteria scores between baseline and the three monthly evaluations after transplantation, using the Wilcoxon signed-rank test. The effectiveness of total body irradiation (TBI) was evaluated by comparison of core set criteria scores between patients treated with TBI and patients treated without TBI, using a Mann-Whitney U test. In order to evaluate the extent and duration of immunosuppression, the Wilcoxon signed-rank test was used to assess whether there were significant differences between baseline and the three monthly measurements of laboratory parameters. To test whether there was a difference in T cell recovery between patients with a complete response and patients with no or partial response, or between patients treated with different conditioning regimens, a Mann-Whitney U test was used. Correlations were calculated by linear regression.

Results

Clinical characteristics before ASCT

The clinical characteristics before ASCT in all children were a polyarticular course complicated by erosions, osteoporosis and stunted growth (**Table 1**). In addition, all children with systemic JIA suffered periods of spiking fever, exanthemata and severe corticosteroid related side effects. Ten of the 34 children had failed treatment with anti-TNF therapy. The mean time interval between diagnosis and transplant was 5 years, 4 months (SEM ± 6 months). More details are shown in **Table 1**.

Inclusion and exclusion criteria for ASCT

In order to be eligible for ASCT, each center used the agreed inclusion and exclusion criteria [18,19]. The inclusion criteria were failure to respond to high dose MTX given by the i.m. or s.c. routes (1 mg/kg/wk or 15 mg/m^2), in addition to failure to respond to at least 1 other disease modifying anti-rheumatic drug (DMARD), corticosteroid dependency and/or unacceptable toxicity to DMARD's or corticosteroids. After anti-TNF therapy became available failure to these agents was added to the inclusion criteria. Exclusion criteria were cardio-respiratory insufficiency, chronic active infection, persistent fever and other signs of systemic disease activity despite corticosteroids at time of transplant, end stage disease (Steinbrocker IV) or poor compliance.

Bone marrow harvest and T cell depletion

In 25 patients, bone marrow was harvested at least one month prior to ASCT. In nine patients autologous hematopoietic stem cells were mobilized into the peripheral blood using a single infusion of cyclophosphamide at 1.5-3.0 g/m^2 and granulocyte colony-stimulating factor (G-CSF) at 10 µg/kg/d. The graft was either purged by T cell depletion with CD2 and CD3 antibodies or by positive stem cell selection using CD34 selection devices. In the nine cases of peripheral bone marrow collection these techniques yielded a final suspension with a CD34$^+$ cell count of 2.9–10.9x10^6 cells per kg (mean 5.2x10^6 cells per kg) and with a CD3$^+$ cell count of 0–1.4x10^5 cells per kg (mean 0.3x10^5 cells per kg). In the 25 cases of bone marrow collection these techniques yielded a final suspension with a CD34$^+$ cell count of 0.44–6.0x10^6 cells per kg (mean 2.2x10^6 cells per kg) and with a CD3$^+$ cell count of 0–3.5x10^5 cells per kg (mean 0.7x10^5 cells per kg). The cell suspensions were frozen and stored until reinfusion [31].

Conditioning for ASCT

In all, three different conditioning regimens were used (**Table 2**). Conditioning regimen A (23 patients) included 4 days of anti-thymocyte rabbit immunoglobulin (ATG,

SangStat, Fremont, CA, USA), 5 mg/kg from day –9 to –6, cyclophosphamide, 50 mg/kg/day from day –5 to –2 and low dose total body irradiation (TBI, 4 gray, single fraction) on day –1. Conditioning regimen B (8 patients) included the above scheme without TBI. Conditioning regimen C (3 patients) included fludarabine (30 mg/m2) on day –7 and –6, cyclophosphamide (50 mg/kg/day) from day –5 to –2, ATG (5 mg/kg) from day –6 to –3, methylprednisolone (1 g/m2) from day –4 to –2 and no TBI. In all 34 children the frozen stem cell suspension was thawed and infused at day 0. Methotrexate and Cyclosporin A (CsA) were stopped before ASCT and prednisone was tapered, when possible, starting not earlier than 2 months after ASCT. Disease flares or relapses were treated as before ASCT.

Engraftment and immunological reconstitution

Engraftment occurred in all 34 patients with full hematological recovery. Detailed immunological reconstitution data were available of 29 patients (85%). In these 29 patients neutrophil recovery (> 0.5 x 10^9/l) occurred at day 8 to 30 post ASCT (mean ± SEM; 16 ± 1.1 days). Details on T cell recovery are given in **Fig. 1** [32]. All children suffered prolonged depression of $CD4^+$ T cells, a common immunological feature also seen after intense chemotherapy [33,34]. 12 months after ASCT (range 6-18 months) numbers of $CD4^+$ T cells were normal when compared to baseline (Wilcoxon-signed rank test).

A relatively fast reconstitution of $CD8^+$ T cells resulted in an inverted $CD4^+$/$CD8^+$ T cell ratio in 24 of 29 patients (83%) lasting 3-18 months (mean ± SE; 9.2 ± 1.1 months). An early predominance of $CD45RO^+$ memory cells was followed by the reappearance of $CD45RA^+$ naïve cells after 6-9 months (**Fig. 1**). 3 to 18 months after ASCT (mean ± SE; 10.5 ± 1.6 months) in vitro mitogenic responses were normal. No differences were seen in T cell recovery between patients with a complete response and patients with no or partial response or between patients treated with different regimens.

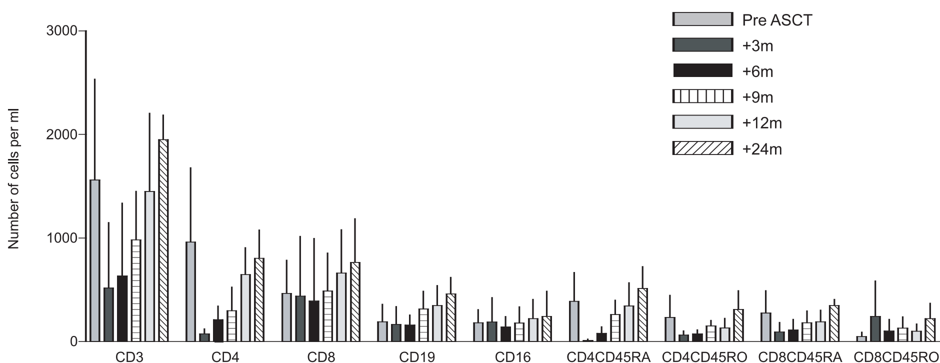

Figure 1. Reconstitution of lymphocyte subsets in 29 JIA patients after ASCT for 24 months (mean ± SE).

General outcome

Complete data on 6 core set criteria (baseline and at 3 months interval) were available for 22 of the 34 patients (65%). For the other 12 patients data on 3 to 5 core set criteria at 3 months intervals were available, always including the number of active joints. In these patients only three or more criteria were used to make an estimation of the improvement. The general outcome of the patients is summarized in **Table 2**. Eighteen of the 34 patients (53%) with a follow up of 12 to 60 months (mean ± SD; 29 ± 12 months) showed a complete drug free remission. Seven of these patients had previously failed treatment with anti-TNF therapy. Five patients, who later achieved complete drug-free remission, had flares of disease 3-6 months post ASCT; these occurred either spontaneously while tapering the prednisone or following infection. Slower tapering of prednisone and/or low dose of NSAID, and in one case intra-articular corticosteroids, was enough to control the flare.

Table 2

Clinical improvement after ASCT at maximal follow up

Center	Protocol	Complete remission	Partial response			No response	TRM**	Total
			30%	50%	70%			
Utrecht [1]	A	5	-	3	1	2*	1	12
Leiden [2]	A	3	1	-	-	2	1	7
London [3]	A	1	-	-	-	-	-	1
Gothenburg [4]	A	1	1	-	-	1*	-	3
Brussels [5]	B	3	-	-	-	-	-	3
Halle-Wittenberg [6]	B	1	-	-	-	-	-	1
Paris [7]	B	1	-	-	-	1	1	3
Newcastle [8]	B	1	-	-	-	-	-	1
Jena [9]	C	2	-	-	-	1	-	3
Total		**18**	**2**	**3**	**1**	**7**	**3**	**34**

Table 2: Clinical improvement after ACST at maximal follow up

[1] Wilhelmina Children's Hospital, Utrecht, The Netherlands. [2] Leiden University Medical Center, Leiden, The Netherlands. [3] Great Ormond Street Hospital, London, Great Britain. [4] The Queen Silvia Children's Hospital, Gothenburg, Sweden. [5] University Hospital of Pediatrics, Brussels, Belgium. [6] Martin-Luther-University, Halle-Wittenberg, Germany. 7Necker- Enfants Malades, Paris, France. [8] Newcastle General Hospital, Newcastle, Great Britain. [9] Children's University Hospital, Jena, Germany.
* One case in Utrecht and 1 case in Gothenburg of Disease Related Mortality (DRM). **TRM = Transplant Related Mortality

No response was noted in 7 of the 34 patients (21%). After an initial phase of improvement during immunoablation, these patients showed a complete relapse of the disease, 3 to 17 months after ASCT, which was as severe as before and needed treatment with high doses of prednisone, MTX, anti-TNF therapy, CsA and in one case even cyclophosphamide pulse therapy. In these patients the disease was as refractory to corticosteroids and other antirheumatic drugs as before ASCT. Two other patients who showed a complete relapse died 13 and 16 months after ASCT. The first patient developed a full-blown relapse 13 months after ASCT and was treated with high dose prednisone,

MTX and cyclophosphamide pulse therapy. While waiting for HLA-matched allogeneic bone marrow he developed an EBV infection and died of hepatic failure, being severely immune suppressed. The second patient developed a primary varicella zoster virus (VZV) infection 13 months after transplantation. At that time she was being treated with anti-TNF therapy (etanercept) and MTX. Antirheumatic drugs were stopped and the patient was treated successfully with aciclovir. However, 2 months later she unexpectedly developed jaundice and died of liver failure of unknown etiology.
Six of the 34 patients (18%) showed a partial response. One of these patients had previously failed anti-TNF therapy. In these 6 patients the treatment with either low dose of prednison (2 patients, 2.5 and 5 mg prednisone daily) or other antirheumatic drug was reïnstituted at time of the relapse, resulting in a subsequent improvement of the disease. Using the Giannini core set criteria for improvement of disease (with modifications as above), these patients, being refractory to these drugs before ASCT now showed a 30-70% improvement in their disease. **Fig. 2** shows Kaplan Meier curves with the proportion of surviving patients and the proportion of surviving patients free of either a partial or complete recurrence of the disease. Interestingly, all cases of mortality and all partial and complete relapses of disease occurred in the first 18 months after ASCT.

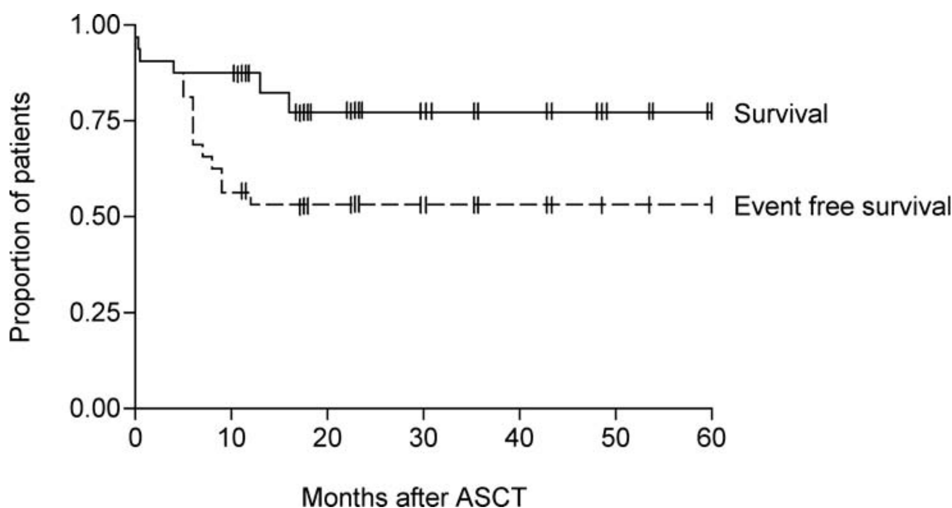

Figure 2. Kaplan Meier curves. The curves show the proportion of surviving patients and the proportion of event-free surviving patients. An event is defined as either a partial or complete recurrence of disease. Each bar mark represents the maximal follow up per patient.

Rheumatological follow up

Fig. 3 shows the available rheumatological follow up data for all patients (mean ± SD). With the exception of EPM-ROM scores at time points 24, 36 and 48 months post-ASCT, there was a significant decrease in all core set criteria scores at time points 3, 6, 12, 24, 36 and 48 months after ASCT ($p \leq 0.04$).

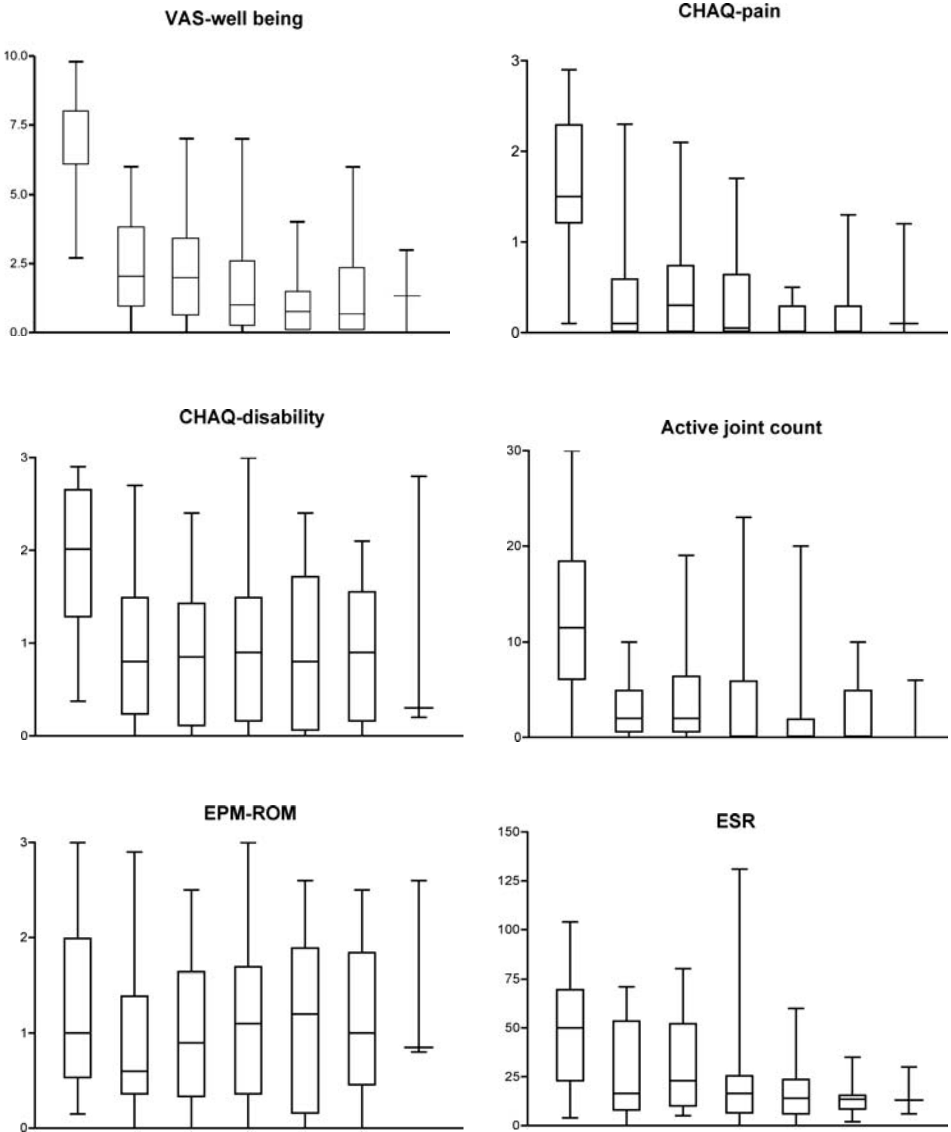

Figure 3. Rheumatological follow up before and at three monthly intervals after ASCT (mean ± SD). Error bars = SD. Numbers below the x-axis indicate ratios of the available data (number of data available/number of patients with indicated follow-up). With the exception of EPM-ROM scores at time points 24, 36 and 48 months post-ASCT, all scores post ASCT were significantly decreased compared with baseline (Wilcoxon signed-rank test, all p values ≤ 0.04). *Top left:* physician's global assessment of overall well-being (VAS, range 0-10); top right: parent/patient assessment of pain (VAS, range 0-3); *middle left:* functional disability (CHAQ, range 0-3); middle right: number of active joints (0-54); *bottom left:* number of joints with limited range of motion (pediatric EPM-ROM, range 0-3); *bottom right:* erythrocyte sedimentation rate (ESR, in mm/hour).

No correlation could be found between stem cell source, number of reïnfused T cells or engraftment with clinical outcome. No statistically significant difference was seen in core set criteria scores obtained 12 and 24 months after ASCT between patients conditioned with protocol A (no TBI) and protocol B or C (with TBI).

Table 3. Infectious complications

Pt	Infections	T
1	VZV, skin	< 3m
2	Mycobacterium Szulgai, eye	6m
3	Xanthomonas maltophilia, stool	< 3m
	HSV, hepatitis	24m
4	EBV, dissiminated (*)	12m
	Streptococcus viridans, blood	< 3m
5	VZV, skin	< 3m
6	VZV, skin	6m
	Hepatitis A	18m
7	Streptococcus, blood	< 3m
	VZV, skin	18m
8	CMV reactivation	4m
	EBV (*, MAS)	4m
	Candida oesophagitis	< 3m
9	CNS, blood	< 3m
	VZV, skin	9m
10	None	
11	H. Influenzae, blood	< 3m
	CNS, blood	< 3m
	VZV, skin	9m
12	none	
13	VZV skin	< 3m
	CMV reactivation	< 3m
14	none	
15	CNS, blood	< 3m
	Adenovirus type 3, faeces (*)	< 3m
16	HSV type 1, throat	12m
	Influenza A, nose	< 3m
	VZV, skin	4m
17	Streptococcus mitis, blood	< 3m
	CMV reactivation	< 3m
	VZV, skin	12m
18	HSV type 1, throat	< 3m
19	Streptococcus mitis, blood	< 3m
	Generalized VZV	< 3m
	Primo CMV	< 3m
	Candida, oesophagitis	< 3m
20	none	
21	VZV, skin (*)	12m
22	none	
23	Cryptosporidium, stool	< 3m
24	none	
25	Candida, oesophagitis	< 3m
26	Toxoplasmosis, generalized (*, MAS)	< 3m
27	none	
28	Parainfluenza, blood	< 3m
	CMV reactivation, blood	< 3m
29	Legionella pneumonia	< 3m
	CMV reactivation	< 3m
30	CNS, blood	< 3m
	CMV reactivation, blood	< 3m
	HSV, stomatitis	< 3m
	Parainfluenza, blood	< 3m
31	CNS, blood	< 3m
32	none	
33	none	
34	EBV genome copies	< 3m

Table 3. Infectious complications after ASCT
Pt = patient, T = timepoint post ASCT, EBV = Epstein Barr Virus, VZV= Varicella Zoster Virus, CMV= Cytomegalovirus, CNS = Coagulase Negative Staphylococcus, HSV = Herpes Simplex Virus, H. Influenzae = Haemophilus Influenzae. * = Mortality cases, MAS = Macrophage Activation Syndrome.

Transplant related complications and mortality

All children developed chills, fever and malaise during infusion of ATG. After ASCT 24 of the 34 children (71%) developed at least one infection, mainly during the aplastic period (**Table 3**). During the aplastic period 6 children developed septicemia; 4 blood cultures were positive for coagulase negative Staphylococcus and 2 for Streptococcus mitis. These cases all responded well to intravenous antibiotics. Ten patients developed a limited and one a generalized VZV eruption, 3 to 18 months after ASCT, successfully treated with aciclovir. One patient showed a primary CMV infection and six patients showed CMV reactivation 1 to 12 months post ASCT.

One case each of localized atypical Mycobacterial infection (6 months after ASCT), Legionella pneumoniae (3 months after ASCT), hepatitis A infection (18 months after ASCT) and herpes simplex virus (HSV) hepatitis (24 months after ASCT) were seen and all resolved completely.

Three patients with systemic JIA died shortly post-ASCT of infectious complications. The first died of an infection associated hemophagocytic syndrome (IAHS), also called macrophage activation syndrome (MAS), induced by EBV 4 months after ASCT. At the time of the EBV infection her JIA was in remission. The second fatal IAHS occurred 18 days post transplant, while the patient was still in aplasia and a third fatality, resembling IAHS, occurred 10 days after ASCT and was caused by a disseminated toxoplasmosis infection [35,36]. Two further patients died 13 and 16 months after ASCT as described above.

Our study indicates that ASCT in severely ill JIA patients induces a drug-free complete remission in a substantial proportion of the patients (18 of 34 patients, 53%), even after prolonged withdrawal of anti-rheumatic drugs. Also the patients who achieved a partial response (six of 34 patients, 18%) showed a remarkable improvement in most core-set criteria, indicating a profound increase in general well being. The limited improvement in pediatric EPM-ROM and CHAQ-disability scores after ASCT illustrates that erosive joint destruction that existed prior to ASCT was not reversed during the post ASCT follow up in this study and might well turn out to be irreversible over a longer follow up. Yearly x-rays of the joints in a substantial part of the children that gained complete remission (data not shown) suggest, however, that the progression of destruction has arrested after ASCT.
The high mortality rate in this study raises the issue of benefit versus risk of this procedure. Though mortality is seldom an imminent issue in most patients with JIA, various series published between 1981 and 1991 report mortality rates between 8 and 14% in children with systemic onset JIA (10% of all cases) [37-41]. There is no doubt that due to better treatment strategies this mortality rate in systemic onset JIA has decreased in the last decade, but is still estimated to be higher than 2% [6]. The causes of death are quite evenly divided between disease-related causes and treatment-related causes. The high total mortality in the group of children treated with ASCT is therefore partly due to the selection of most severely ill patients. Infection Associated Hemophagocytic Syndrome (IAHS) caused three of the 5 fatality cases in this study. There is circumstantial evidence for a relation between systemic JIA and IAHS [42,43]. IAHS and systemic JIA share symptoms such as spiking fever and untreated systemic JIA can progress to IAHS with a high mortality rate [44,45]. The occurrence of IAHS in systemic JIA after ASCT may be caused by the stringent T cell depletion resulting in inadequate control of macrophage activation [42]. In 1999 an international agreement was achieved on adaptation of the protocol, consisting of a less profound T cell depletion, control of systemic disease prior to transplant and a slow tapering of corticosteroids after ASCT [46]. After adaptation of the protocol no further cases of IAHS have occurred.
The high frequency of infection in this study, including two deaths, illustrates the vulnerability of the patient group after ASCT. Whether the two patients dying of infection at 13 and 15 months after ASCT need to be considered as cases of transplant related mortality or disease related mortality is questionable. Both patients were severely immune suppressed as a result of high dose immunosuppressive drugs, which were restarted because of a full-blown relapse. Furthermore, though the immune system had fully reconstituted, it has been shown that after autografting severe immunodeficiency associated with persisting oligoclonality may continue for many years [33,47,48]. Awareness that both factors amplify the immune suppression is important. Early recognition with immediate treatment of infections in this group of

patients is even more important than before ASCT. Future protocols therefore need to contain the prophylactic administration of antiviral drugs and intravenous immunoglobulines until normal $CD4^+$ T cell counts and frequent EBV monitoring by polymerase chain reaction. We believe that with the adaptations made in the protocol in 1999 to reduce MAS, the administration of prophylactics after ASCT and the awareness of the risks of restarting immunosuppressive drugs shortly after ASCT mortality rates can be reduced significantly.
Since the availability of anti-TNF therapy, failure of response to this treatment has been added as an inclusion criterion for future cases. The effects of etanercept in polyarticular JIA are impressive with a response rate up to 80% of MTX-resistant patients with polyarticular JIA [9]. So far no formal study has been undertaken for children with systemic JIA, but first experiences with anti-TNF therapy in patients with persistent systemic features show several treatment failures [11,12]. Furthermore, the potential for development of severe adverse events with anti-TNF agents serves as an unfortunate limitation to this therapy [49-51]. Among the 34 children in this study were 8 children with systemic JIA who had previously failed treatment with anti-TNF therapy; all 8 children did respond extremely well to ASCT, confirming the possible role of this procedure in patients with severe disease.
The mechanism underlying the occurrence of complete remission after ASCT seems obvious. The rational of the treatment is eradication of the autoreactive lymphocytes and memory cells by a lymphoablative conditioning regimen and subsequent rescue with T cell depleted autologous bone marrow. Regeneration of the T cell population occurs in the thymus by a process of proliferation and tolerization to self, resulting in a “reset” immune system. Though it is generally thought that the recurrence of disease post-ASCT either reflects pathogenic cells in the stem cell graft or those remaining in the host, evidence is mounting that further depletion of T cells is not the answer to improving the outcome. In our study no correlation could be found between the number of reinfused T cells and clinical outcome. This finding is consistent with a recent study by Moore et al. showing similar outcomes in severe rheumatoid arthritis patients undergoing hemopoietic stem cell transplantation with unmanipulated cells and those receiving CD34-selected cells [52]. Furthermore, five patients in our study showed a benign, transient, oligoarticular synovitis before achieving complete remission, suggesting that some autoimmune T cells are still present after ASCT. The self-limiting character of the synovitis favors the concept that ASCT not only resets autoimmune T cells, but also restores the regulation of these cells [53].
The above issue raises the question whether T cell depletion of the marrow is crucial to the process of the transplant, or whether an intense but non-myeloablative regimen (with or without stem cell support) would be just as effective. Thus far, the conditioning agents used were selected to maximize T cell eradication. Comparison of various conditioning regimens in animal models indicates that a combination conditioning with high dose cyclophosphamide and low dose TBI is optimal [54]. The use of TBI in children remains, however, controversial because of concerns about

long-term safety. Though the limited number of patients does not allow us to draw definitive conclusions, this study suggests that children not given TBI have equally good outcome as those treated with irradiation. Given the obvious concerns over the use of TBI we propose to eliminate TBI from future conditioning regimens. **Table 4** shows a proposed new protocol. A good controlled, prospective study is now needed to investigate the effectiveness of the new protocol.

Conclusions

Autologous stem cell transplantation in severely ill JIA patients induces drug-free remission of the disease and profound improvement in general well being in a substantial proportion of patients. However, the procedure carries a significant mortality risk, making it necessary to weigh carefully the morbidity and mortality risks of the prolonged immunosuppression of "conventional" treatment against those of the short but intense immunosuppression of ASCT.

Table 4a. Currently used protocols of ASCT for JIA

No	Conditioning	TCD	Follow up (mo)	Inclusion criteria	Exclusion criteria	Supportive care
A	Cy, ATG, TBI (low dose, 4 Gy)	CD2/3 or CD34	60	Drug resistant, including anti-TNF	Cardio-respiratory insufficiency, chronic infections, active systemic disease at time of ASCT, end stage disease, poor compliance.	No specific rules for IVIG or antimicrobial prophylaxis
B	Cy, ATG	CD34	42			
C	Cy, Flu, ATG	VCR or CD34	24			

Table 4b. Proposed new protocol

Conditioning	TCD	Inclusion criteria	Exclusion criteria	Supportive care
• Cy (50mg/kg/day at day –4 and –1). • ATG 5 mg/kg/day at day –8 to –5). • Reïnfusion of stem cells at day 0.	CD34 selection. CD34> 1x 10^6/kg and add back CD3 1x10^5/kg	Same as above	Same as above	• Isolation during aplastic period. • IVIG (0.4 mg/kg x 3 weeks) until CD3 count > 500/mL • Pentamidine (PCP prophylaxis, avoid acotrimoxazol).

Table 4. Currently used protocols (a) and proposed new protocol (b) of ASCT for JIA.
ATG = anti-thymocyte rabbit immunoglobulin, SangStat, Fremont, CA, USA; CD2/3 = negative selection by monoclonal antibodies to CD2 or CD3 positive lymphocytes; CD34 = positive selection of $CD34^+$ stem cells; Cy = Cyclophosphamide; Flu = Fludarabin; IVIG = intravenous immunoglobulin suppletion; PCP = Pneumocystis Carinii Pneumonia; TBI = total body irridiation; VCR = ex vivo T cell depletion using Vincristin; TCD = T cell depletion.

Acknowledgements

U. Neudorf, T. Fiselier and M. van Santen, pediatric rheumatologists are acknowledged for patient referral. The clinical studies in the Netherlands were supported in part by a grant from the Dutch League against Rheumatism and the Dutch Organization for Scientific Research (NWO).

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6

Autologous stem cell transplantation tolerizes autoreactive T cells and restores the $CD4^+CD25^+$ immune regulatory network in juvenile idiopathic arthritis.

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Summary

There are currently no data offering insight into how immune tolerance may be reconstituted in patients who received Autologous Stem Cell Transplantation (ASCT) for severe autoimmune disease. To gain further knowledge on this subject the immune reconstitution of 12 patients was studied who received ASCT for Juvenile Idiopathic Arthritis. The data show that two mechanisms are attributable to the induction of tolerance by ASCT. Before ASCT the patients showed a severely reduced frequency of peripheral blood $CD4^{+}CD25^{bright}$ Treg cells, which was completely restored by the treatment with ASCT. This recovery of the $CD4^{+}CD25^{+}$ regulatory network was the result of an increased preferential expansion of $CD4^{+}CD25^{+}$ regulatory T cells early after ASCT and a highly increased output of regulatory cells by the thymus. Besides recovering the $CD4^{+}CD25^{+}$ regulatory network, ASCT induced persistent changes in arthritis related autoimmune T cells.

Introduction

Patients with autoimmune diseases refractory to conventional therapy suffer considerable morbidity and have in the long term a significantly shortened life expectancy. For this group of treatment resistant patients myelo- and/or lymphoablative chemotherapy followed by autologous bone marrow- or blood derived stem cell transplantation (ASCT) is a relatively new treatment option. While we and others have convincingly described the beneficial effect of ASCT for some forms of refractory autoimmune disease [1-5] exactly how autografting achieves remission has yet to be defined. The initial effect is likely to be attributable to the eradication of autoreactive lymphocytes and memory cells due to the high-dose lymphoablative-conditioning regimen. Subsequently, there may be a significant contribution from altered immune reconstitution that occurs after autologous transplantation. Isolated observations including alterations in CD4:CD8 ratios, decreased mitogenic responsiveness, restoration of reduced perforin expression and changes in the T cell repertoire have been made following autologous transplantation [6-8]. Furthermore, fetal animal work is supportive of the hypothesis that, exposure of the developing immune system to neoantigens, in a period when the immune system is developing its repertoire, leads to tolerance [9]. Altogether these observations suggest that the success of ASCT is not only based on the loss of autoreactive T cell clones, but also on the complete re-assignment of imbalanced cellular and soluble networks, including those that regulate the autoreactive T cells.

To assure the timely and efficient dampening of immune responses the immune system harbors a network of regulatory T cells (Tregs). The most important group of Tregs is currently identified by the expression of CD25 and the transcription factor FoxP3 [10]. $CD4^+CD25^+$ Tregs play a key role in the maintenance of immunologic tolerance to both self- and foreign antigens. This occurs by suppressing aggressive T cell responses and in multiple experimental animal models it has been shown that, in the absence of these so-called $CD4^+CD25^+$ Tregs the risk of developing autoimmunity is significantly increased [11,12]. Studies on $CD4^+CD25^+$ Tregs in human disease are still limited. The authors recently published data showing that a lower number of $CD4^+CD25^+$ Tregs in the synovial fluid of children with Juvenile Idiopathic Arthritis (JIA) is correlated with the development to a less favorable clinical course of the disease [13].

JIA is the most frequent rheumatic disease in childhood [14,15]. Since 1997 ASCT has been applied in a small group of treatment resistant JIA patients[1]. In this study the extent to which $CD4^+CD25^+$ Tregs play a role in the induction of tolerance by ASCT in JIA was questioned. The number, phenotype and functionality of $CD4^+CD25^+$ Tregs was analysed before and at different time points after ASCT. Furthermore, using a newly developed technique to identify peptide specific $CD4^+$ T cells, it was possible to analyze autoreactive T cells with the same specificity before as well as after ASCT. The data show that two mechanisms are attributable to the induction of tolerance by transplantation: persistent changes in autoreactive T cells and the recovery of the $CD4^+CD25^+$ regulatory network.

Methods

Patients and transplant characteristics

Twelve patients who received ASCT for refractory JIA and 8 JIA patients on conventional therapy were analysed (**Table 1**). The inclusion- and exclusion criteria for ASCT as well as the clinical follow up of the 12 transplanted patients is described in detail in earlier publications [1,46]. The graft, harvested at least one month prior to ASCT, was either purged by T cell depletion with CD2 and CD3 antibodies or by positive stem cell selection using CD34 selection devices. These techniques yielded a final suspension with a $CD34^+$ cell count of 0.44 – $6.0x10^6$ cells per kg (mean $2.2x10^6$ cells per kg) and with a $CD3^+$ cell count of 0.5 – $28.4x10^5$ cells per kg (mean $5.6x10^5$ cells per kg). The conditioning regimen included 4 days of anti-thymocyte rabbit immunoglobulin (ATG, SangStat, Fremont, CA, USA), 5 mg/kg from day –9 to –6, cyclophosphamide, 50 mg/kg/day from day –5 to –2 and low dose total body irradiation (4 gray, single fraction) on day –1. Informed consent was obtained either from parents/guardians or from the children directly when they were older than 12 years old (The Netherlands).

Table 1

Clinical characteristics

	ASCT patients (n = 12)	Conv. treated patients (n = 8)
Gender, male	6	5
Onset type JIA		
Systemic	9	8
Polyarticular	3	
Time of onset JIA	3y,10m (11m-7y7m)	5y,9m (1y10m-16y1m)
Age at ASCT	9y,10m (5y2m-14y11m)	n.a.
Follow up (f.u.)	4y, 6m (4m-7y)	n.a.
Outcome at max. f.u.		n.a.
Complete remission	6	
Complete relapse	1	
50-70% response	4	
Deceased	1	

Table 1. Clinical characteristics of ASCT and conventionally treated patients. Age at time of onset JIA, age at ASCT and duration of follow up are given in mean plus range years (y) and months (m). n.a. = not applicable, Conv. = conventionally, max. f.u. = maximal follow up. Patients that are in complete remission are drug-free and fulfill at least 5 or more of the following requirements, for the last two consecutive months; morning stiffness <30 min, no fatigue, no joint pain, no joint tenderness or pain on motion, no soft tissue swelling in joints or tendon sheaths and ESR <20 mm/h.

Blood samples and cell separation

PBMC were isolated using Ficoll Isopaque density gradient centrifugation (Ficoll-Paque, Pharmacia, Sweden). $CD4^+$ T cells from PB were purified by using magnetic bead-activated cell sorting (MACS, Miltenyi Biotec, Bisley, Surrey, GB). In brief, PBMC were incubated for 20 min at 4°C with CD4-coated magnetic beads (10 µl/10x10^6 cells). After washing, the cells were passed through LD-columns within the MACS device. The resulting $CD4^+$ T cell fraction was subsequently sorted into $CD4^+CD25^-$, $CD4^+CD25^{bright}$, $CD4^+CD25^+$CD45RO and $CD4^+CD25^+$CD45RA T cells by FACS (FACS Vantage, Becton-Dickinson, San Jose, CA). The buffer used throughout the whole procedure was PBS supplemented with 2% FCS and 2 mM EDTA. T cell purity was >97%.

Functional assays

For functional assays 5x10^3 MACS sorted $CD4^+$ T cells or FACS sorted $CD4^+CD25^{bright}$ T cells were cultured in a 96-well plate, coated with anti-CD3 (OKT-3, 1 µg/ml) with or without the addition of IL-2 (10 ng/ml, Eurocetus, Amsterdam, The Netherlands). The negative fraction of cells obtained after MACS sorting were used as antigen-presenting cells (APC) after depletion of T cells using anti-CD3 beads followed by irradiation (3500 Rad). 3x10^4 APC were added to each well. The cells were incubated at 37°C for 6 days, the last 18 h in the presence of [^{3}H]thymidine (1 µCi/well). Proliferative responses were calculated as the mean [^{3}H]thymidine incorporation (cpm) of triplicate wells.

Flow cytometry

The following PE, FITC, CY or APC labeled mAbs were used: anti-human CD4 (RPA-T4), CD25 (2A3), CD45RA (L48), CD45RO (4CHL-1), CD44 (G44-26), CCR4 (1G1), GITR (110416), CTLA4 (BN13) and Ki67 (MIB-1). For intracellular staining of CTLA4 and Ki67, the cells were first surface stained, then fixed in Cytofix/Cytoperm solution (20 min, 4°C) and washed twice in Perm/Wash solution (Cytofix/perm kit, BD, San Jose, CA), followed by incubation with anti-CTLA4 or Ki67 mAb. GITR specific mAb was obtained from R&D (Germany) and Ki-67 from Immunotech (Marseilles, France). All other mAbs were obtained from Becton Dickinson (San Jose, CA, USA). Stained mononuclear cells were diluted in sheath fluid and run on a FACSCalibur (Becton Dickinson). CellQuest software (BD Biosciences) was used for analysis.

mRNA analysis by quantitative PCR

Total RNA was isolated using Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized from total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV, Promega, Madison WI, USA) with 1 µg/µl Oligo (dT) and 10 mM dNTPs (both Amersham Pharmacia Biotech AB, Uppsala, Sweden). The reaction mixture was incubated at 40°C for 90 minutes followed by incubation at 70°C for 15 minutes. Messenger RNA expression levels of FoxP3, IL-10, IFN-ϒ, GATA3 and T-bet were determined by real-time quantitative PCR on a LightCycler (Roche Diagnostics, Mannheim, Germany). ß2-microglobulin (ß2M) was analysed as a housekeeping gene. The following combination of primers and probes were used: FoxP3 Forward 5’ TCA AGC ACT GCC AGG CG 3’, FoxP3 Reverse 5’ CAG GAG CCC TTG TCG GAT 3’, IL-10 Forward 5’ TGA GAA CAG CTG CAC CCA CTT, IL-10 Reverse 5’ GCT GAA GGC ATC TCG GAG AT, IFN-ϒ Forward 5’ GCA GAG CCA AAT

TGT CTC CT, IFN-ϒ Reverse 5′ ATG CTC TTC GAC CTC GAA AC, T-bet Forward 5′ CCC CAA GGA ATT GAC AGT TG, T-bet Reverse 5′ GGG AAA CTA AAG CTC ACA AAC, Gata3 Forward 5′ CTG CAA TGC CTG TGG GCT C, Gata3 reverse 5′ GAC TGC AGG GAC TCT CGC T, ß2m Forward 5′ CCA GCA GAG AAT GGA AAG TC 3′, ß2m Reverse 5′ GAT GCT GCT TAC ATGTCT CG 3′. To quantify mRNA amounts the following protocol was used. A standard curve was generated with a dilution series of a reference cDNA sample, which was run at the same time as the unknown samples. Data are expressed as normalized gene expression, which was obtained by dividing the relative quantity of the gene of interest for each sample by the relative quantity of ß2M for the same sample.

T cell capture and artificial APC

This technique is extensively described earlier [47]. Compared to the previously described protocol a few improvements were made. In short, PBMC of a DR4 homozygotic transplanted JIA patient obtained before and after transplantation were cultured with or without a peptide of human heat shock protein 60, HSP60 280-294 (GEALSTLVLNRLKVG). After 4 days the cells were prestained with anti-CD4-CY and subsequently incubated with Artificial Antigen-Presenting Cells (aAPCs) for 2 hours at 37 ºC. Before sorting the cells on the FACSvantage (Becton Dickinson), cells and aAPCs were washed twice and resuspended in FACS buffer. The aAPCs were prepared as follows. Phosphatidylcholine and cholesterol (Sigma) were combined in a glass tube at a molar ratio of 7:2. The solvent was evaporated under an Argon stream for 30 min and dispersed at a final concentration of 10 mg/ml in 140 mM NaCl and 10 mM Tris·HCl, pH 8 (buffer A) containing 0.5% sodium deoxycholate. Monosialoganglioside-GM1 (Sigma G-7641) was added at a final concentration of 0.28 mM. The solution was sonicated until clear and was stored at -20°C. Liposomes were formed through dialysis at 4°C against PBS in a 10-kDa Slide-A-Lyzer (Pierce) for 48h. Biotinylated recombinant MHC was incubated with the peptide (6h, room temperature). The resulting MHC-peptide complexes were incorporated in rafts, engineered on the aAPC surface. The rafts were constructed by mixing biotinylated HLA-DR4 molecules, biotinylated antibodies
to CD28 and anti-LFA-1, and biotinylated Cholera toxin subunit B-FITC conjugated (CTB-FITC; Sigma) in the appropriate (equal) molar ratio. Next, neutravidin (NA; Pierce) was added in a molar ratio of four biotinylated moieties per molecule of NA. CTB-FITC was used to visualize T cells bound by the aAPCs. After incubation (1.5h at room temperature), the Raft-NA mixture was added to the liposomes for 2h, again at room temperature and washed 3 times in PBS. Finally, once the aAPCs were generated, they were incubated with the stained cells.

Statistical analysis

Basic descriptive statistics were used to describe the patient population. A Wilcoxon-signed rank test was used to compare numbers of and the expression of molecules on $CD4^+CD25^+$ T cells before and at different time points after ASCT. A Mann-Whitney U test was used to compare the frequency of $CD4^+CD25^+$ T cells between the different patient groups.

Rapid reconstitution of CD4CD25^{bright} T cells after ASCT

Frequencies of CD4^{+}CD25^{bright} T cells were determined in 8 JIA patients on conventional therapy and 12 JIA patients who received ASCT for JIA (**Table 1**). Both conventionally treated and ASCT treated patients before transplantation displayed significantly lower frequencies of CD4+CD25^{bright} T cells (**Fig. 1a**) than previously found in healthy children (mean ± SEM; 1.6% ± 0.1%) and children with oligoarticular JIA (mean ± SEM: 1.2% ± 0.2%), a subtype of JIA with a much milder clinical course and without the systemic features [13]. As early as 6-8 weeks post transplant, the transplanted patient group showed a significant increase in the relative number of CD4^{+}CD25^{bright} T cells (**Fig. 1a**). Interestingly, the one patient in the study group that suffered a complete relapse after ASCT showed a clear decrease in CD4^{+}CD25^{bright} T cell frequency at the time of relapse, 6 months after ASCT (**Fig. 1b**). None of the patients that gained a long-lasting and drug-free remission by ASCT showed this phenomenon. In evaluating absolute numbers of CD4^{+} and CD8^{+} T cells after ASCT the findings were consistent with published literature on patients treated with intensive chemotherapy [16,17] (data not shown).

(a)

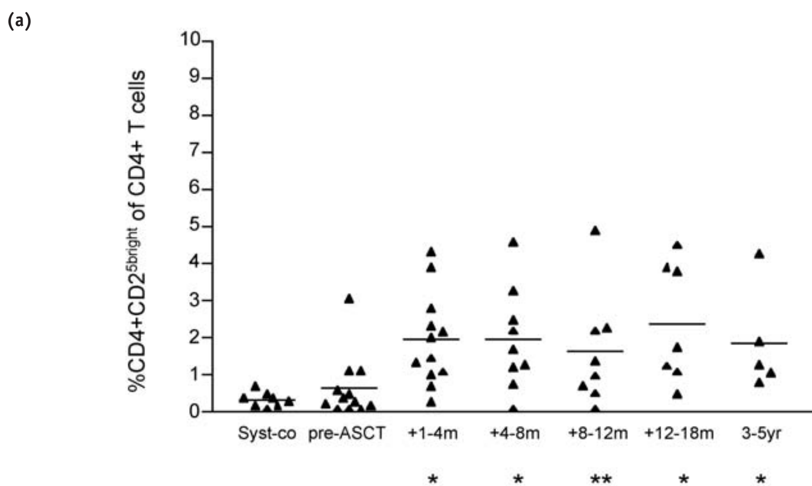


Figure 1. Recovery of CD4^{+}CD25^{bright} T cell frequency after ASCT. (a) The relative number of CD4^{+}CD25^{bright} T cells in 8 systemic JIA patients on conventional therapy (syst-co) and in 12 children who received ASCT for refractory JIA was measured by FACS-staining.

(b)

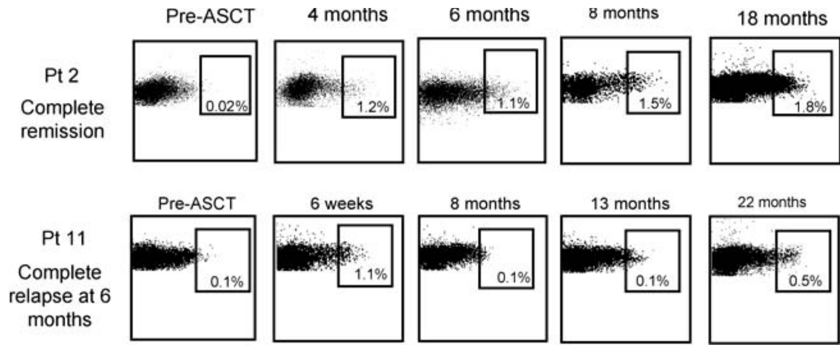


Figure 1. Recovery of CD4⁺CD25^{bright} T cell frequency after ASCT. (b) Since it has been shown that the regulatory CD4⁺ T cells preferentially reside within the CD4⁺CD25^{bright} population, only the CD4⁺CD25^{bright} T cells and not the CD4⁺CD25^{total} T cells were analyzed[48].
*,P < 0.05, **,P = 0.06. Dotplots of two representative patients (pt).

Early reconstituted CD4⁺CD25⁺ T cells express high levels of CTLA4, GITR and mRNA FoxP3

FoxP3 has been identified as a specific marker of CD4⁺CD25⁺ Treg cells, distinguishing them from recently activated, non-regulatory CD4⁺CD25⁺ T cells [10,18]. The early-reconstituted CD4⁺CD25^{bright} T cells expressed high amounts of mRNA FoxP3 and thus can be considered as professional regulatory T cells. The levels of mRNA FoxP3 in these CD4⁺CD25⁺ T cells were significantly higher than expressed by their counterparts obtained from time points before transplantation (**Table 2a**). Furthermore, the early-reconstituted CD4⁺CD25^{bright} T cells (1 month after ASCT) showed an increased expression of CTLA4, GITR and CCR4 compared to their counterparts before ASCT (**Table 2b**). CTLA4, GITR as well as CCR4 are constitutively expressed on CD4⁺CD25⁺ Treg cells [19-21]. It is conceivable that the increase found in CTLA4, GITR and CCR4 expression represents the increased proportion of professional CD4⁺CD25^{bright} Treg cells within the analysed CD4⁺CD25⁺ T cells.

Table 2a

Expression of mRNA FoxP3 in sorted CD4⁺CD25^{bright} T cells pre and post ASCT

	Pre ASCT	3m post ASCT	6m post ASCT	9m post ASCT	1y post ASCT	2y post ASCT
mRNA FoxP3	12.7 ± 6.6	19.8 ± 5.4	29.2 ± 7.8*	29.3 ± 7.8*	36.4 ± 4.8*	40.2 ± 15.1*

Table 2b. Phenotype of CD4$^+$CD25$^-$ and CD4$^+$CD25^{bright} T cells pre and post ASCT

	Pre ASCT		1 month post ASCT	
	CD4$^+$CD25$^-$	**CD4$^+$CD25^{bright}**	**CD4$^+$CD25$^-$**	**CD4$^+$CD25^{bright}**
CTLA4 (MFI)	11.0 ± 1.7	36.0 ± 11.5	29.2 ± 6.0	68.5 ± 11.6 *
GITR	0.7 ± 0.2	9.9 ± 2.1	2.6 ± 1.1	21.1 ± 7.7
CCR4	11.4 ± 3.8	33.1 ± 5.5	11.8 ± 1.1	65.4 ± 10.8 *
CD44 (MFI)	5.9 ± 0.5	8.2 ± 0.5	9.6 ± 0.9	14.7 ± 1.9 *

	6 months post ASCT		1 year post ASCT	
	CD4$^+$CD25$^-$	**CD4$^+$CD25^{bright}**	**CD4$^+$CD25$^-$**	**CD4$^+$CD25^{bright}**
CTLA4 (MFI)	14.4 ± 2.2	56.5 ± 9.6	10.0 ± 1.1	38.5 ± 9.9
GITR	1.5 ± 0.5	14.9 ± 3.4	2.7 ± 0.9	19.2 ± 3.0
CCR4	19.7 ± 2.7	64.8 ± 7.9 *	18.2 ± 2.0	69.8 ± 4.4 *
CD44 (MFI)	7.6 ± 1.0	9.3 ± 1.2	9.4 ± 1.2	13.1 ± 1.8 *

Table 2: (a) MRNA FoxP3 expression in sorted CD4$^+$CD25^{bright} T cells before and after ASCT. CD4+CD25^{bright} T cells were isolated from the PB of 3 transplanted patients before and at different timepoints after ASCT. MRNA FoxP3 was measured by quantitative PCR. Data are expressed as the mean normalized gene expression (±SEM) of the three patients at the indicated time points pre and post ASCT. *Significant ($p<0.05$) compared to the same population before ASCT.
(b) Phenotype of CD4$^+$CD25$^-$ and CD4$^+$CD25^{bright} T cells pre and post ASCT. The indicated markers were measured by FACS. Except when MFI (= Mean Fluorescence Intensity) is indicated numbers are given as percentages (mean ± SEM of 8 patients). *Significant ($p<0.05$) compared to the same population before ASCT

The division rates of CD4$^+$CD25^{bright} Treg cells and CD4CD25$^-$ T cells are not equally increased early after ASCT

After ASCT the immune system is reconstituted via two distinct pathways [22,23]. In the first two months after transplantation the T cell compartment repopulates rapidly through lymphopenia induced expansion of mature T cells that have survived the preconditioning and/or the residual T cells coinfused with the graft. The alternative way of reconstitution is thymic dependent and might be considered as a recapitulation of ontogeny. It was hypothesized that the rapid recovery of the CD4$^+$CD25^{bright} T cell frequency after ASCT is the result of a preferential proliferation of CD4$^+$CD25^{bright} T cells in the early phase of the immune reconstitution. This was supported by the finding that CD4$^+$CD25^{bright} T cells showed a significantly higher expression of CD44 in the first three months after transplantation compared to CD4$^+$CD25$^-$ T cells and compared to their counterparts before transplantation (**Table 2b**). To further substantiate this hypothesis cell proliferation in CD4$^+$CD25^{bright} and CD4$^+$CD25$^-$ T cells were measured separately by the analysis of Ki67. Ki67 is a protein pivotal for cell division and is expressed exclusively by cells that are in cell cycle [24]. Since infections may induce increased proportions of T cells to divide only patients that did not show any sign

of an infection in this early period after transplantation were analysed on Ki67. In each patient analysed the same pattern of Ki67 staining was seen. As could be expected25, before ASCT $CD4^{+}CD25^{bright}$ T cells showed a higher division rate compared to $CD4^{+}CD25^{-}$ T cells (Ki67+ mean ± SEM, pre ASCT: 3.8% ± 0.5% versus 0.8% ± 0.1%). Due to lymphopenia induced homeostatic proliferation in the early period after ASCT the division rate was highly increased in both populations (25.3% ± 9.4 versus 14.7% ± 4.9%). Confirming the hypothesis, the most pronounced increase in division rate was seen in the $CD4^{+}CD25^{bright}$ T cell population (+21.5% versus +13.9% in $CD4^{+}CD25^{-}$ T cells). Given the assumption that $CD4^{+}CD25^{-}$ and $CD4^{+}CD25^{bright}$ T cells equally survived the conditioning regimen, it can be calculated from these division rates that after ASCT less than 35 cycles of proliferation are needed to result in a 15 times increase in $CD4^{+}CD25^{bright}$ T cell frequency. Thirty-five cycles of proliferation are easily accomplished within 6 weeks[26]. Thus it is very likely that the more pronounced increase in division rate in the $CD4^{+}CD25^{bright}$ T cells explains the recovery of the $CD4^{+}CD25^{bright}$ T cell frequency as seen within 6 weeks after ASCT.

The thymus contributes substantially to the reconstitution of $CD4^{+}CD25^{+}$ regulatory T cells after transplantation

It has previously been reported that after transplantation the expression of CD45RA can be used as a surrogate approximation of T cell neogenesis and confound accurate estimates of thymic activity [27-29]. To determine the contribution of the thymus to the reconstitution of $CD4^{+}CD25^{+}$ Tregs the frequencies of $CD4^{+}$CD45RA and $CD4^{+}CD25^{+}$CD45RA cells and the expression of mRNA FoxP3 by these cells were analysed at different time points post transplant.

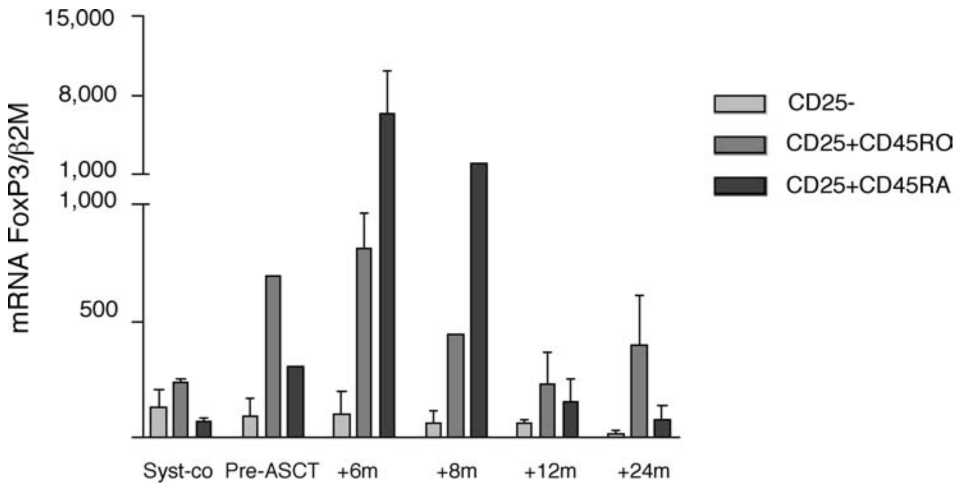


Figure 2. MRNA FoxP3 expression levels in sorted cells pre and post ASCT

$CD4^{+}CD25^{-}$, $CD4^{+}CD25^{+}$CD45RO and $CD4^{+}CD25^{+}$CD45RA T cells were isolated from the PB of three patients by FACS sorting. MRNA FoxP3 was measured by quantitative PCR. Data are expressed as the mean normalized gene expression (±SEM) of the three patients at the indicated time points pre and post ASCT.

The first $CD4^{+}CD45RA$ cells were detectable 3-9 months post transplant and in the 12-18 months post transplant, the ratio of naïve and memory T cells resembled the ratio before transplantation. CD25 expression on $CD4^{+}CD45RA$ T cells was readily detectable and expressed in the same percentage as on $CD4^{+}CD45RO$ cells. However, while the CD25 density on the $CD4^{+}CD45RA$ cells was less bright than on $CD4^{+}CD45RO$ cells, as reflected by a lower mean fluorescence intensity, $CD4^{+}CD25^{+}CD45RA$ cells sorted at time points 6 and 9 months after transplantation, expressed extremely high levels of mRNA FoxP3 (**Fig. 2**). Hereafter mRNA FoxP3 levels rapidly decreased again till pre-transplant levels, most conceivably, due to the conversion of $CD25^{+}CD45RA$ to $CD25^{+}CD45RO$ memory cells as well as to a decreased output of Tregs by the thymus once the immune reconstitution was complete.

IL-2 reverses the hyporesponsive state of $CD4^{+}CD25^{-}$ T cells early after transplantation

T cells of, allogenic as well as autologous transplanted patients, show a markedly decreased mitogenic responsiveness in vitro during the first 6-12 months after transplantation 30,31. In the patient group in vitro mitogenic responses had not returned to normal until a mean of 12 months (± 1.3 months) after ASCT. It was questioned whether or not the rapidly reconstituting $CD4^{+}CD25^{+}$ Tregs attribute to this T cell hyporesponsiveness in the first months after ASCT. Since $CD4^{+}CD25^{+}$ Tregs exert their regulatory function by blocking mRNA IL-2 transcription in the responder cells [32] -and impaired by the low cell counts early after ASCT- this hypothesis was tested by adding IL-2 (10 ng/ml) to anti-CD3 stimulated CD4+ T-cell cultures, obtained 3-6 months post ASCT. In each of the 4 patients tested the addition of IL-2 resulted in a complete reversion of the hyporesponsive state of the cells (Fig. 3). These data suggest that, the rapidly reconstituting $CD4^{+}CD25^{+}$ Tregs might be responsible for the hyporesponsive state of the remaining $CD4^{+}$ T cells during the lymphopenic period after transplantation.

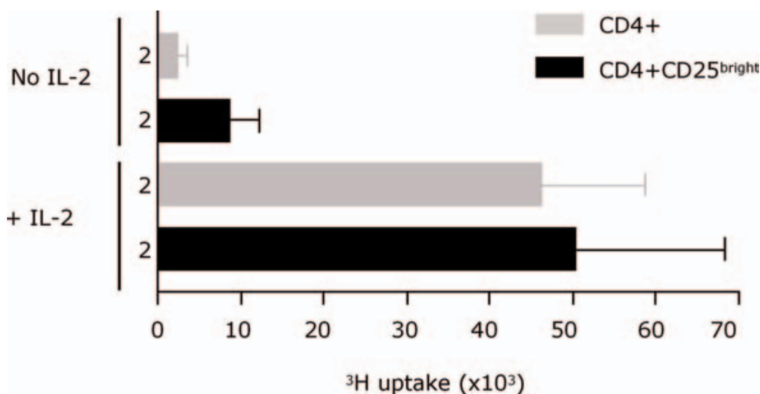
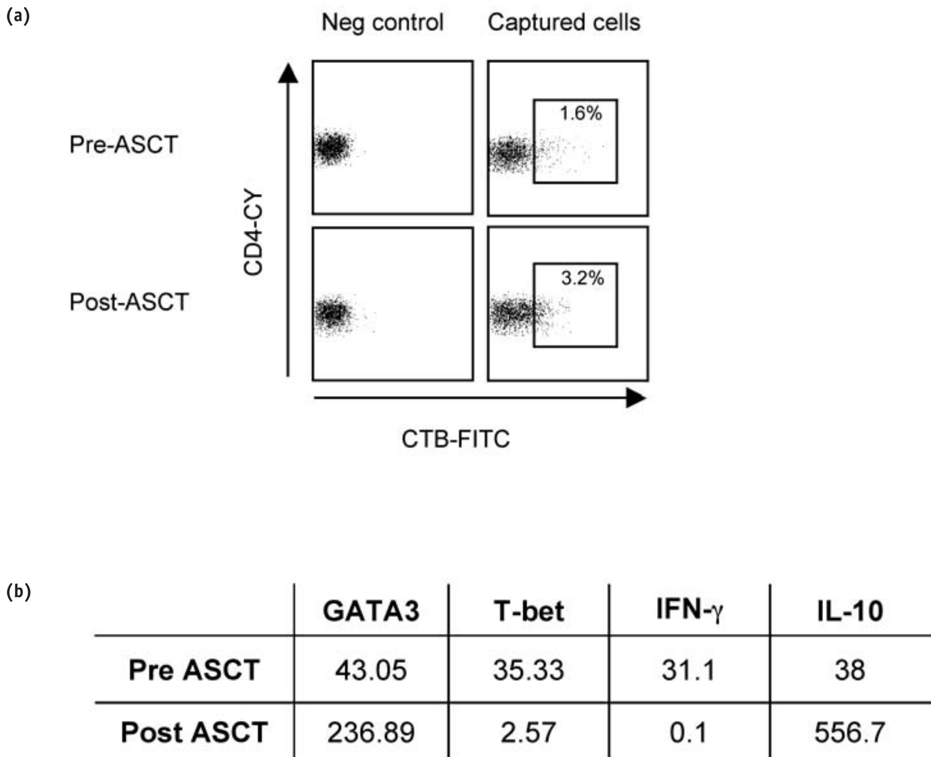


Figure 3. IL-2 reverses the hyporesponsive state of $CD4^{+}CD25^{-}$ T cells early after transplantation

$CD4^{+}$ and FACS-sorted $CD4^{+}CD25^{bright}$ T cells of 4 patients obtained 3-6 months after ASCT were cultured in the presence of plate-bound anti-CD3, APC and with or without the addition of IL-2 (10 ng/ml). Proliferation was measured after 5 days by ^{3}H-incorporation.

Change in arthritis-related autoreactive T cells after ASCT

Seven to 18 months after transplantation the hyporesponsive state of $CD4^+$ T cells had completely reversed. Although theoretically the change in the $CD4^+CD25^{bright}$ Treg cell population observed after ASCT alone may explain the induction of tolerance by ASCT, evidence exists that autoreactive T cells themselves may have undergone persistent intrinsic changes as well. Since changes in the T cell repertoire have been observed following ASCT the only way to investigate this hypothesis properly is by comparison of antigen specific T cells before and after ASCT. A newly developed technique was used, that uses artificial APC (aAPC), and is designed to capture peptide specific $CD4^+$ T cells. Artificial APC containing HLA-DR4 molecules were loaded with a peptide of human heat shock protein 60 (HSP60).



	GATA3	T-bet	IFN-γ	IL-10
Pre ASCT	43.05	35.33	31.1	38
Post ASCT	236.89	2.57	0.1	556.7

Figure 4. Differences in mRNA expression in peptide-specific T cells before and after ASCT

(a) Sortgates of captured peptide specific $CD4^+$ T cells using aAPC. PBMC of a DR4-heterozygotic patient, from time points before and 2 year after ASCT, were stimulated with a peptide derived of human HSP60. After 4 days peptide-specific $CD4^+$ T cells were captured and sorted, using the indicated gates. CTB-FITC and CD4-CY were used to visualize $CD4^+$ T cells bound by the aAPCs. Liposomes containing complete rafts but no MHC-peptide complexes were used as negative controls. **(b)** The sorted peptide specific $CD4^+$ T cells were lysed and analyzed on the mRNA expression of the transcription factors T-bet and GATA-3 and the cytokines IL-10 and IFN-ϒ by quantitative PCR. MRNA levels are expressed as the mean percentage of expression compared to a standardized control cDNA.

Previous studies have extensively described the involvement of T cell reactivity to autologous HSP60 in Adjuvant Arthritis [33,34], as well as Juvenile Idiopathic Arthritis [35-37]. Using the aAPC loaded with a self-HSP60 epitope it was possible to sort antigen specific T cells from a DR4 heterozygotic patient from timepoints before and 2 years after ASCT (**Fig. 4a**). Analysis of the mRNA expression levels revealed major differences between the HSP60 specific T cells before and after transplantation (**Fig. 4b**). An increased expression of mRNA GATA-3 and IL-10 and a decreased expression of mRNA IFN-ϒ clearly indicated a more Th2 or regulatory phenotype of the human HSP60 specific T cells present after ASCT. Thus, besides the recovery of the $CD4^{+}CD25^{+}$ regulatory network, ASCT also induces tolerising changes in the autoreactive T cell clones.

Discussion

ASCT has emerged in recent years as the first opportunity to offer patients with refractory forms of autoimmune disease a potentially curative treatment. While clinical experience with this relatively new treatment is rapidly accumulating, still very little is known about which changes in the immune system induced by ASCT are responsible for the favorable effect.
Three non-mutually exclusive hypotheses can be formulated: 1) immune ablation eliminates autoreactive T cell clones; 2) autoreactive T cell clones are rendered tolerant; 3) regulatory networks controlling the autoreactive T cells are restored.
In an attempt to address this issue a study was conducted of both the immune reconstitution and function of $CD4^+CD25^+$ Treg cells, and self-antigen specific T cells before and after ASCT in a group of 12 patients who received ASCT for JIA.
Normally, for a given age and genetic background, $CD4^+CD25^{bright}$ Treg cells represent a stable proportion of the $CD4^+$ T cells in the steady state, suggesting that the homeostasis of Treg cells is tightly regulated. The data from this study showed a severely reduced frequency of $CD4^+CD25^{bright}$ Treg cells in the peripheral blood of systemic JIA patients, which in all but one patient was completely restored, even after long-term follow up, by the treatment with ASCT. This one patient was the only patient that showed a complete relapse of the disease, all others gained a partial, or complete remission of the disease after ASCT. Since ASCT obviously is not successful in the restoration of genetic defects, it can now be concluded that the low frequency of $CD4^+CD25^{bright}$ Treg cells found in systemic JIA patients is the result of either environmental factors envisioned before the onset of disease, the disease itself or the immunosuppressive drugs the patients received in an attempt to control the disease.
After ASCT the $CD4^+CD25^+$ Tregs reconstitute via the same two pathways as described for $CD4^+$ T cells in general [22,23,29]. Thus, in the first period post-transplant, $CD4^+CD25^+$ Tregs reconstitute via clonal expansion, while in the course of several months, a thymic dependent regeneration of naïve $CD4^+CD25^+$ Treg cells is seen.
As early as 2-3 month after ASCT, during this first period of clonal expansion, the severely reduced $CD4^+CD25^+$ Treg cell frequency, as seen in each of our patients before ASCT, had completely recovered and reached percentages of $CD4^+$ T cells as found in healthy controls (1.5-3%) [13]. This early recovery of the $CD4^+CD25^+$ Treg frequency is at least partly the result of a highly preferential proliferation of $CD4^+CD25^+$ Treg cells above $CD4^+CD25^-$ T cells. It is conceivable that this preferential proliferation of $CD4^+CD25^+$ Tregs during lymphopenia is explained by differences in response to TCR versus lymphopenia induced signals between conventional $CD4^+CD25^-$

T cells and CD4+CD25+ Treg cells [38,39]. Besides the preferential proliferation of CD4+CD25+ Tregs post ASCT, the CD4+CD25+ Treg cells may have selectively survived the conditioning regimen, a mechanism that was recently shown in a murine model for chronic Graft-vs-Host-Disease [40].

After the first period of clonal proliferation the first naïve, thymus derived T cells appeared. These naïve T cells showed an intermediate expression of CD25, but nevertheless extremely high levels of mRNA FoxP3, reaching levels even 10 times higher than found in CD4+CD25bright T cells of healthy children [13]. Thus both an increased preferential proliferation during lymphopenia and an increased output by the thymus, contribute to the reconstitution of CD4+CD25+ Tregs after ASCT and thus to the recovery of these cells in these patients.

During the first 12 months after ASCT, the regenerated CD4+ T cells showed an impaired proliferation to polyclonal stimulators in vitro. This hyporesponsive state was completely abrogated by the addition of IL-2 to the cultures. Given the fact that CD4+CD25+ Tregs function by suppressing the transcription of IL-2 mRNA in their target cells, this finding seem to suggest that the preferentially reconstituting CD4+CD25+ Tregs have a suppressive effect on the CD4+CD25- T cells during the immune reconstitution of our patients [41]. This finding is completely in line with the previous observation by Gavin et al., showing that homeostatic proliferation of CD4+CD25+ Tregs does not abolish but rather augments the suppressor function of CD4+CD25+ Treg cells [42]. This way rapidly reconstituting CD4+CD25+ Treg cells may provide a tolerant environment in which the reconstitution of the rest of the immune system can subsequently take place.

After 12 months the hyporesponsive state was reversed, heralding the time the immune system had fully reconstituted. Importantly, analysis of the same autoantigen specific T cells before and 24 months after ASCT, thus at a timepoint no additional suppressive effect of homeostatic proliferation could be expected, showed a clear deviation to a more regulatory phenotype after ASCT. Thus besides restoring the CD4+CD25+ regulatory network, ASCT brought about persistent changes in autoreactive T cells as well.

The success rate of ASCT in JIA-patients is relatively high [43], when compared to ASCT in other autoimmune diseases like rheumatoid arthritis [3], systemic lupus erythematosis [5] and multiple sclerosis [4]. The main difference between the described trials is the age of the transplanted patients. It has been shown that children recover faster and have a better reconstitution of their T cell repertoire than adults [22,44,45]. This observation seems to be attributed to the age related involution of thymic tissue. Interestingly, on evaluation of the clinical follow up of 34 JIA patients who received ASCT, 90% of all partial and complete relapses of disease occurred in the first 9 months after ASCT. Each child that remained disease free here after remained so till maximal follow up [1]. These observations correlate markedly well with the recovery of naïve CD4+CD25+ Tregs by the thymus that reaches a maximum in this same period, suggesting that once the thymus has been able to reconstitute the regulatory repertoire sustained remission is guaranteed.

In conclusion, ASCT for refractory JIA can induce persistent changes in arthritis related autoimmune T cells, as well as a persistent recovery of the $CD4^{+}CD25^{+}$ regulatory network. These observations could guide the design of new protocols for ASCT aiming at higher remission rates. The data suggest that dose intensification, or new preparative regimens that cause a further depletion of T cells, is not the answer to improving outcomes. Directing the reconstitution and/or activity of regulatory T cells during the first 9 months of immune reconstitution, may provide a mechanism by which a higher incidence of patients can be cured.

Acknowledgements

The authors wish to thank Wilco de Jager and Brenda Hendriks for their help on the molecular work, Erica Roks for excellent secretarial assistance and Lindi Belfield for reading and editing the manuscript.

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7 Summary and general discussion

Developing antigen specific therapy for Juvenile Idiopathic Arthritis:

A tale of Tantalus or Odysseus?

Tantalus was the son of Zeus and was the king of Sipylos. He was uniquely favored among mortals since he was invited to share the food of the gods. However, he abused the guest-host relationship and was punished by being "tantalized" with hunger and thirst in Tartarus: he was immersed up to his neck in water, but when he bent to drink, it all drained away; luscious fruit hung on trees above him, but when he reached for it the winds blew the branches beyond his reach.

The Odyssey, meaning 'the story of Odysseus', takes place ten years after the Trojan War in the Mediterranean Sea. Odysseus, who has been away from his homeland for over twenty years, struggles to return. His tale of the hardships he encountered is told and the fate of his homeland is resolved. When in the last tale he finally returns home, he murders all of the suitors plaguing his household, returns to his wife and son, and restores peace to his island of Ithacs.

Summary

Understanding the complex cellular and molecular mechanisms that regulate the immune response remains one of the major challenges in immunology. A key question is how the immune system is regulated in order to control a protective immune response and prevent chronic and destructive immunopathology. Within the adaptive immune system $CD4^+$ T cells are keyplayers in the initiation and orchestration of immune responses. However, there is now increasing evidence that subsets of $CD4^+$ T cells are also important negative regulators. Amongst these, the so-called naturally occurring $CD4^+CD25^+$ Tregs and IL-10 producing Tr1 cells are the best characterized. The ability of regulatory T cells (Tregs) to control many facets of the immune response suggests that they might be used as targets for new therapeutic strategies.
In the first part of this thesis we explored the presence and functionality of different subsets of Tregs in Juvenile Idiopathic Arthritis (JIA). We identified and characterized self heat shock protein 60 (HSP60) specific T cells (**chapter 2**) and we studied the frequency, phenotype and functionality of naturally occurring $CD4^+CD25^+$ Tregs in JIA patients (**chapter 3**). Subsequently, we explored the role of human HSP60 in the induction and function of $CD4^+CD25^+$ Tregs using PBMC of healthy controls and tried to further classify the human HSP60 specific T cells (**chapter 4**). In the second part of this thesis we evaluated the safety and effectiveness of Autologous Stem Cell Transplantation (ASCT) for refractory JIA by studying 34 JIA patients transplanted in 9 different transplantation centers within Europe (**chapter 5**). Furthermore, we explored the mechanisms that are attributable to the induction of tolerance by ASCT and identified $CD4^+CD25^+$ Tregs as one of the keyplayers in this induction (**chapter 6**).
Taken together the studies described in this thesis may contribute to our understanding of the pathophysiology of JIA as well as of the physiology of regulatory immune responses in general. We identified different subsets of Tregs as crucial players in the tolerization process. Furthermore, we identified human HSP60 being the first known physiological human antigen, naturally present at inflammatory sites and able to activate Tregs. This knowledge may be pivotal in designing the new immunoregulatory strategies for JIA and other autoimmune diseases.

Developing antigen specific therapy for Juvenile Idiopathic Arthritis: a tale of Tantalus or Odysseus?

Identification of regulatory T cells (Tregs) in JIA: CD4+CD25+ naturally occurring Tregs

Considering the efficient innocuous immune response mounted by healthy individuals it is likely that immune suppression mediated by Tregs is not an all-or-nothing event but rather operates in a quantitative manner, which would depend on the balance between effector cells and regulatory cells. In **chapter 3** we studied the presence and functionality of $CD4^{+}CD25^{+}$ Tregs in persistent oligoarticular (pers-OA) and extended oligoarticular (ext-OA) JIA patients. Pers-OA JIA is a subtype of JIA with a relatively benign, self-remitting course while ext-OA JIA is a subtype with a much less favorable prognosis. Despite a significantly reduced number of peripheral $CD4^{+}CD25^{+}$ Tregs when compared to healthy controls a highly increased frequency of Tregs was found in the synovial fluid (SF) of both groups. In addition, a comparison of the patient groups revealed that pers-OA JIA patients displayed the highest number of mRNA FoxP3 expressing $CD4^{+}CD25^{+}$ Tregs in both the PB as well as at the site of inflammation. Thus, the number of $CD4^{+}CD25^{+}$ Tregs clearly correlated with the clinical course of the disease. Since the SF $CD4^{+}CD25^{+}$ Tregs of the ext-OA-JIA patients showed good suppressive capacity in vitro it can be concluded that the progression of disease in ext-OA JIA patients is not the result of an intrinsic defect of the $CD4^{+}CD25^{+}$ Treg cells but rather a failure to either home to or expand at the site of inflammation. In addition, abrogation of the suppressive capacity of $CD4^{+}CD25^{+}$ Tregs by third factors present at the site of inflammation, but not in the simplified in vitro assays[1-3], may play a role in the perpetuation and progression of the disease in the ext-OA JIA patients as well.

The importance of knowing the antigen specificity

Since evidence is accumulating that the outcome of an immune response depends on the balance between Tregs and effector T cells future studies need to be focused on finding ways to enhance local proliferation of $CD4^{+}CD25^{+}$ Tregs without abrogating their regulatory capacity. $CD4^{+}CD25^{+}$ Tregs as well as Tr1-type Tregs require recognition of their antigen through their TCR to be able to retain their suppressive activity. Through a process coined "linked suppression" both types of Tregs need only recognize a limited number of antigens from the tissue to be able to regulate responses to other antigens in that tissue[4,5]. Thus, the in vitro or in vivo establishment of Treg cell lines to predefined antigens presented at the site of inflammation might be useful in harnessing such linked suppression. In contrast, a random and thus polyclonal stimulation of Tregs may result in unwanted suppression of immune responses, leading to the induction of tumors and chronic infections.

Not much is known about the antigen specificity of naturally occurring $CD4^+CD25^+$ Tregs. $CD4^+CD25^+$ nTregs have a polyclonal TCR repertoire and could conceivably recognize a wide spectrum of antigens [6]. It is generally believed that the repertoire of nTreg is biased toward self-antigens. However, thus far no physiological present antigen has been identified that is recognized by human naturally occurring $CD4^+CD25^+$ Tregs.

Identification of Tregs in JIA: human HSP60 specific Tregs

Previous studies by our group have suggested that T cell reactivity against autologous HSP60 in pers-OA JIA patients forms part of regulatory processes leading to down regulation of inflammation and thus to suppression of disease. In **Chapter 2** we further characterized the T cells reactive to human HSP60 in OA as well as polyarticular (PA) JIA patients. We found that in pers-OA JIA patients, T cells responding to human HSP60 express CD30, and during active phases of the disease, these T cells have a cytokine profile with a high IL-10/IFN-ϒ ratio. In contrast, PA JIA patients responded to human HSP60 with virtually no expression of CD30 and a low IL-10:IFN-ϒ ratio. The high IL-10:IFN-ϒ ratio and the up-regulation of CD30 in response to HSP60 in pers-OA JIA patients are consistent with the benign clinical course of the subgroup.
As a consequence of the unique degree of evolutionary conservation of HSP, HSP specific T cells are repeatedly, almost continuously exposed to their antigen: to bacterial HSP during infections and through exposure to the indigenous bacterial flora or to autologous HSP at times of tissue damage. Though the cytokine profile and the regular exposure to their antigen highly suggests that the HSP60-specific T cells in pers-OA JIA constitute an example of human Tr1 cells, we further tried to characterize the HSP60 specific T cells by analyzing the expression level of mRNA FoxP3 (**chapter 4**).
To achieve this we used a newly developed technique (S. Albani, University of California, San Diego), to capture low affinity, HLA class II restricted peptide specific T cells (T cell capture technique)[7]. The used peptide was identified by using a computer algorithm and had previously shown to be recognized in the majority of JIA patients (S. Kamphuis, submitted). With this technique we were able to sort $CD4^+$ T cells specific for a previous selected peptide of HSP60 from 2 JIA patients. Interestingly, the sorted HSP60 specific T cells expressed significant levels of mRNA FoxP3 (**chapter 4**), as well as high mRNA IL-10:IFN-ϒ ratio's (data not shown). Notably, it has recently been shown that the expression of mRNA FoxP3 is not only confounded to naturally occurring Tregs but that TR1-cell clones may express mRNA FoxP3 upon activation as well (Unpublished data, Roncarolo MG and others, International Immunology Congres, Montréal, July 2004). So the question whether the HSP60-specific cells are classical Tr1 like cells, which gained their regulatory function in the periphery or originated as FoxP3 expressing regulatory cells from the thymus and thus need to be classified as $CD4^+CD25^+$ nTregs, cannot be solved. Fortunately, this unknown does not undermine the therapeutic potential of HSP60 specific Tregs. Tr1 cell clones as well as $CD4^+CD25^+$ Tregs have both shown to be highly suppressive, to be able to induce linked suppression and to be able to reverse ongoing immunopathology.

The peripheral induction of Tregs by human HSP60

Since both, T cell reactivity to human HSP60 (**chapter 2**) and the frequency of $FoxP3^+CD4^+CD25^+$ Tregs (**chapter 3**) in the joints of patients with JIA clearly correlated with the clinical course of the disease, the question was raised what factors are

involved in the accumulation of Tregs at the site of inflammation. Is the high incidence of Tregs at the site of inflammation determined by migration and proliferation from the periphery to the site of inflammation alone or is it possible that part of the Tregs have been formed locally? Indeed, it has only recently been shown, that besides the thymically derived $CD4^+CD25^+$ Tregs, $FoxP3^+CD4^+CD25^+$ T cells can be induced in the periphery [8]. The upstream signals that activate FoxP3 transcription in the periphery are largely unknown. High avidity TCR interactions, mediated by self-antigens [9-11] and the interference of TGF-ß have both shown to play a role [12,13].
In **chapter 4** it is shown that human HSP60 is able to induce the differentiation of naïve $CD4^+CD25^-$ T cells into mRNA FoxP3 expressing $CD4^+CD25^+$ Tregs. Though the exact processes driving this differentiation are still unclear, a dual mechanism seems to be responsible (**Figure 1**). It has previously been shown that the activation of PBMC with HSP60 induces the production of TGF-ß [14,15]. Thus, the induction of FoxP3 expression in $CD4^+CD25^-$ T cells might be driven by HSP60 induced TGF-ß [16]. Besides this innate effect through the production of TGF-ß, blocking experiments showed that HSP60 is processed and presented in the context of MHCII. It is conceivable that the $FoxP3^+CD4^+CD25^+$ T cells induced through TCR interactions with MHCII-HSP60 epitopes may have evolved from activation and proliferation of a small population of HSP60 specific Tregs, while TGF-ß caused a more polyclonal differentiation of Tregs.

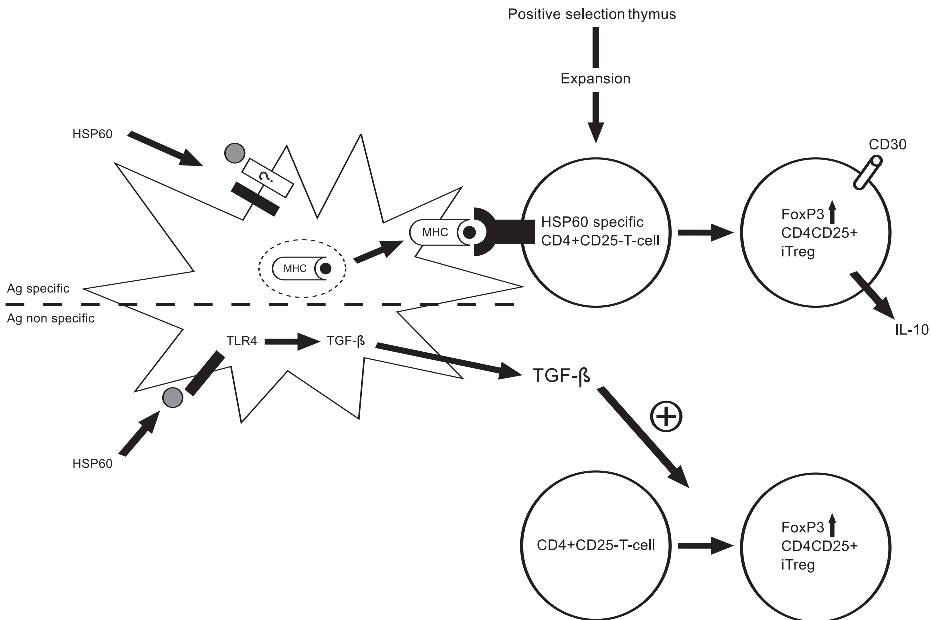

Figure 1. Human HSP60 mediates the induction of regulatory T cells by two pathways.
Ligation of human HSP60 with TLR4 on APC results in an antigen (Ag) specific and Ag nonspecific induction of $FoxP3^+$ Tregs. HSP60 is processed and presented in the context of MHCII to HSP60 specific T cells. The HSP60 specific T cells become activated and upregulate FoxP3. The Ag nonspecific pathway is mediated through the production of TGF-ß. TGF-ß mediates a polyclonal expression of FoxP3.

The finding that HSP60 (as well as LPS), through TLR ligation, can both activate the innate immunesystem and induce regulatory cells seems contradictory but can be understood by a recent observation. Engagement of TLRs on APCs leads to the up-regulation of MHC and co-stimulatory molecules on dendritic cells (DCs), a process called DC maturation and needed to efficiently prime naïve T cells. Pasare et all recently showed that the induction of DC maturation alone, as measured by the upregulation of CD80 and CD86, is insufficient to induce T cell priming in vivo: Besides DC maturation, IL-6 together with other cytokines is necessary to overcome $CD4^+CD25^+$ Treg mediated control of the priming process [1]. IL-6, produced by the DCs, act by rendering responder T cells refractory to suppressors and not by shutting of the ability of suppressors to carry out their function.
Taking into account that TLRs are primary sensors of microbial infection, the two contradictory results of TLR4 ligation, namely activation of the innate immune system and induction of Tregs, now make sense: At the start of an infection ligation of TLRs should be able to overcome the function of Tregs so that pathogen specific T cell responses can be initiated. However, when the pathogen has been cleared and the production of IL-6 decreased, inflammatory cells regain susceptibility to suppression by naturally as well as induced Tregs and the inflammation will be downregulated. Situations, in which this feedback mechanism is defective, for example by the increased production of IL-6, may lead to chronic inflammation. Indeed, high levels of Il-6 can be found in the peripheral blood of patients with systemic JIA [2]. Furthermore, the above-described mechanism may form a plausible explanation for the success of anti-IL-6 therapy, currently tested in clinical trials [17].

ASCT: an effective though risk full way to restore the balance

ASCT induces clinical remission in as many as 50% of the treated JIA patients (max follow up 7 year) (**chapter 5**). While before ASCT the patients showed a severely reduced frequency of peripheral blood $CD4^+CD25^{bright}$ Treg cells, this frequency was completely restored by the treatment with ASCT. This recovery of the $CD4^+CD25^+$ regulatory net-work was the result of a preferential expansion of $CD4^+CD25^+$ Tregs early after ASCT and a highly increased output of regulatory cells by the thymus. Furthermore, we showed that besides recovering the $CD4^+CD25^+$ regulatory network, ASCT induced persistent changes in human HSP60 specific T cells (**chapter 6 and figure 2**). While before ASCT the HSP60-peptide specific T cells, as analysed by using the T cell capture technique, expressed a low mRNA IL-10:IFN-ϒ ratio and a low GATA3:Tbet ratio, after ASCT both ratio's were reversed, suggesting the restoration of the regulatory capacity of HSP60 specific T cells. Altogether, these data suggest that ASCT is an effective way to restore the balance between regulation and (auto)immunity and that this balance is indeed needed to gain tolerance.
Interestingly, the one patient in the study group that suffered a complete relapse after ASCT showed a clear decrease in $CD4^+CD25^{bright}$ T cell frequency at the time of relapse, 6 months after ASCT. None of the patients that gained a long-lasting and drug-free remission by ASCT showed this phenomenon. Furthermore, upon evaluation of the clinical follow up of 34 JIA patients who received ASCT, 90% of all partial and complete

relapses of disease occurred in the first 9 months after ASCT (**chapter 5**). Each child that remained disease free here after remained so till maximal follow up. This observation emphasizes the importance of the first period of reconstitution, which seems to determine the level of regulation in the fully reconstituted immune system (**figure 2**). Importantly, though in our patient group with the present follow up no relapses occurred in the period between 9 months post ASCT and maximal follow up, this cannot be considered a guarantee for a life-long remission. The disruption of the newly regained balance between effector functions and regulation is probably not an one-event process, but rather the result of encountering just those multi-step environmental triggers that cause tolerance to break: A process that might take much longer than the present follow up.

Though the success rate of ASCT is relatively high, the procedure carries a significant mortality risk as well. Besides the "minor" adjustments in the protocol as described in **chapter 5**, meant to reduce mortality rates, new therapeutic strategies using less radical mechanisms of ablation of autoreactive cells, combined with a more selective stimulation of the regulatory network need to be explored.

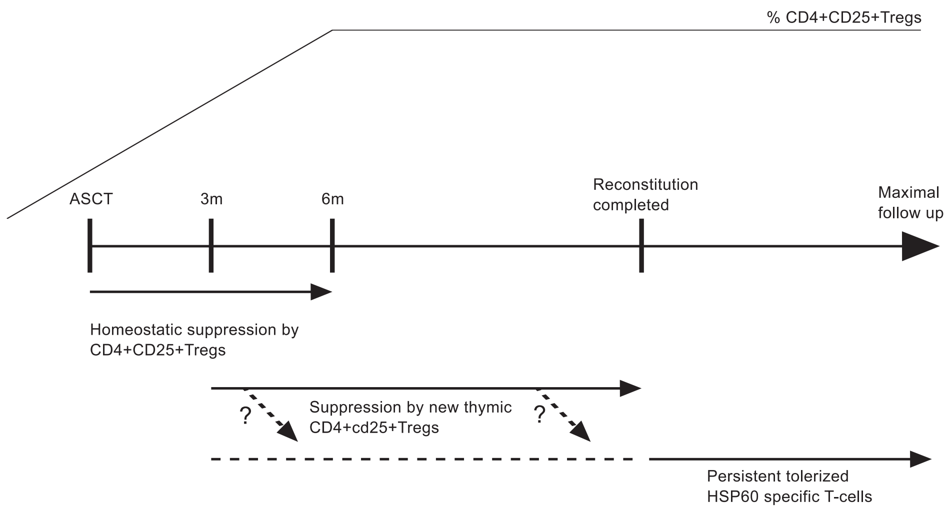

Figure 2. Recovery of the regulatory network after ASCT for JIA

Three mechanisms account for the recovery of the regulatory network after ASCT. In the first 3-6 months after ASCT the number of $CD4^{+}CD25^{+}$ nTregs rapidly accumulate due to a selective homeostatic proliferation of nTregs (1). Three to 18 months post ASCT a highly increased output of nTregs by the thymus is observed (2). In addition, ASCT resulted in the recovery of the regulatory phenotype of HSP60 specific Tregs (3).

Future therapeutic strategies: Tipping the balance at will

The stimulation of Treg cells seems to be an extremely attractive strategy, complementing and hopefully substituting for conventional Ag-non-specific immunosuppression with all of its hazards. However, a major hurdle in the application of Treg cells thus far is to selectively target antigen-specific Tregs and disarm the pathogenic T cells, while leaving undisturbed T cells directed against infectious pathogens and/or tumors. To this end antigens, even epitopes, need to be identified that are selectively recognized at the site of inflammation. The antigen specific, and consequently site specific, activation of Tregs may then induce bystander suppression and the local control of inflammation. This thesis, in addition to extensive previous work shows that HSP60 represents an important antigen that can potentially be used for the in vivo manipulation of the balance between regulatory and effector T cell function in JIA and presumably also in other chronic inflammatory diseases. HSP60 is selectively expressed at the inflammatory site, recognized by Tregs and able to induce the expansion of Tregs. Even more, epitopes of human HSP60 that are recognized by the majority of JIA patients have recently been identified (S. Kamphuis, submitted).
Another hurdle is the development of methods to manipulate and regulate the quality of Tr1 and/or $CD4^{+}CD25^{+}$ Treg suppressor function in vivo. Though nowadays there is great interest in exploiting in vitro expanded Treg cell lines as therapeutic agents, this seems not a very realistic approach. Besides being unattractive for commercial development, it would require a major commitment of transplant centers to cellular manipulations and to the storage of preexpanded T cell lines. A more realistic and presumably less risk-full way to harness the therapeutic use of Tregs is by promoting their antigen specific expansion in vivo. Three possible therapeutic strategies to achieve this goal are described below.

Mucosal tolerance approach

Antigens elicit qualitatively distinct immune responses based on their portal of entry [18]. Systemically introduced antigens, whether by injection or injury, leads to local infiltration of inflammatory cells and specific immunoglobulin production. By contrast antigens introduced at mucosal surfaces (such as the gastrointestinal and the respiratory tract) elicit tolerance to those antigens, systemically. In adjuvant arthritis [14,19], as well as in other animal models of autoimmune diseases such as diabetes [20], myasthenia gravis [21,22], experimental allergic encephalomyelitis [23,24], mucosal tolerance has proved to be effective in suppressing the disease. From these studies we know now that mucosal tolerance occurs after either administration of a single high dose of antigen or repeated exposure to lower doses [25-27]. These two forms of tolerance, now termed high- and low-dose tolerance, are mediated by distinct mechanisms. It has been shown that high doses of oral antigen induces anergy and/or deletion [28,29], while low-dose tolerance is now known to be mediated by active suppression of immune responses by regulatory T cells [30]. $CD4^{+}CD25^{+}$ nTregs [30,31] and Th3 cells [32,33] have mainly been implicated to play a role in mucosal tolerance. The contribution of Tr1 cells to mucosal tolerance is still unclear, though Tr1 cells have shown to suppress inflammation in mouse models of inflammatory bowel disease [34,35].
As discussed above each of the described regulatory T cell subsets needs to be activated in an antigen-specific manner, but is subsequently able to suppress immune

responses in the immediate surrounding area in an antigen-non-specific manner (linked suppression). Expansion and/or reactivation of HSP60 specific regulatory T cells, through the mucosal administration of peptides derived from HSP60, can be a potentially powerful therapeutic tool for restoring the immune balance and thus controlling autoimmune/inflammatory conditions. Potential peptides that can be used for this purpose were recently identified by using a novel computer algorithm for the analysis of potential pan-DR binding epitopes (A. Sette, Epimmune, La Jolla, CA). Testing of the selected peptides revealed clear T cell responses, as measured by T cell proliferation and antigen specific cytokine production, in a vast majority (60-90%) of patients with JIA (S. Kamphuis, submitted).

Combining anti-TNFα treatment and antigen specific therapy

The induction of mucosal tolerance with HSP-derived peptides has proven to be effective in the Adjuvant Arthritis model: not only in the prevention but also in the treatment of ongoing arthritis [14]. The question is whether, in the more complex human situation, self-HSP60 specific T cells will be sufficient in downregulating ongoing disease, especially once massive inflammation and joint destruction has taken place. When this is not the case, a combination therapy of anti-TNFα therapy followed by the mucosal administration of HSP derived peptides might be the solution. While long-term side effects and limited efficacy in certain groups of patients set bounds to the use of anti-TNFα therapy, anti-TNFα therapy does induce impressive immunological changes: Besides the induction of a “ceasefire” by a general suppression of pro-inflammatory pathways, an increase in the frequency of $CD4^+CD25^+$ Tregs was reported after treatment with infliximab [36]. It is very well possible, that HSP specific regulatory T cells, induced by mucosal administration during this “ceasefire”, have more regulatory impact than when induced during ongoing inflammation. The newly induced HSP60 Tregs might even be powerful enough to lead to sustained remission of the disease. Encouraging are the results in an animal model of experimental arthritis: A single, sub-optimal dose of etanercept, combined with oral tolerance induction with an HSP peptide was more effective than treatment with an HSP peptide alone (S. Roord et al., submitted).

Combining anti-CD4 MoAB’s and antigen specific therapy

When T cells recognize their specific peptides presented by APC, several additional receptor-ligand interactions transduce additional signals and stabilize the binding process, permitting T cell activation and clonal expansion. Blocking some of these critical ancillary signals at the time of T cell receptor engagement by antigen may lead to the induction of Tregs. This effect was first shown by Waldmann’s group, in the mid-1980’s. He discovered that antibodies against CD4, one of the co-stimulatory molecules, can induce immunological tolerance without depleting $CD4^+$ T cells. Most of the research concerning this tolerising effect of CD4 non-depleting antibodies has been concentrated in allogeneic transplant models (heart, skin, bone marrow) [37-39]. In these models the administration of anti-CD4 antibodies not only creates an “immune ceasefire” by blocking effector function, blocked $CD4^+$ T cells even gain a regulatory phenotype. In this regulatory environment, graft specific T cells that have never been exposed to CD4 antibodies, may gain regulatory properties as well, by experiencing antigen in the microenvironment of regulatory T cells. These graft specific, regulatory T cells are subsequently responsible

for a dominant tolerance that is reflected in findings of linked suppression.
Importantly, it has been shown that the resulting block of the rejection process persists after the physiological clearance of the CD4 MoAB's.
The same mechanistic and therapeutic principle as defined in these transplant models seems also applicable in autoimmunity. Several reports (animal models) have described extended remissions or tolerance to auto-antigens induced by treatment with CD4 antibodies [40-43]. The combination of CD4 MoAB and peptides of autologous HSP60 might form an effective treatment for JIA. A short period of CD4 MoAB therapy, ensuring not only a "ceasefire" but even a regulatory environment, and meanwhile exposing the immune system to peptides of autologous HSP60, by for example prolonged subcutaneous infusion of low doses of peptide [44], should theoretically result in the selective expansion of HSP60 specific regulatory T cells. Interestingly, besides CD4+ antibodies, CD3, CD11a, CD40L, CD25 and CTLA4-Ig have all shown capable of facilitating tolerance.

In a broader perspective

This thesis handles the concept of reprogramming immune cells in a way that would eliminate pathogenic responses while preserving normal immune function. Establishing such a "re-education" of the immune system is a desirable goal, not only for autoimmune diseases such as JIA, but also for many other immune mediated diseases. For example, the future focus for treating allergies and asthma moves beyond symptomatic therapies and aims at the replacement of allergen-specific Th2-driven processes with allergen-specific, protective immune responses, mediated by regulatory cells. Furthermore, the ultimate goal of organ transplantation is to establish graft tolerance as stably and naturally as in natural tolerance to self-constituents, without continuous general immunosuppression. One way of achieving this is to induce graft-specific regulatory T cells, sufficiently potent to maintain long-term graft acceptance. And finally, regulatory T cells can be exploited to tune the intensity of antimicrobial immune responses in acute and chronic infections and to develop effective ways of vaccination against microbes. Thus, there is little doubt about the enormous potential value of antigen-specific Tregs in the treatment of human immune mediated diseases. The biggest hurdle in the development of Tregs mediated immunotherapy for most diseases is, however, the identification of antigens/epitopes, specifically expressed at the site of interest. Interestingly, HSPs are upregulated at sites of tissue breakdown, not only at sites of autoimmune-inflammation, but also at sites of graft rejection or infections. This site-specific and stress-induced upregulation of HSPs and the, in this thesis described, capacity to induce regulatory T cells, suggests that HSPs could be immunoregulatory agents with more potent and widely-applicable therapeutic uses than in arthritis only. Therefore, future studies may be broadened and investigate the potential of HSP specific regulatory T cells in the regulation of other immune mediated diseases as well.

Conclusions

The findings described in this thesis suggest that HSP60 can cause a polyclonal induction of Tregs at sites of tissue damage. Furthermore, they provide the first evidence that $CD4^+CD25^+$ Tregs respond to autoantigens and add to previous reports demonstrating that Tregs play an important role in the detection and prevention of deleterious tissue damage. In JIA the quality (HSP60 Tregs) and quantity ($CD4^+CD25^+$ Tregs) of regulatory T cells present in the peripheral blood and the synovial fluid of the patient clearly correlates with the clinical course of disease. The in vivo expansion of antigen-specific (and thus site-specific) regulatory T cells is therefore an attractive focus of therapeutic strategies. Epitopes of human HSP60 repeatedly administered via the mucosa or in combination with anti-TNFα or CD4 MoAB therapy seem promising ways to achieve this feat. Together these data add to others and seem to give faith that, like the Odyssea, the exploration of immuneregulatory processes will come to a good end. Rather than being tantalized, new immunotherapies for chronic inflammatory diseases like JIA seem a realistic goal and only a few years away.

Utrecht, 1 September 2004

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Nederlandse samenvatting

Inleiding

Ons afweersysteem herkent en elimineert potentieel schadelijke micro-organismen en bestaat dan ook uit vele onderdelen die nauw samenwerken in de verdediging van ons lichaam. Om er voor te zorgen dat een afweerreactie op het juiste moment en de juiste plaats verloopt beschikt het lichaam over regulatiemechanismen. Soms falen deze regulatiemechanismen waardoor normale afweerreacties te hevig verlopen (bijvoorbeeld in allergie en/of asthma) of het afweersysteem zich richt tegen lichaams-eigen eiwitten, waardoor zgn auto-immuun ziekten ontstaan zoals Diabetes Mellitus of Reumatoïde Artritis.

Regulatoire cellen

Eén van de belangrijkste regulatiemechanismen waarover het immuunsysteem beschikt, is het systeem van de zgn regulatoire cellen. Dit zijn cellen van het afweersysteem (meestal T cellen), die in staat zijn onstekingsbevorderende of wel pro-inflammatoire cellen te remmen in hun activiteit. Eén type regulatoire cel is de "$CD4^+CD25^+$ regulatoire T cel (Treg)". $CD4^+CD25^+$ Tregs zijn in allerlei diermodellen in staat gebleken ontstekingscellen te deactiveren en daarmee ontstekingsprocessen af te remmen en zelfs te voorkomen. Hoewel inmiddels vrij veel onderzoek naar $CD4^+CD25^+$ Tregs is verricht in diermodellen is er nog bitter weinig bekend over of en hoe deze cellen in humane ziekten functioneren. Naast de ontdekking van $CD4^+CD25^+$ Tregs heeft onderzoek in een diermodel voor reuma (Adjuvant Arthritis), aangetoond dat T cellen specifiek voor "heat-shock eiwitten" (HSPs) regulatoire functies kunnen hebben. Heat shock eiwitten zijn eiwitten die een belangrijke intracellulaire rol spelen op het moment dat cellen aan stress van bijvoorbeeld hitte, infecties of UV-straling, onderhevig zijn. Op HSPs reagerende cellen zijn ook aangetoond in kinderen met Juveniele Idiopathisch Arthritis (JIA, jeugdreuma) en opvallend genoeg met name in kinderen met de meest gunstige vorm van de ziekte (zie beneden).

Juveniele Idiopathische Arthritis (JIA)

JIA is de meest voorkomende reumatische aandoening bij kinderen jonger dan zestien jaar, één op de duizend kinderen lijdt eraan. Ondanks alle obstakels die men tegenkomt bij het verrichten van basaal wetenschappelijk onderzoek in de mens, vooral als het gaat om kinderen, heeft JIA een aantal voordelen ten opzichte van andere humane ziekten die onderzoek naar immuun-regulatiemechanismen juist vergemakkelijken. Ten eerste is het, in het geval van reuma, mogelijk om materiaal te verkrijgen van de plek van ontsteking. Doordat de gewrichten tijdens actieve ziekte dik en gezwollen zijn, kan er tijdens actieve ziekte ontstekingsvloeistof uit de gewrichten worden afgenomen en kunnen de cellen die werkelijk betrokken zijn bij de ontsteking, dan wel de regulering ervan, bekeken worden. Ten tweede, bestaat jeugdreuma uit verschillende subtypes met zeer uiteenlopend ziektebeloop. Eén vorm van jeugdreuma, de zgn persisterende oligo-articulaire JIA (pers-OA JIA), heeft over het algemeen een uitstekende prognose. Per

definitie zijn er bij deze patiënten minder dan 4 gewrichten aangedaan en veel van deze kinderen blijken naar verloop "over de ziekte heen te groeien". Een ander subtype, de zgn extended oligoarticulaire JIA (ext-OA JIA) begint, net als de gunstige vorm, met minder dan 4 aangedane gewrichten, maar ontwikkelt zich na 6 maanden naar een veel ernstiger vorm van jeugdreuma, waarbij per definitie meer dan 4 gewrichten zijn aangedaan (polyarticulaire JIA). In dat geval is de prognose veel slechter. De ziekte is dan aanzienlijk moeilijker te behandelen en door de continue chronische ontsteking ontstaat er meestal veel schade aan de gewrichten.

Huidige behandelingen voor JIA

Momenteel worden kinderen met JIA behandeld met middelen als corticosteroiden, methotrexaat en anti-TNFα therapy; allemaal middelen die het hele immuunsysteem onderdrukken en niet alleen het kleine groepje cellen dat de ziekte veroorzaakt. Deze middelen hebben relatief ernstige bijwerkingen en de arts moet dan ook steeds een afweging maken tussen de nadelige effecten van de ziekte zelf en die van de medicatie. Hoewel de meeste kinderen met JIA redelijk tot goed reageren op de huidig beschikbare middelen, geven ze alleen onderdrukking van de ziekte en daarmee verlichting van de symptomen, maar genezen ze de patiënt niet. Bovendien is er een kleine groep patiënten waarbij de ziekte helemaal niet reageert op de beschikbare conventionele middelen. Het gaat dan om ernstig zieke kinderen die naast ernstige symptomen van de ziekte ook nog eens lijden aan ernstige bijwerkingen van de medicatie. Bij deze kinderen wordt er sinds 1997 een nieuwe therapie toegepast: de autologe stam cel transplantatie (ASCT). Dit is een vooralsnog experimentele en redelijk riskante therapie die bestaat uit het volledig uitschakelen van de immuuncellen van de patiënt en het daarna terugplaatsen van schone bloedvormende stamcellen.

Dit proefschrift

Regulatoire cellen in JIA

Hoofdstuk 2 beschrijft de typering van HSP60 specifieke T cellen in kinderen met JIA. HSP60 specifieke cellen blijken vooral in kinderen met de meest gunstige vorm van JIA (oligoarticulaire JIA) een duidelijk regulatoir karakter te hebben: in respons op HSP60 maken deze cellen het regulatoire cytokine IL-10 en brengen ze de "regulatoire membraanmarker" CD30 tot expressie. Kinderen met een ongunstige vorm van JIA (polyarticulaire JIA) daarentegen, reageerden niet of nauwelijks op HSP60 met de productie van IL-10 of de expressie van CD30.

Naast HSP60 specifieke T celen onderzochten we de frequentie en het voorkomen van $CD4^{+}CD25^{+}$ Tregs in JIA (**hoofdstuk 3**). Kinderen met de meest gunstige vorm van JIA (pers-OA JIA) blijken een significant hogere frequentie van $CD4^{+}CD25^{+}$ Tregs te hebben dan kinderen met de ernstiger vorm van JIA (ext-OA JIA); in zowel het bloed als de gewichtsvloeistof. Er is dus sprake van een duidelijke correlatie tussen de gevonden frequentie van $CD4^{+}CD25^{+}$ Tregs en het klinisch beloop van de ziekte. Behalve een verschil in het aantal $CD4^{+}CD25^{+}$ Tregs vonden we geen verschil in het phenotype en/of de functie van de cellen.

Het beinvloeden van de balans als nieuwe therapie voor JIA

Onze bevindingen dat het aantal regulatoire T cellen correleert met het klinische beloop van JIA ondersteunt de hypothese dat autoimmuniteit kan worden veroorzaakt door een relatief tekort aan regulatoire cellen en dus door een disbalans tussen ontstekingscellen enerzijds en regulatoire cellen anderzijds. Uitgaande van deze hypothese schuilen er mogelijkerwijs nieuwe en veel belovende therapieën in het selectief activeren en/of induceren van regulatoire cellen en daarmee het herstellen van de balans.
Het is echter belangrijk te realiseren dat het niet-antigeen specifiek stimuleren of een transfusie van regulatoire cellen daarvoor niet de oplossing is. Behalve het onderdrukken van ontstekingscellen kunnen regulatoire cellen namelijk ook effector cellen tegen bijvoorbeeld micro-organismen en tumoren onderdrukken. Zo maar een transfusie met polyclonale regulatoire cellen geven kan dus leiden tot ernstige bijwerkingen, zoals de ontwikkeling van tumoren en/of het persisteren van infecties.
Om de balans effectief te beinvloeden is een antigeen specifieke tactiek nodig: alleen die regulatoire cellen activeren/induceren die een antigeen herkennen dat selectief tot expressie wordt gebracht op de plek van ontsteking. Omdat regulatoire cellen hun regulatoire functie pas uitoefenen na het herkennen van hun antigeen, leidt dit tot een selectieve regulatie op de plek van ontsteking, dus alleen daar waar het nodig is.

De inductie van regulatoire cellen

Om in de toekomst een verstoorde balans tussen regulatoire cellen enerzijds en inflammatoire cellen anderzijds te herstellen moet er worden gezocht naar factoren die de deling en/of inductie van regulatoire cellen kunnen bevorderen.
Hoofdstuk 4 laat zien dat HSP60 de differentiatie van gewone, niet regulatoire $CD4^+$ cellen naar $CD4^+CD25^+$ regulatoire cellen stimuleert. Dat doet het via twee mechanismen: een aspecifiek mechanisme door binding aan zgn. “toll-like-receptoren” op antigeen presenterende cellen en een specifiek mechanisme, dwz via herkenning van HSP60 epitopen door de T cell receptor.
Dat dit laatste mechanisme een rol speelt bewezen we middels een nieuw ontwikkelde techniek waarmee HSP60-epitoop specifieke T cellen direct uit het bloed van JIA patiënten kunnen worden geisoleerd. De geisoleerde HSP60 specifieke cellen bleken een grote hoeveelheid mRNA FoxP3 tot expressie te brengen: een zeer specifieke marker voor regulatoire cellen.
Onze bevindingen dat HSP60 regulatoire T cellen kan induceren en dat $CD4^+CD25^+$ regulatoire cellen in staat zijn epitopen van HSP60 te herkennen lijken erg belangrijk. HSP60 is bij uitstek een antigeen dat tot expressie wordt gebracht op plaatsen van ontsteking. Eerder onderzoek heeft de expressie van grote hoeveelheden HSP60 in de gewrichten van kinderen met JIA aangetoond, maar ook bijvoorbeeld in de darm van patiënten met de ziekte van Crohn. In theorie, zou de activatie/inductie van HSP60 specifieke regulatoire T cellen dan ook moeten leiden tot regulatie op de plek van ontsteking alleen, dus alleen daar waar het nodig is. Bovendien belooft de universele rol van HSPs dat de activatie en/of inductie van regulatoire cellen met HSP60 ook een potentiële behandelingen voor meer aandoeningen dan JIA alleen kan zijn. In de discussie van het proefschrift worden drie mogelijkheden beschreven waarop HSP60 specifieke T cellen in vivo geinduceerd kunnen worden.

Autologe stam cel transplantatie voor onbehandelbare JIA

In 1997 werd er in het Wilhelmina kinderziekenhuis gestart met een pilot studie naar het effect van ASCT in kinderen met verder therapie resistente JIA. Na het succes van de eerste transplantaties (beschreven in de Lancet) werd de behandeling door meerdere ziekenhuizen, wereldwijd, overgenomen. **Hoofdstuk 5** van dit proefschrift beschrijft een follow-up studie van 34 kinderen met JIA die in 9 Europese transplantatie centra zijn getransplanteerd. De behandeling heeft in 50% van deze kinderen geleid tot een volledige, medicatieloze, remissie van de ziekte (maximale follow up 7 jaar). Bij een autologe transplantatie wordt de patiënt getransplanteerd met de eigen (autologe) stamcellen. De patiënt krijgt dus niet een "nieuw" immuunsysteem, maar het eigen blanco immuunsysteem, in "ge-resette" versie, terug. Waarom met het teruggeven van het eigen immuunsysteem de behandeling toch in 50% van de gevallen een volledige remissie tot gevolg heeft (tenminste zolang de follow-up duurde), onderzochten we in **hoofdstuk 6.**

Autologe Stam Cel Transplantatie herstelt de balans

Alle JIA patiënten behandeld met ASCT vertoonden voor ASCT een ernstig verlaagde frequentie van $CD4^{+}CD25^{+}$ regulatoire cellen in het perifere bloed. Dit aantal herstelde zich zeer vroeg na ASCT en bleek het resultaat van twee mechanismen (**hoofdstuk 6**). Ten eerste toonden regulatoire T cellen die de conditionering overleefden of teruggegeven werden met het transplantaat een snellere deling na transplantatie dan niet-regulatoire T cellen. Ten tweede leidde een verhoogde output van regulatoire T cellen door de thymus tot een snelle normalisering van de Treg frequentie. Naast een herstel in $CD4^{+}CD25^{+}$ Treg frequentie leidde ASCT tot blijvende veranderingen in HSP60 specifieke T cellen. ASCT lijkt dus een effectieve manier om de balans tussen regulatie en (auto)immuniteit te herstellen. Het is echter ook een zeer robuuste manier met invloed op het gehele immuunsysteem. Bovendien blijkt uit het onderzoek beschreven in **hoofdstuk 5** dat ASCT in deze zeer kwetsbare groep kinderen een mortaliteitsrisico heeft van 15%.

Conclusies

De bevindingen in dit proefschrift laten zien dat HSP60 regulatoire T cellen kan induceren. De studie levert bovendien het eerste bewijs dat $CD4^{+}CD25^{+}$ regulatoire T cellen autoantigenen herkennen en een rol spelen in de detectie en preventie van weefselschade. In JIA correleert de kwaliteit (HSP60 Tregs) en de kwantiteit ($CD4^{+}CD25^{+}$ Tregs) van regulatoire cellen in het bloed en de gewrichten van de patiënten met het klinische beloop van de ziekte. Daarom is de in vivo expansie van antigeen specifieke en dus plaatsgebonden regulatoire T cellen een belangrijke focus voor nieuwe therapieën. Middels epitopen van humaan HSP60, toegediend via de mucosa of in combinatie met anti-TNFα therapie of therapie met anti-CD4 monoclonale antilichamen lijkt dit doel binnen bereik.

List of abbreviations

AA	adjuvant arthritis
APC	antigen presenting cell
ASCT	autologous stem cell transplantation
ATG	anti-thymocyte rabbit immunoglobulin
CFSE	carboxyfluorescein diacetate succinimidyl ester
Con A	concanavalline A
CY	cy-chrome
Cy	cyclophosphamide
Ext-OA	extended oligoarticular
FITC	fluorescencein isothiocyanate
FLU	fludarabin
GITR	glucocorticoid induced tumor-necrosis-factor receptor
HSP	heat shock protein
IFN	interferon
IL	interleukin
iTreg	induced regulatory T cell
ILAR	International League Against Rheumatism
IVIG	intravenous immunoglobulin suppletion
JIA	juvenile idiopathic arthritis
LPS	lipopolysaccaride
MAb	mononuclear antibody
MAS	macrophage activation syndrome
MRNA	messenger ribonucleic acid
MTX	methotrexate
NSAID	non steroidal anti-inflammatory drugs
nTreg	naturally occurring regulatory T cell
PA	polyarticular
PB	peripheral blood
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered salt
PE	phycoerythrin
Pers-OA	persistent oligoarticular
RA	rheumatoid arthritis
SEM	standard error of mean
SF	synovial fluid
SFMC	synovial fluid mononuclear cells
TCD	T cell depletion
TCR	T cell receptor
TGF	transforming growth factor
Th1/2/3	T helper 1/2/3
TNF	tumor necrosis factor
Tr1	regulatory T cell type 1
Treg	regulatory T cell

Acknowledgements

This work is a synergistic product of many minds
It began in 1996 when an exciting period of clinical research, under supervision of Berent Prakken and Wietse Kuis, at that time still in the meanders of the "old WKZ", hooked me on research. It was not a coincidence that this period was followed by an inspiring period of eight months in San Diego in the lab of Salvatore Albani, and subsequently by the research that ended in this thesis: I am grateful for having found the best supervisors I could wish, in the most inspiring scientific field in pediatrics nowadays.

Berent, dat ik afgelopen jaren als een intense, plezierige en leerzame periode heb ervaren, heb ik vooral aan jou te danken. Door het vertrouwen en de vrijheid die je me gegeven hebt, maar ook door je gave (mijn) grenzen haarfijn aan te voelen was samenwerken een plezier en is dit onderzoek een succes geworden. Vol bewondering heb ik de afgelopen jaren kunnen toezien hoe jij in onafzienbare tijd "de Prakken-groep" hebt weten op te zetten. Wat ben ik trots de eerste in het promotierijtje te mogen zijn!

Wietse, bijzonder is jouw vermogen vanaf een afstandje steeds weer die vragen te stellen waar het om draait. Bedankt voor alle steun, je enthousiasme op gepaste tijden en je kritisch-analytische begeleiding. Een betere promotor heb ik mij niet kunnen wensen.

Nico, bedankt voor je enthousiaste begeleiding op het klinische gedeelte van het proefschrift. Je netwerken, potjes en creatieve inbreng waren een welkom aspect van de totale supervisie.

Salvo, you were the one who introduced me into basic immunology. More than anyone else you seem to understand that "Education is not filling a bucket, but lighting a fire". Besides that you taught me one of the most important aspects in science; to think "outside the box". Thank you for an inspiring time to which I am looking back with tremendous pleasure.

For the development and production of the book I owe many thanks
to the whole "Prakken"-group
Leuke, interessante, gemotiveerde en gezellige collega's om je heen hebben is niet vanzelfsprekend maar, tot mijn grote dankbaarheid, wel mijn dagelijkse ervaring.
Wilco en Mark, jullie zijn de twee rotsen in de branding van het Prakken-lab. Het is heerlijk om voor technische vragen altijd eerst bij jullie aan te kunnen kloppen en experimenten te kunnen doen in een lab dat loopt als een trein. Bovendien is jullie hulp met de computer, Elisa's, Lightcycle, en T cell capture van groot belang geweest voor vele delen van dit proefschrift.
Sylvia, samen met Wilco, erbij vanaf het begin. Met weemoed denk ik nog wel eens terug aan de "triplo" besprekingen en de bespreking van de trypan-blue pipetteer

techniek. “Evidemment”, is er heel wat gebeurd in de tussentijd. Ondanks dat de combinatie kliniek, onderzoek, Nina en Floris niet altijd mee valt schiet jouw boekje nu toch ook aardig op. Heel veel succes met het laatste deel en hopelijk kunnen we gauw nog eens op congres.
Yvonne Vercoulen, my one and only student. Ik ben het hele jaar trots op je geweest. In no time wist je alles over de regulatoire T cel en had je alle technieken onder de knie. Bovendien bracht je een hoop gezelligheid met je mee. Vetcoolie dus! Hoofdstuk 4 draag ik aan jou op. Heel veel succes en plezier in Londen en wie weet kruisen onze paden elkaar nog eens.
Huib, onze big guy. Ook jij bent er één van het eerste jaar. Terwijl het mij logistiek voor de wind ging, zat het jou niet altijd mee. Die tijd lijkt nu veranderd: met rasse schreden haal je nu alles en iedereen in. Veel succes met de laatste experimenten dit jaar.
Bas, met jou heb ik carpoolstrook en bench gedeeld. Ik weet nu dat de snelheid waarmee we naar Eindhoven reden slechts begrensd werd door de ouderdom van je auto. Je tempo en creatieve manier van denken zijn erg verkwikkend en aanstekelijk. Hopelijk kunnen we in de toekomst nog veel van gedachten wisselen binnen groepje 3, maar ook daarbuiten. Bedankt voor je hulp als paranimf.
Alvin, van jou heb ik geleerd dat gedrevenheid en rust prima samen gaan. Ook van jou hoop ik dat we ook na mijn promotie kennis en ideeën kunnen blijven uitwisselen.
Sarah, je bent de lijm in de groep en altijd gezellig. Dat tijd en energie overhebben zonde is ben ik met je eens. Bedankt voor het meedenken over alles, de paranimfen incluis.
Lianne, steeds weer vind ik je hartelijke lach en zwaai op het moment dat we elkaar tegenkomen in lab of gang bijzonder. Het is ook prettig, met jou lunchend me nooit schuldig te hoeven voelen over kroketten, kaassoufflés of toetjes. Bedankt voor die dingen en voor het zijn van de trouwste artikel doorstuurder.
Evelien, grote waardering heb ik voor jouw verantwoordelijkheidsgevoel. Schemaatjes, kadootjes en kaartjes; jij regelt het wel. Fijn, zo iemand in de groep! Bedankt ook voor je interesse en succes met je cavia’s, muizen en andere beesten.
Lize, tijdens het schrijven van dit dankwoord maak je een spannende tijd door. Ik wens je heel veel sterkte en ben vol vertrouwen dat je straks een goede balans zult vinden.
Kolbrun, the latest recruit of the Prakken-group. Thank you for your interest and your gezelligheid in the lab. I am looking forward to share the dance floor with you again, this time on my own party.
Suzanne Berlo, ook jij bent nu volledig lid van de Prakken-groep en voor ons allemaal een voorbeeld van innerlijke kracht en vechtlust. Ik hoop innig dat je je proefschrift vanaf nu zonder al te veel obstakels kunt afronden. Ook hoop ik nog regelmatig met je te kunnen afspreken om onder het genot van een kop thee, met of zonder één van die fantastische baksels, bij te kletsen.
Erica, bedankt voor al je hulpvaardigheid op het secretariaat en balvaardigheid op de tennisbaan. Deze winter gaan we er weer voor!

to all members of the IACOPO-institute
Gisella, thank you for all your help on the FoxP3 analysis. Good luck as the first post-doc in the Prakken-lab. Gijs bedankt voor je hulp tijdens je verblijf in San Diego en ook voor de gezelligheid op het lab in Utrecht. Eva, veel succes in San Diego; we houden contact!

to members of the Veterinary Faculty

Willem van Eden, je bent de grondlegger van het HSP-regulatie imperium. Bij jou was ik kind aan huis afgelopen maanden. Bedankt voor de gastvrijheid en bedankt voor het kritisch reviewen van meerdere van de artikelen uit dit proefschrift.

Ger Arkesteijn, heel wat uurtjes heb ik met jou doorgebracht achter de FACS. Het grootste, of in ieder geval meest interessante deel van dit proefschrift is voortgekomen uit de data verkregen in die uren. Bedankt voor het delen van je expertise en ook voor de gezellige gesprekken, vakantie- en muziektips.

Peter van Kooten, die hybridoma gaat nog zeker een keer lukken. Bedankt voor alles wat je Yvonne en daarmee ook mij hebt geleerd over cel kweek. Enne...volgend jaar schrijven we ons in!

to all others that were of importance for the quality of this thesis

Ger Rijkers, bedankt voor je scherpe bijdragen, feedback en ideeën; tijdens werkbesprekingen maar ook tijdens al die keren dat ik gewoon even je kamer kon binnen lopen.

Grada van Bleek, ook jij bedankt voor het delen van je ervaring, je opbouwende kritiek en scherpe vragen tijdens de werkbespreking.

Brenda, hoewel je geen lid bent van de Prakken-groep hoor je daar toch een beetje bij. Bedankt voor al je hulp met de Lightcycle, onze FoxP3 perikelen en met name ook voor je hulp met het verdrijven van het lab-spook. Hopelijk blijft ie voorlopig even weg!

De mensen van het “routine lab”. Met velen van jullie heb ik de afgelopen jaren met plezier samengewerkt. Met name Wil, Pirkko en Koos; bedankt voor het vele geduld wanneer ik weer met mijn vraagjes aankwam. Ook jullie interesse heb ik erg gewaardeerd. Via de kliniek houden we voorlopig contact!

Angela, je bent de onmisbare spreekbuis van Wietse. Bedankt voor alle ondersteuning.

En natuurlijk Martin, jij bedankt voor de prachtige kaft en al je hulp met de lay-out en het drukken van het proefschrift. Beter had je het niet kunnen regelen.

For the understanding of the necessity of these and future studies I am very thankful

to the many patients, parents and healthy volunteers who have cooperated

For the sharing of gossips, coffee breaks, the printer and finally the air-conditioning I am thankful

to all members of the “aio-kamer”

Wendy, Cora, Niels, Maaike, Selma, Patricia, Loes en voorheen Anne, Harriët en Mark, bedankt voor jullie interesse in de laatste fase van mijn proefschrift en de gezelligheid op de aio-kamer.

Selma, dat laatste productieve uurtje als alle PNI-ers en RSV-ers al de deur uit zijn, was best een beetje speciaal. Evelyn, weliswaar geen aio-kamergenoot maar wel een regelmatige bezoeker; het was leuk onze eindperikelen zo af en toe te kunnen delen. Veel succes met jouw afronding.

For the fact that I am looking forward to go back into clinics I am thankful

to colleagues like Mieke, Jaime and Gijs

Nu ik na twee jaar onderzoek ons “studieclubje” eigenlijk het hardst nodig heb, zijn we inmiddels uitgewaaierd over drie verschillende ziekenhuizen. Ik heb onze avondjes erg gezellig gevonden en hoop dat we zo af en toe nog kunnen blijven afspreken om al onze dromen, ziekenhuis roddels en (jullie) opvoed perikelen uit te kunnen wisselen.

to the pediatricians and residents of the Catharina Ziekenhuis in Eindhoven
Dr. Waelkens, Jan, Rolf, Hein, Angelique, Edwin, Willem en Carien, bedankt voor de klinische basis die ik in Eindhoven heb meegekregen. Een betere perifere opleiding kan ik mij niet voorstellen. Die overgang terug naar de kliniek zal nu vast meevallen. Raffi, Victorien, Bas, Mieke, Anne-Jet, Tom, Karin en Monique met jullie heb ik het langst de carpool strook bij Everdingen gedeeld. Dat reizen kostte wat tijd, de files wat frustraties; toch mis ik af en toe onze intieme uurtjes in Bas zijn rooie rakker of Karins polootje. Bedankt hiervoor.

For the joy I have in life and the people who save it from dullness I am thankful

to many friends/family, among them
Lidewij, mijn uiterste en soulmate tegelijk. Maar ook degene met wie ik ooit begonnen ben bij de pediatrische immunologie. Uiteraard dus ook mijn paranimf! Bedankt voor alles wat we tot nu toe gedeeld hebben en alle mooie dingen die we samen nog gaan meemaken en delen in de toekomst.
Cécile, Kralingen is nog steeds dichtbij. Bedankt voor de vriendschap tot nu toe en in de toekomst.
Suzan en Maria, en Roel en Marijn, het is super tof jullie om de hoek te hebben wonen. Ons contact is me erg dierbaar. Maria en Marijn, veel plezier in Zuid-Amerika. Blijf niet te lang weg! Suzan, even part-time een studietje erbij en ook nog clubkampioen; ik ben trots!
Maurice, Marjolein, Annemijn, Mats, jullie heb ik de afgelopen periode veel te weinig gezien en dus ook gemist. Ik kom gauw weer naar jullie toe!
Cor, Greetje, Johan, Guusje, Hannah en Daniël, bedankt voor jullie warmte en interesse.

Since thanks are the highest form of thought and gratitude is happiness doubled by wonder, I feel a deep sense of gratitude

to my parents
Pap, mam, dit proefschrift was er natuurlijk nooit gekomen zonder alle belangrijke bagage die jullie me meegegeven hebben. Bedankt voor jullie continue steun.

and to David
Daaf, er zijn twee ereplaatsen in dit dankwoord: de eerste en de laatste. Jouw steun was onmisbaar en je liefde en vriendschap veel belangrijker dan dit proefschrift.

Curriculum Vitae

Ismé de Kleer (Oosterbeek, 1973) behaalde in 1991 haar Gymnasium-ß diploma aan het Stedelijk Gymnasium te Arnhem. Datzelfde jaar begon zij aan de studie Geneeskunde in Utrecht. Tijdens deze studie heeft zij zich onder andere ingezet als bestuurslid van de medische studenten vereniging MSFU-Sams en als studentlid van het Faculteitsbestuur. Als wetenschappelijke stage verrichtte zij onderzoek aan de afdeling Pediatrische Immunologie van het Wilhelmina Kinderziekenhuis (Prof. dr. W. Kuis) naar longfunctie-stoornissen bij kinderen met Juvenile Idiopathische Arthritis (1996). Alvorens aan haar co-schappen te beginnen werkte zij 8 maanden in het labarotorium van Prof. Dr. S. Albani aan de University of California, San Diego (1996-1997). Als keuze co-schap genoot zij een stage tropische kindergeneeskunde in een ziekenhuis in Kola N'doto, Tanzania. Direct aansluitend aan de studie geneeskunde, in oktober 1999 werd zij AGIKO kindergeneeskunde (opleider Prof. dr. J.J. Kimpen). Inmiddels heeft zij 2,5 jaar van de klinische opleiding doorlopen, onder andere in het Catharina ziekenhuis in Eindhoven (2000-2001, opleider dr. J.J.J. Waelkens). Vanaf oktober 2000 werkte zij op de afdeling Pediatrische Immunologie aan het onderzoek dat geresulteerd heeft in dit proefschrift. Voor dit onderzoek ontving zij in 2001 de "Young Investigators Award" van de Pediatric Rheumatology European Society.

List of publications

I.M. de Kleer, Y. Vercoulen, M. Klein, G. Rijkers, W. van Eden, S. Albani, W. Kuis, B.J. Prakken. Human heat shock protein 60 regulates immune responses by the induction of $FoxP3^{+}CD4^{+}CD25^{+}$ regulatory T cells. Submitted.

I.M. de Kleer, B. Vastert, M. Klein, G. Teklenburg, G. Arkesteijn, G. Puga Yung, S. Albani, W. Kuis, N. Wulffraat and B. Prakken. Autologous stem cell transplantation tolerizes autoreactive T cells and restores the $CD4^{+}CD25^{+}$ immune regulatory network in juvenile idiopathic arthritis. Submitted.

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HAD I the heavens' embroidered cloths,
Enwrought with golden and silver light,
The blue and the dim and the dark cloths
Of night and light and the half light,
I would spread the cloths under your feet:
But I, being poor, have only my dreams;
I have spread my dreams under your feet;
Tread softly because you tread on my dreams.

W.B. Yeats

Finis operis.

(Aristoteles, 384-322 BC)

Notes

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