Heat Shock Proteins are Targets of Regulatory T cells

The studies described in this thesis were performed at the Division of Immunology, Department of Infectious Diseases and Immmunology, Faculty of Veterinary Medicine, Utrecht University, the Netherlands

Printed by Wöhrmann Print Service

ISBN 978-94-6203-491-4

Heat Shock Proteins are Targets of Regulatory T cells

Heat Shock Eiwitten als doelwit voor Regulatoire T cellen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
dinsdag 26 november 2013 des middags te 2.30 uur

door

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geboren op 3 mei 1983 te ‘s-Hertogenbosch

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General Introduction

Heat shock proteins can be targets of regulatory T cells for therapeutic intervention in rheumatoid arthritis

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Int J Hyperthermia 2013; 29; 448-54

Abstract

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by excessive immune responses resulting in inflammation of the joints. Although current therapies can be successful in dampening inflammation, a long-lived state of tolerance is seldom achieved. Therefore, novel therapies are needed that restore and maintain tolerance in patients with RA. Targeting regulatory T cells (Tregs) is a successful strategy to achieve tolerance, as was shown in studies performed in animal models and in human clinical trials. The antigen-specificity of Tregs is crucial for their effectiveness and allows for very specific targeting of these cells. However, which antigen is suitable for autoimmune diseases like RA, for which the autoantigens are largely unknown? Heat shock proteins (Hsp) are ubiquitously expressed and can be upregulated during inflammation. Additionally, Hsp, or Hsp-derived peptides are immunogenic and can be recognized by a variety of immune cells, including Tregs. Therefore, this review highlights the potential of Hsp-specific Tregs to control inflammatory immune responses. Targeting Hsp-specific Tregs in RA can be achieved via the administration of Hsp (derived peptides), thereby controlling inflammatory responses. This makes Hsp attractive candidates for therapeutic intervention in chronic autoimmune diseases, with the ultimate goal to induce long lasting tolerance.

Introduction: Immune tolerance by regulatory T cells

In order to maintain immunological self-tolerance under homeostasis and control autoreactive or exaggerated immune responses, regulatory T cells (Tregs) have evolved to keep unwanted responses in check (1). Tregs are specialized suppressor cells that constitute 5-7% of the peripheral CD4+ T cell population. Loss of Treg function leads to multi-organ autoimmune disease (2), while Treg dysfunction can lead to autoimmune diseases like diabetes and rheumatoid arthritis (RA) (3;4). CD4+ Tregs are characterized by high expression of the IL-2 receptor alpha chain (CD25) (2) and most Tregs express the transcription factor forkheadbox P3 (FoxP3) (5). There are several subsets of Tregs, which can be divided into natural Tregs (nTregs) and induced Tregs (iTregs) (6). nTregs leave the thymus as mature Tregs and predominantly recognize self-antigens, while iTregs differentiated from CD25- precursors in the periphery in response to foreign antigens. It is thought that these subsets complement each other in antigen-specificity (7): nTregs are specific for self-antigens (8) and circulate in the periphery, while iTregs are specific for foreign antigens (allergens, commensal microbiota, pathogens, alloantigens and altered self-antigens like tumor antigens and inflammatory antigens) (9) and can be found mainly at mucosal sites.
Both nTregs and iTregs share a variety of mechanisms to suppress the function of target cells upon activation via their T cell receptor (TCR) (10). Basically, there are four mechanisms of suppression (11). The first mechanism of suppression is the production of anti-inflammatory cytokines, of which transforming growth factor beta (TGF-β) and interleukin (IL)-10 are best defined (12). Recently the inhibitory cytokine IL-35 was discovered and was added to this list (13). The second mechanism of suppression is the cytolysis of target cells. Although several other immune cells were known to cause cytolysis of target cells, surprisingly this characteristic was also seen in Tregs. Tregs express Granzyme A and B and perforin, which can induce the lysis of CD4+ or CD8+ effector T cells (14). The third mechanism of suppression is the metabolic disruption of target cells. For instance, via their high expression of CD25, Tregs consume vast amounts of IL-2, thereby depriving other cells from this important growth factor (15). Finally, the fourth mechanism of suppression is the direct down-modulation of antigen presenting cells (APC) via inhibitory receptors like cytotoxic T lymphocyte antigen-4 (CTLA-4) that binds B7 (16), or lymphocyte activation gene 3 (LAG-3) that binds major histocompatibility complex (MHC)-class II (17).
In order to become suppressive, Tregs need to be activated via their TCR in an antigen-specific fashion. However, once activated, Tregs can suppress immune responses also to other antigens which is called 'bystander suppression' (10). Targeting antigen-specific Tregs with the appropriate antigen can therefore result in dampening of inflammation, even in conditions where the disease inducing antigens are unknown, such as RA. Moreover, Tregs can enable the induction of new suppressor cells by creating an anti-inflammatory environment. This 'infectious tolerance' can be of great benefit for long term suppression (18), making antigen-specific Tregs ideal targets for therapy in RA.

When tolerance fails: Rheumatoid Arthritis

Although central tolerance aims to eliminate high affinity autoreactive cells, some potentially dangerous cells do escape deletion. The role of Tregs is to maintain

homeostatic tolerance by controlling such cells, however, different circumstances can permit the breach of Treg mediated tolerance, as will be discussed below. In RA, autoreactive immune responses occur in which different immune cells infiltrate the joint which ultimately leads to bone erosion. The role of Tregs in RA has been extensively studied and has focused on the presence and function of these cells.

One possible explanation for the impaired immunotolerance in RA could be the lack of sufficient Tregs numbers in patients. However, several studies have demonstrated that cell numbers of CD4+CD25high Tregs in peripheral blood of active RA patients are comparable to healthy individuals (3;19-21). Moreover, one study has shown that Treg number in peripheral blood of RA patients is even increased as compared to healthy individuals (22). Additionally, CD4+CD25high cells have been shown in the synovial fluid (SF) of RA patients and express the proliferation marker Ki-67 (23). The influx of Tregs into the synovium can be explained by the expression of chemokine receptors like CXCR4, CCR4 and CCR8 (24) on Tregs, and that the ligands for CCR4 (CCL17 and CCL22) are highly expressed in the synovium (25). Studies in animal models have shown that Tregs can migrate to the site of inflammation (26;27). On the other hand, identification only of CD25 and FoxP3 is insufficient for the analysis of Tregs, since recently activated Teff also upregulate these markers (28). Therefore phenotypical analysis of Treg associated markers is not sufficient and the *in vitro* suppressive function of SF CD4+CD25high cells needs to be tested.

Several studies have looked into the suppressive capacity of SF Tregs. On the one hand, Tregs isolated from the synovial fluid are suppressive *in vitro* (19;22;29-31). On the other hand, Tregs isolated from the SF of juvenile idiopathic arthritis (JIA) patients are incapable of suppressing responder T cells from the SF (32;33), while in fact these Tregs were capable of suppressing responder T cells from peripheral blood. Others have shown that Tregs from RA patients are unable to suppress pro-inflammatory cytokine production (3;20), and that pro-inflammatory mediators such as tumor necrosis factor alpha (TNFα) can actually impair human Tregs (3;34). Thus, the proinflammatory environment in the inflamed joints leads to Treg dysfunction (23;35). Together with the unresponsiveness of effector T cells to Treg suppression, this leads to uncontrolled inflammation. Overcoming this imbalance is a major challenge for developing new therapies with long lasting effect. Interestingly, for the induction of therapeutic tolerance, Hsp peptide therapy shows promising results.

Restoring tolerance in RA: Peptide therapy with Heat shock proteins

Although current treatment for RA is successful in controlling inflammation, patients require life long treatment and can face severe side effects ranging from opportunistic infections to tumor development. Inducing long lasting tolerance by targeting antigen-specific Tregs via the administration of suitable antigens is considered as the next generation in therapy (36). For autoimmune diseases like RA, the disease-inducing antigen(s) are unknown. Therefore, target antigens should be selected on other characteristics. Considered as candidate antigens are those that are: 1) immunogenic, so that they can activate antigen-specific Tregs efficiently. For instance, it has been shown that heat shock protein (Hsp)-derived peptides are recognized by T cells, and that especially bacterial Hsp peptides that cross-react with self-HSP peptides induce stronger anti-inflammatory responses than non-conserved epitopes (37).

Hsp are major components of bacteria and immune responses against Hsp can have a protective effect in infectious diseases (38). For instance, protective Hsp-specific T cell responses have been found after bacterial infections with *Listeria* (39), *Mycobacterium* (40) and *Wolbachia* (41). However, in these cases, the Hsp responses included both effector and regulatory responses (see table 1 for Treg associated responses found after infection). This indicates that both Hsp-specific effector T cells and Tregs are induced or activated during infections. It has been shown that iTregs directed against pathogenic peptides can suppress pathogen-induced immunopathology (9), giving a possible explanation for the induction of Hsp-specific Tregs during infection Interestingly, although the Hsp-specific Tregs induced after *Listeria* infection made rats more susceptible for infection (39), these cross-reactive antigen-specific T cells were capable of suppressing AA in rats upon transfer (42). This shows that T cells raised against bacterial Hsp that are cross-reactive with self Hsp are capable of suppressing experimental arthritis. Therefore, bacterial Hsp peptides that have human homologues are particularly suited for selection as potential candidate antigens. Several animal models of experimental arthritis that are induced by non-bacterial antigens (e.g. pristane, collagen type II, proteoglycan) show spontaneous responses against Hsp, indicating their immunogenicity and relevance especially in inflammation (27;43;44) 2) candidate antigens for peptide therapy should be expressed at the site of inflammation, in the case of RA, that would be the synovial tissue. The local expression of target antigens will allow for the activation of antigen-specific Treg only there where suppression of the immune system is needed, reducing non-specific immune suppression. Hsp are ubiquitously expressed and can be found at sites of inflammation, for instance Hsp are expressed in inflamed synovial tissue of RA patients (45-47) 3) Next to the local expression, target antigens need to be specific for inflammation itself (i.e. upregulated as a consequence of inflammation). In this manner Treg activation only occurs in the presence of inflammation. In this case, Treg activation will no longer occur once tolerance has been established, which will reduce continued immuno suppression when not needed. For Hsp it is known that cells upregulate Hsp under conditions of stress (48), for which inflammation also qualifies, thereby enhancing their expression. In this case, stress (including inflammation) inducible Hsp is the best candidate antigen, because it is a 'bystander' antigen that is expressed at sites of inflammation and it is immunogenic in the sense that it is recognized by the immune system. Peptide therapy with bacterial Hsp peptides can induce or expand cross-reactive Hsp-specific Tregs that are activated locally at the site of inflammation (27). It needs to be determined whether the resolution of the inflammation leads to Treg inactivation, leading to a tailor-made immuno suppression with potentially very little side-effects.

It is possible that peptide therapy as a mono-therapy in ongoing inflammation may need additional support in order to be effective. Therefore combination therapy with low doses of anti-inflammatory drugs like disease-modifying anti-rheumatic drugs (DMARDs) or prednisone together with Hsp might be effective. This approach has already been tested and proved effective in a phase II clinical trial (49). In addition, combination therapy with low-dose anti-TNFα together with Hsp40 was able to suppress adjuvant arthritis in rats (50), indicating that combining current therapies in RA with the administration of Hsp can dampen inflammation.

In the case of infection during autoimmunity, the regulation by Hsp-specific Tregs can be difficult to predict. As mentioned, pathogen-specific Tregs can be formed, but their suppressive role is only apparent at the end stage of infection, when the pathogen is

neutralized and the immune system needs to be calmed down. Therefore, it is expected that infection will not suppress autoimmunity immediately, and it is not sure if it will suppress autoimmunity at all. Currently, there is no literature about Hsp-specific Tregs induced during infection in an autoimmunity model.
Thus, Hsp peptide therapy has the potential to activate T cells. However, for successful induction of tolerance these T cells should be able to effectively suppress other immune cells involved in the inflammatory process. The suppressive capacity of Hsp-specific T cells has been shown and several Treg associated markers have been identified on these suppressive Hsp-specific Tregs, which will be discussed next.

Heat shock protein specific Tregs

Hsp responsive T cells were previously identified in animal models for RA (51;52), and in peripheral blood of humans (53;54). Although many effector functions of Hsp-specific T cells were identified, like antigen-dependent proliferation and interferon gamma (IFN-γ) production (54;55), more recently also Treg-associated markers have been identified on Hsp-responsive T cells. Apart from CD25 and FoxP3, which substantially contribute to the suppressive function of Tregs, other markers or characteristics have been identified to be associated with Hsp-responsive Tregs after stimulation with Hsp (table 1). Some of the markers or characteristics identified were actually needed for Hsp-directed suppression. For instance, the lack of CD25 or FoxP3 on Hsp-induced T cells resulted in disease development as compared to control CD4+CD25- cells (27). Additionally, CD30, glucocorticoid-induced TNFR-related protein (GITR), or LAG-3 was required by Hsp-specific Tregs to be suppressive in the models tested (27;56). In transfer experiments performed in a mouse model for experimental arthritis, LAG-3 was necessary for suppression as CD4+CD25+LAG-3+ Tregs were suppressive, while CD4+CD25+LAG-3- Tregs were not able to suppress disease development (27). Additionally, only as little as 4.000 LAG-3+ cells were required to achieve disease suppression, indicating that Hsp-specific Tregs can be suppressive at low numbers. The underlying mechanism could be fast proliferation of the transferred Hsp-specific Tregs (in this case specific against Hsp70 peptide B29), due to the small clonal size which allows for the efficient generation of memory cells (57). This was demonstrated by the increased presence of Hsp-specific Tregs as compared to control Tregs, as well as an increased expression of Ki-67 on the transferred Hsp-specific Tregs. Apart from cell surface molecules, secreted anti-inflammatory cytokines like IL-4, IL-10 and TGF-β have been identified as effector molecules of Hsp-specific Tregs. These cytokines were required for suppression, as was shown in k.o. mice, or by blocking the function by neutralizing antibodies (43;58), proving that some specific Treg characteristics are required for the suppressive function of Hsp-specific Tregs.
Given the number of Treg markers expressed by Hsp-responsive T cells, the mechanism by which Hsp-specific Tregs can suppress inflammation seems rather broad. For instance, CD25 is involved in the metabolic disruption pathway (15), whereas CD30 (59), GITR (60) and LAG-3 (17) are used for cell-cell interactions which can lead to the inhibition of the target cell. The inhibitory cytokines IL-10 and TGF-β are secreted to suppress target cells, and it has been shown that IL-10 is crucial for Hsp70-mediated suppression of PGIA (61). Also, the Th2 cytokine IL-4 has been found to be secreted by Hsp-specific Tregs (43;62;63), which was responsible for the suppression CIA as a result

Table 1: Treg-associated makers expressed on T cells responsive to Hsp

Molecule	Function	Hsp	Model (of disease)
CD25	IL-2 receptor alpha chain	Hsp40 Hsp60 Hsp70 Hsp90	JIA (75), None (76), Lung Cancer (68,77) AA (50,78, AD (79), Atherosclerosis (69), DTH (58) Filariasis (41), Infection (58), None (66,80-82) PGIA (27), None (83) EAE (67), Cancer (84)
CD27	TNF receptor	Hsp60 Hsp70	None (56) None (83)
CD28	co-stimulatory molecule	Hsp60	AA (85)
CD30	TNF receptor	Hsp60	None (56)
CD45RA	on naive cells	Hsp60	None (80)
CD45RO	on memory cells	Hsp60	None 80)
CD69	activation marker	Hsp40	JIA (75)
CD86	co-stimulatory molecule	HS APC	AA (62)
CTLA-4	co-stimulatory molecule	Hsp40 Hsp60 Hsp70	JIA (75) None (56) None (83)
FoxP3	transcription factor	Hsp40 Hsp60 Hsp70 Hsp90	RA (49,74), None (76) AA (50,78), AD (79), Atherosclerosis (69), None (56,66,80) PGIA (27) Cancer (84)
GITR	co-stimulatory molecule	Hsp60	None (56)
IL-4	Th2 cytokine	HS APC Hsp70	AA (62) AA (63), CIA (43)
IL-5	Th2 cytokine	Hsp70	CIA (43)
IL-10	anti-inflammatory cytokine	HS APC Hsp40 Hsp60 Hsp70	AA (62) JIA (75) AA (86), Atherosclerosis (69), DTH (58), Infection (58), None (56,80) AA (63), CIA (43), Listeriosis (39), PGIA (27)
IL-13	Th2 cytokine	Hsp60	None (80)
LAG-3	inhibitory molecule	Hsp70	PGIA (27)
Nrp-1	inhibitory molecule	Hsp70	PGIA (27)
TGF-β	anti-inflammatory cytokine	Hsp60 Hsp70	AA (85-87), DTH (58), Infection (58) Listeriosis (39)

All markers depicted were identified on CD4+ Hsp-responsive T cells that were functionally tested for suppressive capacity in vitro (e.g. suppression assay), or in vivo (adoptive transfer).

Abbreviations: AA: adjuvant arthritis (rat); AD: atopic dermatitis (human); CIA: collagen-induced arthritis (mouse); CTLA-4: Cytotoxic T-Lymphocyte Antigen 4; DTH: delayed-type hypersensitivity reaction; EAE: Experimental Autoimmune Encephalomyelitis; FoxP3: forkhead box P3; GITR: glucocorticoid-induced TNFR family related gene; Hsp: Heat shock protein; HS APC: heat-shocked antigen presenting cells; IL: interleukin; JIA: juvenile idiopathic arthritis (human); LAG-3: Lymphocyte-activation gene 3; None: studies performed without inducing disease; Nrp-1: Neuropilin-1; PGIA: proteoglycan-induced arthritis (mouse); RA: rheumatoid arthritis (human); TGF-β: transforming growth factor beta.

of immune deviation from Th1 to Th2.
As mentioned previously, nTregs and iTregs are two separate subsets that rise from different sites (thymus or periphery) and recognize different sources of antigens (self or foreign). Which subset of Tregs would be Hsp-specific? Since Hsp epitopes can share great homology between bacteria and humans, we hypothesize that nTregs (mostly directed against self-antigens) could be able to cross-recognize bacterial Hsp epitopes at mucosal sites, including the gut. Additionally, iTregs formed after encounter with bacterial Hsp in mucosal tissue can be cross reactive to self-Hsp due to the homology of the sequence. Commensals induce the upregulation of Hsp70 in gut endothelial cells (64;65). In this manner, the Hsp from mucosal bacteria would allow for the maintenance of both Hsp-specific nTregs and iTregs. There is actually one study that shows the induction of Tregs from naïve cells in response to Hsp60 derived from gut micro flora (66). Additionally, we have unpublished data that shows that Hsp-specific Tregs induced *in vivo* after immunization with bacterial Hsp70 peptide B29 can suppress proteoglycan induced arthritis in recipients after adoptive transfer. These studies indicate that induction of Hsp-specific Tregs in response to bacterial Hsp can occur.
Thus, Hsp-responsive T cells can express or produce molecules that enable them to suppress inflammation and the source of Tregs could be the iTreg subset. Adoptive transfer therapy using these Hsp-specific Tregs in animal models for inflammatory diseases has shown the feasibility of using Hsp-specific Tregs for therapy, and could be a new therapeutic approach in the induction of tolerance for patients with RA.

Adoptive transfer therapy with Hsp-specific Tregs

Various studies in animal models have shown the potential of Tregs to suppress inflammation, including RA (27;67-70). Especially antigen-specific Tregs are suited for the control of inflammation since antigen-specific Tregs are functionally superior over the polyclonal Treg population (71;72). Clinical trials with adoptive transfer therapy of autologous polyclonal Tregs are currently studied for graft-versus-host disease, type 1 diabetes and kidney transplantation rejection (73). These involve the isolation and expansion of Tregs and re-infusion into the patient. Therapeutic approaches using Tregs to suppress inflammation currently involve the *in vivo* induction of polyclonal Tregs (for instance via the administration of anti-CD3 (74), or the induction of antigen-specific Tregs via peptide therapy (75). Additionally, ex vivo expansion or induction of polyclonal Tregs which are then reintroduced into the patient is an option. The rationale behind this approach is to increase the total number of Tregs and thereby to tip the balance from inflammation to tolerance. Both protocols require the isolation of ultrapure T cells (e.g. CD4+CD25- cells for induction of Tregs, or CD4+CD25high cells for expansion of Tregs), and sufficient controls to determine Treg function (FoxP3 expression and in vitro suppressive capacity) under good manufacturing practice (GMP) conditions. Due to the lack of well defined and abundantly expressed autoantigens with specificity for RA, antigen specific Tregs have not yet been introduced into the clinic. Nonetheless, antigen-specificity of the generated Tregs can be evaluated after stimulation with antigens through FACS analysis for Ki-67, neuropillin-1 (Nrp)-1 and IL-10 as previously described by us for adoptive transfer therapy for the mouse (27). The outcome of current clinical trials with Treg transfers mentioned earlier will determine the future for the implementation of antigen-specific Treg as a source for T cell therapy. Given the

superior suppressive capacity of antigen-specific Tregs over polyclonal Tregs, and the existence of Hsp-specific Tregs that can suppress chronic inflammation, the introduction of Hsp specific Treg through Hsp vaccination or possibly hyperthermia may lead to innovative therapeutic strategies, including adoptive transfer of Hsp specific Treg.

Conclusion

Autoimmune diseases like RA are characterized by uncontrolled inflammation directed against self tissues. Although current therapies can be successful in suppressing inflammation, RA patients can suffer severe side effects and new therapies are needed that induce long term tolerance. In this respect, peptide therapy with Hsp derived peptides that specifically activate antigen-specific Tregs, shows promising results. Identifying suitable epitopes that can be recognized by suppressive T cells is therefore needed, as well as expansion protocols for Hsp-specific Tregs that can be used for adoptive transfer therapy.

Acknowledgements

This work was supported by Innovation Oriented Programme in Genomics Project Grants IGE3018 and IGE07004, European Union Grant Seventh Framework Programme TOLERAGE: HEALTH-F4-2008-202156, and the Dutch Arthritis Association.

Outline and aim of this thesis

Using Hsp to modulate immune responses during inflammation has great potential. Utilizing Tregs can be a powerful tool to restore balance, however the choice of antigen is important in activating these suppressor cells. The aim of this thesis was to study the immunosuppressive effects of Hsp70-derived peptides on chronic inflammation in a mouse model for rheumatoid arthritis. We have further investigated the role of antigen-specific T cells and Tregs that were activated after administration of Hsp70 peptides.
In c**hapter 2** we review current literature about the use of hsp to restore immunological balance during chronic inflammation and the role of antigen-specific Tregs during this process.
In **chapter 3** we describe the identification of Hsp70 epitope B29 and its homologs, which were shown to suppress chronic inflammation after intranasal application. In this chapter we further investigated the mechanism by which Hsp70 peptides activate immune cells. We found that antigen-specific Tregs were activated after administration of B29 peptide and that adoptive cell transfer of B29-specific Tregs suppressed (already established) arthritis in mice. Transferred cells were long-lived and remained present at the former site of inflammation. Depletion of transferred cells abolished the suppression, resulting in the reoccurrence of the disease. We also found that selecting B29-specific Tregs via LAG-3 enabled us to transfer as little as 4.000 cells to suppress arthritis in mice.
In **chapter 4** we identify the subset of Tregs that is activated by immunization of Hsp70 peptide B29. We show that immunization with B29 induces Tregs in the periphery from CD25- precursors. These induced Tregs were suppressive in vitro and in vivo after adoptive cell transfer in mouse recipients with arthritis.
In **chapter 5** we reveal a new transgenic mouse with CD4+ T cells specific for Hsp70 peptide B29 and its homologs. With this TCR-transgenic mouse model we will be able to further characterize and study Hsp-specific Tregs function under inflammatory conditions and look more specifically into the nature of naive Hsp-specific T cells.
Finally, in **chapter 6** the data from this thesis are summarized and discussed in the context of recent knowledge in the field of antigen-specific immune regulation. We will discuss the potential of Hsp-specific Tregs to suppress inflammation, and the utilization of these cells for future therapies.

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Stress proteins are used by the immune system for cognate interactions with anti-inflammatory regulatory T cells

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FEBS Letters 2013; 587; 1951-1958

Abstract

Since the initial discovery of the protective role of heat shock protein (HSP) 60 in arthritis, T cell recognition of endogenous HSP was found to be one of the possible underlying mechanisms. Recently we have uncovered potent disease-suppressive Tregs (anti-inflammatory immunosuppressive T cells) recognizing HSP70 self-antigens, and enabling selective targeting of such Tregs to inflamed tissues. HSP70 is a major contributor to the major histocompatibility complex (MHC) Class II ligandome and we have shown that a conserved HSP70-epitope (B29) is abundantly present in murine MHC Class II. Upon transfer, B29-induced CD4+CD25+Foxp3+T cells suppressed established proteoglycan- induced arthritis (PGIA) in mice. These self-antigen specific Tregs were activated in vivo and as little as 4.000 cells sufficed to fully inhibit arthritis. Furthermore, in vivo depletion of transferred Tregs abrogated disease suppression. Given that B29 can be presented by most human MHC class II molecules and that B29 inhibited arthritis in HLA-DQ8 (human MHC) transgenic mice, we feel that therapeutic vaccination with selected HSP peptides can be an effective route for induction of anti-inflammatory Tregs as a novel intervention in chronic inflammatory diseases.

Introduction

The fundamental problem of autoimmune diseases is faulty regulation of the inflammatory process. In the past we have regarded autoimmune inflammation as a process initiated by the accidental emergence of a forbidden clone of self-reactive effector T cells; however, it is now clear that the immune systems of healthy people are populated with T cells and B cells bearing receptors that can bind self-antigens. Chronic inflammation and autoimmune diseases result from the chronic activation or repeated reactivation of self-reactive lymphocytes that are an intrinsic and normal element of the healthy immune system. Inflammatory disease results from the failure of the immune system to down-regulate these potentially dangerous cells. Thus, the rational goal of therapy in diseases of unregulated inflammatory activation is to reorganize physiological regulation. Current therapies are oriented towards indiscriminate suppression of immune cells and molecules and are therefore less safe, as they create general immunosuppression with risks of losing resistance against for instance infectious diseases or cancer. For these reasons, the relatively recent discovery of a specialized T cell subset with the capacity to regulate (down-modulate) effector T cells has led to a very active area of research aimed at the exploitation of these so-called Tregs for the cure of autoimmune, atopic (allergy) and other inflammatory diseases. In addition Tregs are analyzed for their potential to promote transplant tolerance. Clinical trials with adoptive transfer therapy of autologous polyclonal Tregs are currently carried out for graft-versus-host disease, type 1 diabetes and kidney transplant rejection [1,2]. These involve the isolation and ex-vivo expansion of Tregs and re-infusion into the patient. The logistics of these forms of cellular therapies are however complicated. A much more attractive and straight-forward approach would be the in vivo expansion of Tregs by the immunization with the relevant antigens. Such an approach would resemble the vaccination approach of infectious diseases: in vivo administration of antigen with the purpose of eliciting cognate interactions with antigen specific receptors leading to an adaptive immune response. Like other CD4+ T cells, Tregs are selected in the thymus on the basis of cognate TcR interactions with self-antigens. Therefore, in theory, antigen specific manipulation of Tregs through a vaccine like approach must be possible. Based on recent findings obtained in a mouse model of autoimmune arthritis we now have evidence that stress proteins may constitute an attractive source for antigens that can be used to engage into cognate interactions with Tregs.

Treg, the physiologic inhibitors of inflammatory diseases

The cellular part of the immune system comprises several specialized subsets of T cells, including regulatory T cells (Tregs) that maintain homeostatic self-tolerance and control excessive immune responses (230 Corthay,A. 2009). Dysfunction of Tregs can lead to autoimmunity [3,4], while absence of Tregs results in multi-organ autoimmune diseases [5]. Tregs are characterized by the expression of CD25, the IL-2 receptor alpha chain [5], and the transcription factor forkheadbox P3 (FoxP3), which regulates the expression of Treg associated molecules. Tregs can be subdivided into natural occurring Tregs (nTregs), that come from the thymus and mainly recognize self-antigens in the tissues [6]. Additionally, induced Tregs (iTregs) [7] are another subset which differentiate from CD25_ precursors in the periphery and are located at mucosal tissue, while these

cells recognize foreign antigens (such as antigens from pathogens and commensals, allergens, alloantigens and tumor antigens, or inflammatory antigens) [8,9]. Tregs express a variety of effector molecules that enable the suppression of target cells [10]. These mechamisms of suppression can be characterized into four types. First, is the suppression via anti-inflammatory cytokines like transforming growth factor beta (TGF-b), interleukin (IL)-10 and IL-35 [11]. Secondly, Tregs are able to lyse target cells via Granzyme A and B, and perforin [12]. Thirdly, Tregs are able to influence the metabolic pathway of other cells, for instance by depriving them from the growth factor IL-2, due to high IL-2 consumption via CD25 [13]. Finally, the fourth mechanism of suppression involves the action of inhibitory receptors like Cytotoxic T lymphocyte antigen-4 (CTLA-4) that binds B7 on antigen presenting cells (APC) [14], which gives an inhibitory signal to the APC. Additionally, lymphocyte activation gene 3 (LAG-3) that binds major histocompatibility complex (MHC)-class II, delivers an inhibitory signal [15]. Tregs are interesting cells to target because they can induce 'bystander suppression': once activated via their TCR, Tregs suppress immune responses to other antigens [16]. Activating Tregs via disease relevant antigens is a suitable approach, however in the case of RA, it is better to select for antigens that are up-regulated during inflammation since the disease inducing antigens are unknown. Not only the antigen specificity of Tregs makes these cells ideal targets for therapy, also the induction of new suppressor cells via activated Tregs helps to control inflammation. This 'infectious tolerance' induced by Tregs can result in long term suppression of inflammation.

Stress proteins and the MHCII ligandome

Being intracellular proteins, textbook immunology would primarily teach that HSP do load MHC class I molecules. And indeed, induction of HSP specific class I restricted CTL responses (Cytotoxic T cell responses) have been documented in many different situations of cell stress. One of the first observations was made with CTL raised against mycobacterial HSP65, as these CD8+ class I restricted T cells recognized macrophages subjected to various forms of cells stress. This was a first demonstration of the fact that HSP are processed in stressed host cells and can be presented in the context of class I molecules [17]. Interestingly, besides class I, also class II molecules are loaded with HSP peptides. In fact, HSP70 peptides were prominently represented in the RP-HPLC profile of the content of class II molecules of a human lymphoblastoid cell line [18]. It is of interest to note that the loading of MHCII by intracellular stress proteins occurs especially proficient in cells under stress. In this case the routing of the intracellular cargo into the MHCII compartment is organized through autophagy [19]. Autophagy consists of a collection of intracellular routing pathways and essential homeostatic maneuvers by which cells break down their own components in lysosomes and direct the fragments for presentation on MHCII molecules. Perhaps a primordial function of this lysosomal degradation pathway is adaptation to various forms of stress such as nutrient deprivation. In addition, in complex multicellular organisms, these pathways or autophagy proteins orchestrate diverse aspects of cellular and organismal responses to dangerous stimuli such as infection [20]. Some HSP70 family members are directly involved with one of the molecular machineries that take care of autophagy, such as so-called chaperone mediated autophagy [21]. Chaperone-mediated autophagy (CMA) refers to the chaperone-dependent selection of soluble cytosolic proteins that are

then targeted to lysosomes and directly translocated across the lysosome membrane for degradation. This may well explain why fragments of HSP70 have been found to dominate the MHCII ligandome of both human and mouse cells [22,23], especially under conditions of cellular stress. See Table 1 for a rather complete listing of HSP70 peptides eluted from MHC Class II molecules. The B29 sequences are in the boxed area. To activate CD4+ T cells, and thus Treg, peptides should be presented by MHC class II molecules. Cytosolic proteins, like HSP70, are by default loaded on MHC class I molecules while extracellular proteins will be presented on MHC class II. The classical ''textbook'' distinction between MHC class I and MHC class II loading pathways has been proven not fully correct because cytosolic proteins have been eluted from MHC class II and vice versa. It is known that natural MHC class II ligands are preferentially generated from longlived cytosolic or nuclear proteins [24], and that long lived proteins are preferentially turned over by autophagy. HSP70 and HSC70 seem to have relatively long half-lives of between 4 and 20 h and are found more frequently in MHCII than in MHCI molecules [25]. Autophagy has been initially found as a process to sustain metabolic fitness during food deprivation through bulk protein degradation [26]. The role of autophagy in the immune system is only now becoming clear [27]. Two pathways can result in loading of intracellular peptides on MHC class II. First, intracellular proteins can be incorporated into autophagosomes that subsequently fuse with lysosomes for degradation of their cargo (macroautophagy). In addition, cytosolic proteins can be transported via LAMP2a directly into the lysosome (chaperone mediated autophagy) [28,29]. Recently, the role of autophagy in loading HSP70 peptides has been described; in human HLA-DR4+ B cells a striking increase of especially HSP70 peptides was eluted from HLA-DR4 upon induction of autophagy by amino acid deprivation [22]. Autophagy induction coincided with elevated HSP70 mRNA levels. In other words, especially under conditions of cell stress, fragments of HSP70 will be presented on antigen presenting cells to T cells, possibly initiating a regulatory T cell response. A recent paper by Costantino et al. [30] analysed peptides obtained from MHCII molecules of human activated T cells. It was already known that antigens derived from CD4+ T cells injected as a vaccine can activate so called antigen-specific idiotypic and ergotypic responses [31,32], which also can have a regulatory activity. HLA-DR+CD4+ T cells themselves have been hypothesized to present T cell-derived proteins such as CD25 or HSP60 [22]. Costantino et al. were unable to identify any peptides derived from HSP60; interestingly, however, they identified peptides derived from heat shock cognate 71 kDa protein (HSPA8), a ubiquitously expressed chaperone protein.

HSP are abundant at sites of inflammation

To understand the pathogenesis of autoimmune diseases an intensive search has been made to delineate specific antigens being involved in the break-down of self-tolerance and leading to these diseases. This has resulted in a broad range of distinct antigens possibly causally involved. On theoretical grounds however one would have doubts whether low numbers of autoantigen specific Tregs in polyclonal T cell populations would be able to exert sufficient effect to suppress ongoing inflammation. For this process to be effective the likely scenario is that only sufficiently prevalent Tregs with the ability to engage into cognate interactions with an abundantly produced autoantigen would be able to have significant impact on such a process. And here heat shock proteins are

Table 1. Hsp70 peptides eluted from MHC Class II molecules

Sequence of eluted peptide	Peptide originating from [(a)]	MHC Class II type	Ref.
QQYLPLPTPKVIGID	human HSPA13 (23-37)	HLA–DR10 (DRB1*1001)	54
IIANDQGNRTTPSY	mouse HSPA1A (28-41), HSPA1B (28-41), HSPA1L (30-43), HSPA2 (29-42), HSPA8 (28-41)	I-Ak	55
ITPSYVAFTPEGERL	mouse HSPA5 (62-76)	I-Ab	56
TPSYVAFTDTERLIG(DA)	human HSPA1A (38-52), HSPA1L (40-54), HSPA2 (39-53), HSPA8 (38-52)	HLA-DR7	57
TPSYVAFTDTERLIGD	human HSPA1A (38-52), HSPA1L (40-54), HSPA2 (39-53), HSPA8 (38-52)	HLA-DQ2	58
DVYVGYESVELADSNPQ	human HSPA13 (77-93)	HLA-DQ2	58
DAAKNQLTSNPEN	mouse HSPA5 (79-91)	I-Ag7	59
PFVEAEKSNLAYD	mouse HSPH2 (78-90)	I-Ab	60
DAAKNQVAMNPTNTVFDAK	human HSPA8 (53-71)	HLA-DRB1*1301; *1501; DRB3*0202; DRB5*0101	61
NPTNTVFDAKRLIGRRFD	human HSPA8 (62-79)	HLA-DRB1*1104	62
LIGRTWNDPSVQQDIKFLP	human HSPA5 (98-116)	HLA-DR7	63
QDIKFLPFKVVEKKTKPY	mouse HSPA5 (111-128)	BoLA-DRB3*1201(in mus line)	64
LTKMKEIAEAYLGKTVTNAV	human HSPA8 (124-143)	HLA-DR7	63
AVVTVPAYFNDSQRQATKDAGTIAGLN	human HSPA8 (142-168)	HLA-DR7	63
LNVLRIINEPTAAAIAYG (NVLRIINEPTAAAIAYG)	rat HSPA1A (167-184), HSPA1L (169-186), HSPA2 (168-185), HSPA8 (167-184)	HLA-DRB1*0401 (in rat line)	65
NVLRIINEPTAAAIAYG	human HSPA1A (168-184), HSPA1L (170-186), HSPA2 (169-185), HSPA6 (170-186), HSPA8 (168-184)	HLA-DRB1*0401/DRB4*0101	66
NVLRIINEPTAAAIA	human HSPA1A (168-184), HSPA1L (170-186), HSPA2 (169-185), HSPA6 (170-186), HSPA8 (168-184)	HLA-DRB1*0401/*02x/DRB5*0101	67
NVMRIINEPTAAAIAYG	human HSPA5 (194-210)	HLA-DRB1*0401/*02x/DRB5*0101	67
VMRIINEPTAAAIAYG	human HSPA5 (195-210)	HLA-DRB1*0401/DRB4*0101	66
IINEPTAAAIAYGLD	human HSPA1A (172-186), HSPA1L (174-188), HSPA2 (173-187), HSPA5 (198-212), HSPA8 (172-186)	HLA-DQ6 (B*602)	68
KREGEKNILVFDLGGGTFD	mouse HSPA5 (214-232)	I-Ab	60
FDVSILTIEDGIFE	human HSPA8 (205-218)	HLA-DQ2	58
GIFEVKSTAGDTHLGGEDFD	mouse HSPA2 (218-237), HSPA8 (215-234)	I-Ab	60
NRMVNHFIAEFKRK	mouse HSPA8 (236-249)	I-Ek	69
RMVNHFIAEFKRKH	mouse HSPA8 (236-249)	I-Ek	70
VNHFIAEFKRKHKKD	human HSPA8 (238-252)	HLA-DR11/w52	18
XDFYTSITRAXFEE	human HSPA1A (291-304), HSPA1L (293-306), HSPA2 (294-307), HSPA6 (294-306), HSPA8 (291-304)	HLA-DR11/w52	18
EGEDFSETLTRAKFEEL	mouse HSPA5 (315-331)	BoLA-DRB3*1201(in mus line)	64
ADLFRGTLDPVEK	human HSPA8 (307-319)	HLA-DQ6 (B*0604)	68
PVEKALRDAKLDKSQIHD	mouse HSPA8 (316-333)	I-Ab	60
DLNKSINPDEAVAYGA	human HSPA1A (358-373), HSPA1L (360-375)	HLA-DRB1*0101; 0301; DRB3*0101	61
KSINPDEAVAYG	human HSPA1A (361-372), HSPA1L (363-374), HSPA2 (364-375), HSPA6 (363-374), HSPA8 (361-372)	HLA-DQ2	58
TIPTKQTQTFTTYSDNQP	rat HSPA1A (419-436), HSPA8 (419-436)	RT1.Bl	71
VPTKKSQIFSTASDNQPTVT	human HSPA5 (443-462)	HLA-DRB1*0401/DRB4*0101	66
GERAMTKDNNLLG	human HSPA1A (445-457), HSPA1L (447-459), HSPA2 (448-460), HSPA6 (447-459), HSPA8 (445-457)	HLA-DR4Dw4	72
GERAMTKDNNLLGKFE	human HSPA1A (445-460), HSPA8 (445-460)	HLA-DRB1*0401/DRB4*0101	66
GERAMTKDNNLLGRFE	human HSPA6 (447-462)	HLA-DRB1*0401/DRB4*0101	66
ANGILNVSAVDKSTGKE	human HSPA8 (482-499)	HLA-DRB*0401	73
GILNVSAVDKSTGK	human HSPA8 (484-497)	HLA-DRB*0401	73
GILNVSAVDKSTGKE	human HSPA8 (484-498)	HLA-DRB1*0401/DRB4*0101	66
CNEIINWLDKNQ	human HSPA8 (574-585)	HLA-DR4Dw10	72
EIINWLDKNQTA	human HSPA8 (576-587)	HLA-DRB1*0402 and -1104	74
ISWLDKNQTAEKEEFE	human HSPA8 (578-593)	HLA-DQ8 (transgenic in NOD)	59
YGSGGPPPTGEEDTSEKDEL	mouse HSPA5 (636-655)	I-Ag7	59

(a) Identification of the Hsp70 proteins with the Entrez Gene ID between brackets:
human HSPA1A (3303), HSPA1L (3305), HSPA2 (3306), HSPA5 (3309), HSPA6 (3310), HSPA8 (3312), HSPA13 (6782),
mouse HSPA1A (193740), HSPA1B (15511), HSPA1L (15482), HSPA2 (15512), HSPA5 (14828), HSPA8 (15481), HSPH2 (15525),
bovine HSPA5 (14828) and rat HSPA1A (24472), HSPA1L (24963), HSPA2 (60460), HSPA8 (24468).
For the Hsp70 nomenclature (including the traditional names) see Kampinga et al., Cell Stress and Chaperones, Volume 14, Number 1, 105-111, DOI: 10.1007/s12192-008-0068-7

interesting candidate antigens to serve as targets for Tregs at sites of inflammation. Inflammation goes together with the production of various mediators that trigger the up-regulation of HSP. Most prominent in this can be the reactive oxygen species (ROS), toxic factors that lead to tissue damage and amplification of the inflammatory reaction. In addition to this the lipid mediators of inflammation (products of the arachidonic acid cascade) and pro-inflammatory cytokines (IL1, IL6, TNFa) are known inducers of the heat shock response [33]. It is probably this up-regulated presence of HSP that induces the production of HSP specific antibodies in patients with chronic inflammatory conditions. Local presence of up-regulated HSP at sites of inflammation was already shown by HSP60 antibody staining in light microscopy immunohistochemistry of synovial membranes of patients with juvenile chronic arthritis. The increased staining for LK1, with a unique specificity for mammalian HSP60, the mitochondrial chaperone, thus unequivocally demonstrated that this is due to a raised expression level of endogenously produced host HSP60 and not to deposition of bacterial antigens [34]. Aging is known to impact the capacity of cells to upregulate stress proteins. A decrease in HSF1 (the transcription factor which organizes HSP expression) activity has been seen in normally aging individuals, and in aging fruit flies and worms, leading to relative loss of the heat shock system [35,36].The reason for the decrease in the HSF1 activity is unknown but it results in an attenuated heat shock response and a decrease in the ability of an individual to cope with stress. Possibly, along similar lines, the relative loss of expressed HSPs can contribute to a loss of Treg activity, resulting in a relative loss of self-tolerance. If so, this may be an explanation for the increased occurrence of chronic inflammatory diseases in aged individuals. Besides reduced expression, also structural changes in HSPs might influence the maintenance of self-tolerance. HSP70 polymorphisms have been associated with inflammatory or autoimmune diseases such as Crohn's disease [37], Alzheimer's disease [38], pancreatitis [39] and with development of graft versus host disease upon allogeneic haematopoietic stem cell transplantation [40]. Interestingly, decreased HSP expression has been observed in some immune disorders. A low HSP70 response has also been described in a subtype of Biobreeding (BB) rats with a high susceptibility for development of autoimmune diabetes [41]. Similar results have been found in human PBMC from patients with newly diagnosed type-1 diabetes. In that study, stress responses were found to become re-established again in patients with longstanding diabetes, more than eight months after disease manifestation. So, defective HSP70 induction coincided with beta cell directed inflammatory activity, and seemed modulated by pro inflammatory cytokines rather than metabolic factors [42]. Additional evidence for the role of HSPs as targets for Tregs may have come from studies of in vivo manipulation of HSP expression levels. In one of our previous studies we have identified carvacrol, one of the main essential oils of many oregano species, as an effective co-inducer for HSP70. Oral administration of carvacrol in mice was found to up-regulate the expression of HSP70 in Peyer's patches, the secondary lymphoid organs of the gut. When lymphocytes were collected from carvacrol treated animals, raised T cell responses to HSP70 were observed. Moreover, the induction of arthritis in carvacrol treated animals was almost fully impossible. The inhibitory effect on arthritis turned out to be transferable with CD4+ T cells obtained from carvacrol fed mice. Altogether, the effects seen with the HSP co-inducer carvacrol were fully compatible with the induction of HSP specific anti-inflammatory Tregs that merely resulted from the upregulated HSP70 in gut lymphoid tissues[43]. Also by other means, such as serological identification of antigens by recombinant expression cloning

(SEREX), stress proteins such as DNAJA1 (a HSP70 associated co-chaperone), were defined as targets for naturally occurring Tregs [75].

Peptide B29: a conserved Treg inducing HSP70 epitope

As mentioned above, fragments of stress proteins, such as HSP70 family members, are frequent and relatively abundant in the MHCII ligandome of cells and stressed cells in particular. This means that HSP epitopes are well represented on cells poised for presenting their internal cargo to T cells. Regulatory T cells are for their function dependent on triggering through their TcR (T cell receptor). That such stress protein fragments can be targeted by Treg was demonstrated recently by van Herwijnen et al. [23]. A conserved mycobacterial HSP70 peptide (B29) (see Fig. 1 for its structure, conservation and position in the HSP70 sequence) was found to have the capacity to induce a very potent regulatory T cell response. Due to its conservation, various self-homologs with amino acid sequences almost identical with B29, were identified in the mammalian HSP70 family members. Interestingly, the mammalian B29 homologs were also found to be present in human HLA DR4 molecules obtained from stressed B cells (130 [22]). Following immunization with B29, responding spleen lymphocytes were selected by cell-sorting for regulatory T cells on the basis of CD4+CD25+Foxp3+expression. By adoptive transfer of these sorted Tregs we found that these cells had a remarkable capacity to suppress (ongoing) disease. The latter was shown in an experimental model of autoimmunity, proteoglycan induced arthritis. By the in vitro re-stimulation of the B29 induced Treg with the mammalian homolog's, the up-regulation of Treg associated activation markers was seen, indicating that indeed the mammalian homologs were in vivo targets of these Tregs. By using a congenic cell marker (CD90.1) is was possible to trace back transferred CD90.1.2 positive T cells in the CD90.2 recipient animals. By doing this the Treg phenotype (Foxp3+, CD25+) was found to remain stable, even until day 50 after the transfer. The cells were found in peripheral blood, bone-marrow, draining lymph-nodes, spleen and also the joint synovium. In addition, by infusing anti-CD90.1 specific antibodies to deplete transferred disease suppressive CD90.1.2+ cells in vivo, it was shown that the disease returned, providing direct proof of the disease suppressive nature of the transferred Treg. Therefore, as it seems, we have identified peptide B29 as an evolutionary conserved HSP70 epitope with homologues abundantly present in human and mouse MHC class II molecules. T cells recognizing B29 and these homologues were found to be strongly disease suppressive and characterized by CD25+Foxp3+LAG3+expression. Exceptionally low numbers of these cells, up to as few as 4000 cells, were capable of preventing induction of disease and of suppressing already established disease. These cells were long-lived and found to reside in the joints and draining lymph-nodes. The findings made with the B29 epitope of HSP70 in Balb/c mice concur with earlier findings of disease inhibition with a conserved mycobacterial HSP70 derived peptide in rats with mycobacteria induced adjuvant arthritis [76]. In this case the peptide, p111, was found to be effective upon nasal and not upon parenteral administration. The analysis of responding T cells following activation with p111, revealed the production of IL10 as the possible disease suppressive mechanism of the p111 specific T cells.

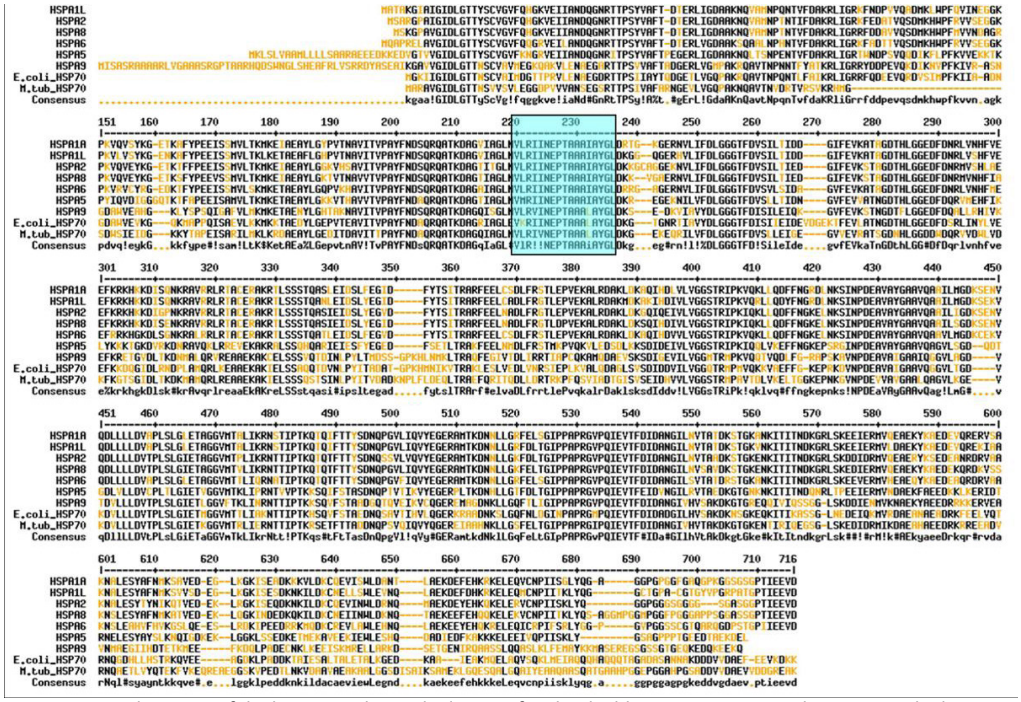

Fig. 1. Protein alignment of the human and microbial HSP70 family. The blue square contains the B29 microbial sequence and the mammalian homologous sequences. The extreme degree of sequence conservation is notable for the B29 regulatory T cell epitope.

Other examples of HSP specific Treg that inhibit inflammatory diseases

In the model of atherosclerosis induced by a, cholesterol rich, western type diet in LDL receptor knock-out mice, oral administration of HSP60 was found to inhibit plaque formation in the clipped (partially obstructed) carotid artery and in the aortic root. Reduction in plaque size correlated with an increase in CD4(+)CD25(+)Foxp3(+) regulatory T cells in several organs and in an increased expression of Foxp3, CD25, and CTLA-4 in atherosclerotic lesions of HSP60-treated mice. The production of interleukin (IL)-10 and transforming growth factor (TGF)-beta by lymph node cells in response to HSP60 was observed after tolerance induction [44]. Similar observations with oral administration of whole (mycobacterial) HSP60 had been made by others earlier, very much in line with the accumulating evidence for a pivotal role of HSP60 in atherosclerosis [45–48]. In the study of van Puijvelde [44], however, the same disease inhibitory effect was seen with both whole HSP60 and a defined HSP60 derived T cell epitope: 253–268. Therefore, the latter study provides additional evidence for a disease suppressive mechanism mediated by HSP specific Tregs. HSP90 was also found to inhibit spontaneous diabetes in NOD mice, although in this case mechanisms may have remained to be solved [49]. A more exciting set of findings was made with an HSP60

peptide, HSP60 peptide 277, in type I diabetes. This peptide, also known as DiaPep277 may well be the first therapeutic vaccine with the capacity to reinstall the HSP-mediated immune regulation in this important clinical entity [50]. The Cohen group (Weizmann Institute) has done pre-clinical studies of HSP60 peptides in NOD mice, the model of spontaneous type I diabetes [51,52] and has gone onto develop DiaPep277 in particular for the treatment of developing diabetes mellitus in humans. DiaPep277 performed very well in phase II [53] and recently in phase III clinical studies: Newly diagnosed patients were randomized to receive injections of 1 mg DiaPep277 or placebo subcutaneously for 2 years at quarterly intervals. Insulin treatment was administered by the patients' physicians as needed. The primary efficacy endpoint was the change from baseline to study end in glucagon-stimulated C-peptide. The study was carried out in 40 centers in Europe, Israel and South Africa. Excitingly enough, DiaPep277-treatment was safe, well tolerated and significant preservation of C-peptide levels was observed. Treated patients experienced fewer hypoglycaemic events with a significant difference in the rate of decline in the hypoglycemic events/month. More patients in the treated group were in partial remission maintaining target HbA1c levels while requiring less insulin. Thus, HSP60 peptide treatment preserved beta-cell function and improved clinical outcomes over 2 years in newly diagnosed type 1 diabetes patients.

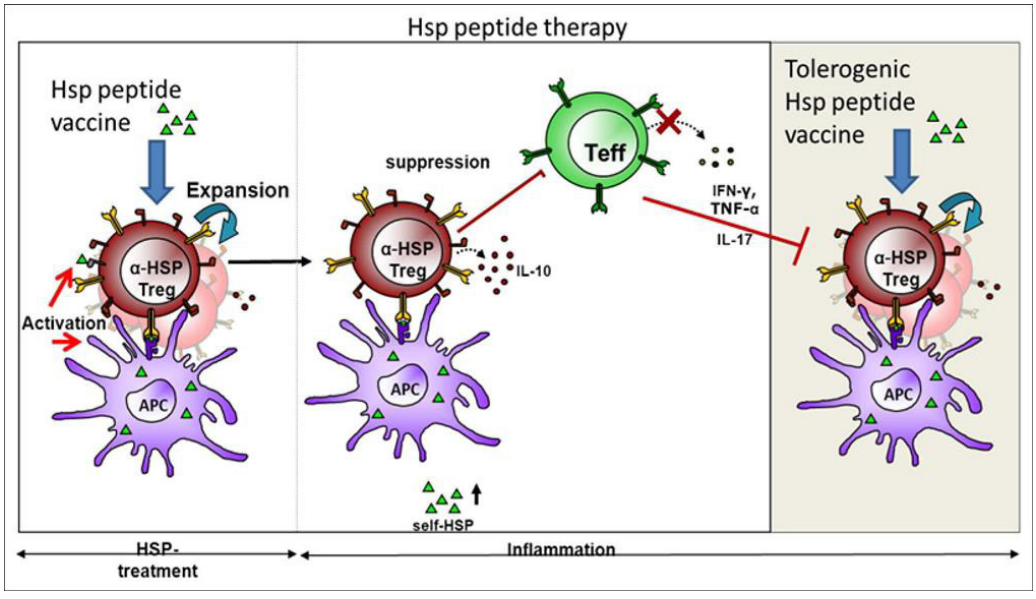

Fig. 2. HSP peptide based vaccines have been shown to enhance regulatory activity in chronic inflammatory disorders through expansion of Tregs amongst others via peptide MHC complex activation. Direct expansion of Tregs is hampered by the pro-inflammatory cytokines produced by the inflammatory T effector cells. To enhance the Treg inducing capacity of the vaccines, tolerogenic adjuvants and routes of administration will be exploited.

Conclusion

Findings on the role of HSP in the induction of anti-inflammatory T cell responses have led to a concept wherein the regulatory T cells of the immune system exploit the abundant presence of stress proteins for their default suppressive activity. The cellular interaction in this concept are presented in Fig. 2. In the occasion of inflammatory stress the further up regulation of stress proteins by antigen presenting cells leads to a further enforcement of the regulatory T cell activity. By artificial immunization with stress proteins such as HSP60 and HSP70 the repertoire of HSP specific Tregs is increased leading to a raised resistance against inflammatory diseases. The first clinical trials with HSP peptides in autoimmune diseases have been promising and therefore lead to the expectation that further development of immuno-modulatory vaccines will be possible with the use of HSP proteins and peptides.

Acknowledgements

This work was supported by Innovation Oriented Programme in Genomics Project Grants IGE3018 and IGE07004, European Union Grant SeventhFramework Programme TOLERAGE: HEALTH-F4- 2008-202156, and the DutchArthritis Association.

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3

Regulatory T cells that recognize a ubiquitous stress-inducible self-antigen are long-lived suppressors of autoimmune arthritis

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Proc Natl Acad Sci USA 2012; 109; 14134–39

Abstract

Re-establishing self-tolerance in autoimmunity is thought to depend on self-reactive regulatory T cells (Tregs). Exploiting these antigen-specific regulators is hampered by the obscure nature of disease-relevant autoantigens. We have uncovered potent disease-suppressive Tregs recognizing Heat shock protein (Hsp) 70 self-antigens, enabling selective activity in inflamed tissues. Hsp70 is a major contributor to the MHC Class II ligandome. Here we show that a conserved Hsp70-epitope (B29) is present in murine MHC Class II and that upon transfer B29-induced $CD4^+CD25^+Foxp3^+$ T cells suppress established proteoglycan-induced arthritis (PGIA) in mice. These self antigen-specific Tregs were activated *in vivo* and when using Lymphocyte Activation Gene-3 (LAG-3) as a selection marker, as little as 4.000 cells sufficed. Furthermore, depletion of transferred Tregs abrogated disease suppression. Transferred cells exhibited a stable phenotype and were found in joints and draining lymph nodes up to two months after transfer. Given that i) B29 administration by itself suppressed disease, ii) our findings were made with wild type (TcR non-transgenic) Tregs and iii) the B29 human homologue is presented by HLA-Class II, we are nearing translation of antigen-specific Treg activation as a novel intervention for chronic inflammatory diseases.

Introduction

Regulatory T cells (Tregs) are a subset of specialized $CD4^+$ helper T cells defined by the expression of the IL-2 receptor α-chain (CD25) (1) and the transcription factor FoxP3 (2). $CD4^+CD25^+FoxP3^+$ Tregs have the capacity to downregulate immune responses and are essential for immune homeostasis. Mechanisms by which they regulate include the secretion of anti-inflammatory cytokines such as IL-10 and IL-35, and the inactivation of antigen-presenting cells through neuropillin-1 (Nrp-1) and Lymphocyte Activation Gene-3 (LAG-3) (3, 4). While polyclonally expanded populations of Tregs exhibit suppressive activity (5), antigen-specific Tregs appear superior in suppressing autoimmune diseases (6, 7). In addition, the antigen-specific targeting of Tregs may avoid induction of a potentially detrimental systemic immunosuppression. Ideally, effective Treg-mediated suppression would be generated by continuous recognition of ubiquitous self-antigens (8, 9), preferentially expressed during inflammation (10-12). For autoimmune diseases like rheumatoid arthritis (RA), it is unknown which antigens are critical for disease. Interestingly, Heat Shock Proteins (Hsps) are relatively abundantly expressed in tissues such as the inflamed synovium (13-15). We and others have shown previously that immunization with *mycobacterial* (*Mt*) Hsp70 can induce an Hsp-specific T cell response with anti-inflammatory activity (16-18).
Therefore, we set out to examine the capacity of Hsp70 to serve as a target for antigen-specific Tregs. In this study we show that immunization or intranasal administration of the Hsp70 epitope B29 induces LAG-3 expressing antigen-specific Tregs that can directly suppress experimental arthritis.

Results

The identification of the Hsp70 epitope B29

First, we identified Hsp70 T cell epitopes using *Mt* Hsp70-immunized Balb/c mice and clustered them according to sequence conservation (Table S1). In subsequent immunizations with pooled Hsp70 T cell epitopes, we observed a strong response against peptide B29 (Fig, 1 A and B). Cross-reactivity between bacterial- and self-Hsp is proposed to be critical for the regulatory potential of Hsp70 (19). Notably, the sequence of B29 shows strong homology with at least two human/mouse Hsp70 family members (Table S2). B29-induced T cells responded to mB29a or mB29b after *in vitro* restimulation (Fig, 1 C and D), indicating cross-recognition of mammalian homologues. Previous studies have shown that Hsp70-derived peptides are abundantly present in MHC Class II molecules (20-22) (Table S3) and length variants of the B29 homologues have been identified frequently in the peptide binding grooves of HLA-DRB*0401 molecules (21, 23-25). In MHC peptide-elution studies we found that also in Balb/c mice homologues of B29 are presented with relative abundance in MHC Class II molecules of bone marrow-derived DC (Table S4), indicating that these peptides are naturally presented by MHC Class II in our model, thereby allowing for cross-recognition by B29-induced T cells.

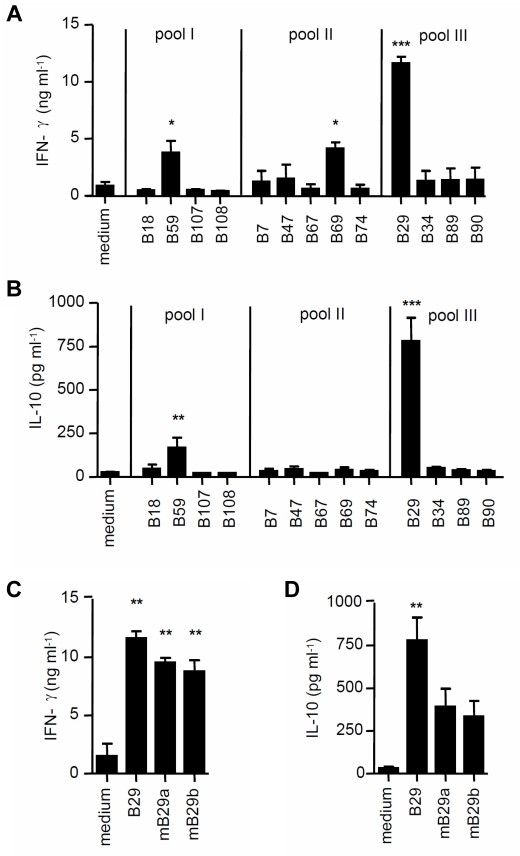

Figure 1. B29 is a dominant T cell epitope of Hsp70. Pools containing 4-5 epitope-peptides were made based on homology between mammalian and bacterial Hsp70 (Table S1). Mice were immunized with one of the pools and splenocytes were restimulated ex vivo with Hsp70 peptides individually. (A) IFN-γ and (B) IL-10 production of spleen cells measured in supernatant after restimulation with medium or bacterial-Hsp70 peptides. Data show the average cytokine production (± s.e.m.) after restimulation with peptides that were present in the immunization pool, n=3 mice per immunization group. (C) IFN-γ and (D) IL-10 production by splenocytes from mice immunized with pool III after restimulation with B29 or mouse homologue peptides mB29a and mB29b. Indicated is the mean cytokine production (± s.e.m.) of n=3 mice. P values are from an unpaired two-tailed Student t-test in which Hsp70 peptides were compared to medium and are representative of two independent experiments. * P < 0.05, ** P <0.001, *** P < 0.001.

B29 induced Tregs are responsive to B29 and homologues *in vitro*

To investigate whether Tregs were included in the B29-induced T cells, we isolated $CD4^+CD25^+$ cells from B29 or pOVA-immunized mice and analyzed their phenotype and regulatory properties. $CD4^+CD25^+$ T cells were suppressive *in vitro* in a standard suppression assay using anti-CD3 stimulation (Fig. S1 A). Phenotypical analysis of $CD4^+CD25^+FoxP3^+$ T cells from B29-immunized mice showed a slight increase in the percentage of IL-10, IL-35 (p35 subunit) and LAG-3 positive cells (Fig. S1 B), which prompted us to use upregulated Treg markers as a readout for *ex vivo* activated B29-specific Tregs. $CD4^+CD25^+FoxP3^+$ cells from B29-immunized mice showed increased expression of the activation marker Ki-67, as well as upregulation of Nrp-1 and IL-10 after restimulation with B29 or homologue peptides mB29a and mB29b (Fig. 2). Tregs from pOVA-immunized mice specifically responded to pOVA, which resulted in increased expression of Ki-67 and Nrp-1 (Fig. S1 C). The upregulation of these markers has been associated with Treg function (3, 4, 11) and therefore these results demonstrate that $CD4^+CD25^+FoxP3^+$ Tregs from B29-immunized mice can respond to B29 *ex vivo* and most importantly also to the mouse homologues mB29a and mB29b.

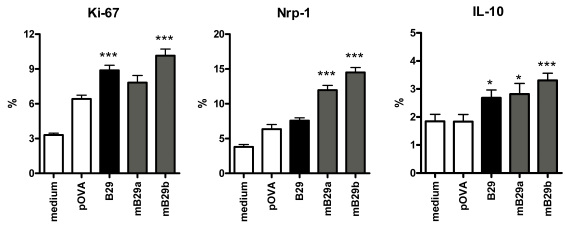

Figure 2. B29 immunization primes for a recall response to B29 and its homologues in CD4+CD25+FoxP3+ T cells. Splenocytes from B29-immunized mice were restimulated in the presence of pOVA (control peptide), B29, or homologous mB29a or mB29b peptides and stained for Ki-67, Nrp-1 and IL-10. Percentage of positive cells (± s.e.m.) is shown within the CD4+CD25+FoxP3+ population. P values are from an unpaired two-tailed Student t-test in which Hsp70 peptides were compared to pOVA. * $P < 0.05$, *** $P < 0.001$. Data are mean of 5-10 animals per group and are representative of two independent experiments.

Adoptive transfer with B29 induced Tregs suppresses arthritis

We next examined whether B29-induced cells were functionally activated *in vivo* after adoptive transfer. For this, $CD4^+CD25^+$ Tregs from B29 or pOVA-immunized donor mice were isolated and injected into recipients with proteoglycan induced-arthritis (PGIA), an experimental mouse model for rheumatoid arthritis (26). Adoptive transfer with B29-specific Tregs suppressed disease both prophylactically and therapeutically (Fig. 3 A) indicating an active suppressive function of the transferred Tregs in response to endogenously presented Hsp70 peptides. Tregs from pOVA-immunized donors were not suppressive, presumably due to the absence of their cognate antigen (ovalbumin) *in vivo*. It has been shown previously that *in vitro* pre-activation of polyclonal Tregs was essential for suppressing experimental arthritis upon adoptive transfer (27). Notably however, the transferred B29-specific Tregs were not *in vitro* pre-activated yet demonstrated very effective suppression *in vivo*. As Tregs must be activated via their TCR in order to become suppressive (7, 28), we may infer *in vivo* antigen recognition by the B29-specific Tregs. To examine if intravenously administered cells act locally at the site of inflammation and remain phenotypically stable over time, we transferred $CD90.1.2^+$ congenic Tregs from B29-immunized, pOVA-immunized or naïve donor mice into $CD90.2^+$ recipients with PGIA. These injected cells were distinguished from host cells by flow cytometry (Fig. 3 B) and were found in various organs at least up to 50 days after transfer (Fig. 3 C). Compared with transferred $CD25^-$ T cells, the $CD25^+$ B29-specific Tregs were relatively enriched, especially in the joints, while the opposite was found in the blood. Additionally, most transferred $CD4^+CD25^+$ Tregs remained $CD25^+$ and $FoxP3^+$ until the end of the experiment (Fig. 3 D). Moreover, the transferred $CD90.1.2^+$ Tregs from B29-immunized donors revealed enhanced Ki-67, Nrp-1 and LAG-3 expression in the dLN of the joints (Fig. 3 D), indicating local activation. These experiments demonstrate that transferred Tregs can migrate to sites of inflammation and this Treg population remains present for up to 50 days while maintaining an activated phenotype. To further investigate if the B29-specific Tregs suppressed disease by direct regulation or through induction of infectious tolerance (29), transferred $CD90.1.2^+$ cells were depleted with anti-CD90.1 antibody. At day 35, when Treg-mediated suppression of disease was established, depletion of B29-specific Tregs abrogated disease suppression (Fig. 3 E). Overall, these data reveal that Tregs from B29-immunized mice are potent suppressors of inflammation and are directly involved in disease suppression.

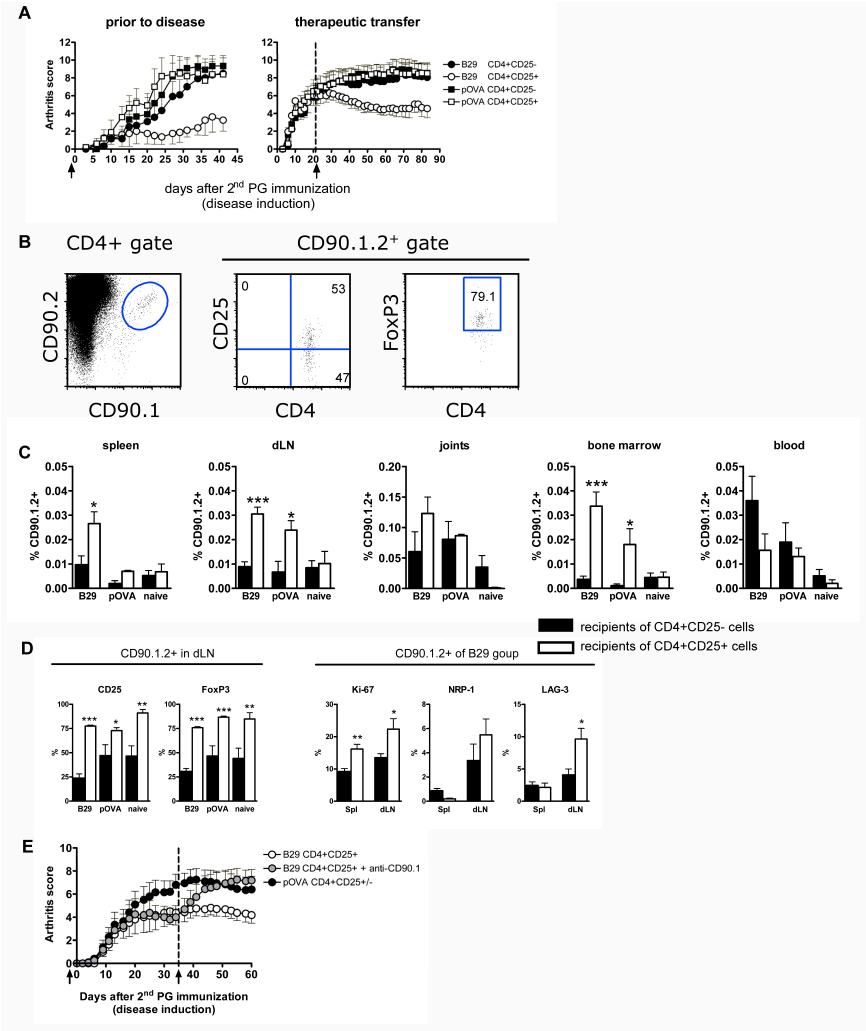

Figure 3. Adoptively transferred B29-induced Tregs are activated and long-lived in vivo, resulting in disease suppression. (A) Mean arthritis scores (± s.e.m.) are depicted for recipients with PGIA receiving either CD4+CD25+ or CD4+CD25- cells from B29 or pOVA immunized donors. Left panel: prophylactic treatment: One day prior to the second PG immunization mice received 3*105 cells i.v. (n=4-6/group) Right panel: therapeutic treatment: three weeks after the second PG immunization 1*106 cells were transferred i.v (arrow). (n=9-10/group). Data are representative of three to four independent experiments. (B) Flow cytometric staining for CD25 and FoxP3 of CD90.1.2+ cells within the splenic CD4+ T cell population 50 days after prophylactic transfer (c) Distribution of CD90.1.2+ cells within CD4+ cells from naïve, B29 and pOVA immunized donor mice 50 days after transfer in spleen, dLN (pooled axillary, brachial and popliteal), joints, bone marrow and blood (n=9-10/group). (D) Expression of CD25, FoxP3, Ki-67, Nrp-1 or LAG-3 in transferred CD90.1.2+ cells (n=6-9/group). In (C and D) P values are from an unpaired two-tailed Student t-test in which recipients of CD4+CD25- cells were compared to CD4+CD25+ recipients. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. The data are representative of four independent experiments. (E) Five weeks after disease induction, animals from the B29 CD4+CD25+ group were randomized and half were given anti-CD90.1 to deplete CD90.1.2+ cells. Mean arthritis scores (± s.e.m.) are depicted. (n=8-10/group). The data shown are representative of two independent experiments.

Selection for FoxP3 or LAG-3 defines B29 induced Tregs

Although 90% of the transferred $CD25^+$ cells expressed FoxP3 (Fig. S2), we can not exclude that the B29-specific T cells were activated T cells without FoxP3 expression. To examine this, we used a FoxP3-green fluorescent protein (GFP) reporter mouse which allowed us to isolate $CD4^+CD25^+FoxP3^+$ cells by the expression of CD25 and GFP (30). Only adoptive transfer of $CD4^+CD25^+GFP^+$ cells from B29-immunized donors resulted in suppression of disease induction (Fig. 4 A). As expected, the $CD4^+CD25^+GFP^+$ T cells from pOVA-immunized mice were not capable of suppressing disease (Fig. S 2). Thus, these data confirm that B29-immunization induces a population of $CD4^+CD25^+FoxP3^+$ Tregs that suppress induction of experimental arthritis upon transfer. Since LAG-3 was slightly upregulated on $CD4^+CD25^+FoxP3^+$ Tregs after immunization with B29 (Fig. S 1) and was enriched on $CD90.1.2^+$ Tregs 50 days after transfer (Fig. 3 D), we hypothesized that LAG-3 was expressed on B29-specific Tregs. To test this, $CD4^+CD25^+LAG\text{-}3^+$ or $CD4^+CD25^+LAG\text{-}3^-$ cells from B29-immunized donors were transferred to mice followed by disease induction. Animals receiving as few as 4,000 $CD4^+CD25^+LAG\text{-}3^+$ Tregs had significantly lower arthritis scores than recipients of $CD4^+CD25^-$ T cells (Fig. 4 B). Interestingly, transferring 300,000 $LAG\text{-}3^-$ Tregs did not suppress disease. The suppression of disease obtained with transferring 4,000 $LAG\text{-}3^+$ Tregs was similar to the suppression with 300,000 $CD4^+CD25^+$ Tregs not specifically sorted for LAG-3, indicating that the suppressive B29-specific Tregs are present in the $LAG\text{-}3^+$ population.

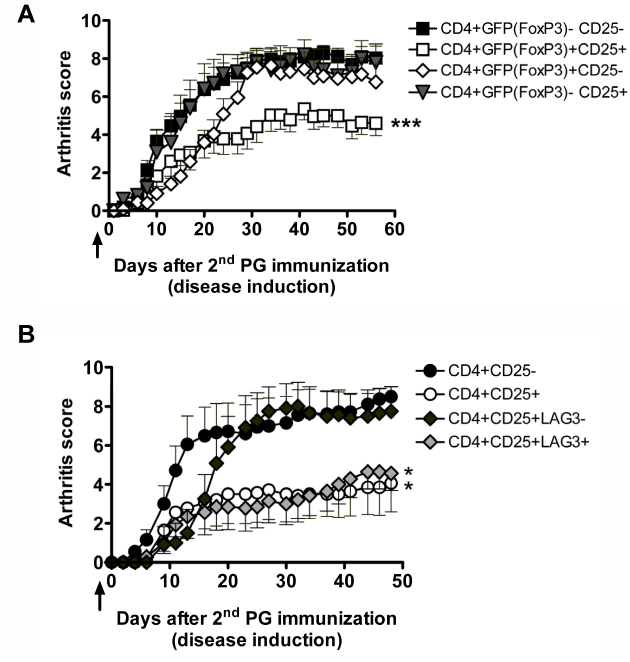

Figure 4. FoxP3 or LAG-3 selected B29-induced Tregs are suppressive in low numbers. (A) Mean arthritis scores (± s.e.m.) of recipients with PGIA after adoptive transfer. One day prior to the second PG immunization, animals received 3*105 CD4+ cells i.v. from B29-immunized FoxP3-GFP reporter donor mice (arrow). Donor CD4+ cells were selected on expression of CD25 and/or GFP (FoxP3). (CD4+GFP(FoxP3)-CD25- n=16; CD4+GFP(FoxP3)+CD25+ n=14; CD4+GFP(FoxP3)+CD25- n=11; CD4+GFP(FoxP3)-CD25+ n=5). The data shown are combined from two independent experiments. (B) Acceptors received CD4+ cells selected on expression of CD25 and/or LAG-3 .i.v. from B29-immunized donor mice (arrow). Mean arthritis scores (± s.e.m) of recipients are depicted. P values are from a two-way ANOVA followed by Bonferroni post hoc comparison. * $P < 0.05$, *** $P < 0.001$. (3*105 CD4+CD25- n=9; 3*105 CD4+CD25+ n=7; 3*105 CD4+CD25+LAG-3- n=6; 4*103 CD4+CD25+LAG-3+ n=7).

Intranasal administration of B29 ameliorates arthritis

Finally, we wanted to test the direct immunosuppressive activity of the B29 peptide. We monitored disease development upon nasal administration (31, 32), also because of successful disease suppression using the whole Hsp70 protein administered intranasally (Fig. 5 A). Nasal treatment with B29 or its homologues diminished disease after administration (Fig. 5 B), although the mB29b homologue was only effective at higher concentrations. At the end of the experiment, restimulated splenocytes taken from treated animals showed peptide-specific and cross-reactive T cell responses as demonstrated by proliferation and cytokine production (Fig. 5 C), indicating a continued presence of B29-specific T cells. To confirm that intranasally administered B29 induced disease-suppressing Tregs, we transferred $CD4^+CD25^+$ Tregs from B29-treated mice into arthritic mice. Recipients of B29-induced Tregs had lower arthritic scores than recipients of Tregs from pOVA-treated donors (Fig. 5 D). These data show that B29 also induces Tregs when nasally administered.

Discussion

The induction or activation of Tregs is considered a promising approach in the treatment of inflammatory diseases (32) . Tregs are selected centrally or in the periphery on the basis of TCR specificity for self antigens. It has been shown that the antigen specific Tregs have a superior potential to down-modulate inflammation (6, 7) . It may seem attractive to use autoantigens in the case of autoimmune diseases to induce or activate disease-antigen specific Tregs. In the case of complex diseases such as RA this may be difficult given the absence of identified critical self antigens. Moreover, it has been argued that disease suppression is unlikely to result from the activities of a relatively small number of auto-antigen specific Tregs present in polyclonal T cell populations (11). A more likely scenario would be the induction of polyclonal Treg control to be activated through MHC class II presentation of ubiquitous self-peptides (11) . Here we show that Hsp70 can be a source of such ubiquitous self-peptides, especially when cells experience stress. Multiple peptides of various Hsp70 family members have been found in various MHC class II molecules (21, 23-25) . A contributing reason for this is that Hsp70 is one of molecular components of the process of so-called chaperone mediated autophagy (33). Under stress, cells exploit autophagy for the economic use of proteins and amino acids. In the case of cellular stress imposed by nutrient deprivation, human cells were found to have uploaded their MHC class II molecules with fragments of Hsp70 (21). Interestingly, in the latter case the B29 self homologues were found to be present among the of HLA-DR eluted peptides. Recently, autophagy was suggested to exert an anti-inflammatory role by controlling inflammasomes, since autophagy was found to accompany inflammasome activation and to temper inflammation by eliminating active inflammasomes (34) . Therefore, it is possible that stress induced autophagy has anti-inflammatory effects at multiple levels varying from inhibition of inflammasome activity to production of stress protein specific Tregs.

Interestingly, as a result of our T cell epitope mapping, we identified the B29 epitope from bacterial Hsp70 to exhibit prominent immunogenicity, despite being very conserved and herewith closely resembling its mammalian homologs mB29a and mB29b. Subsequent intranasal administration or parenteral immunization with B29 was found to induce

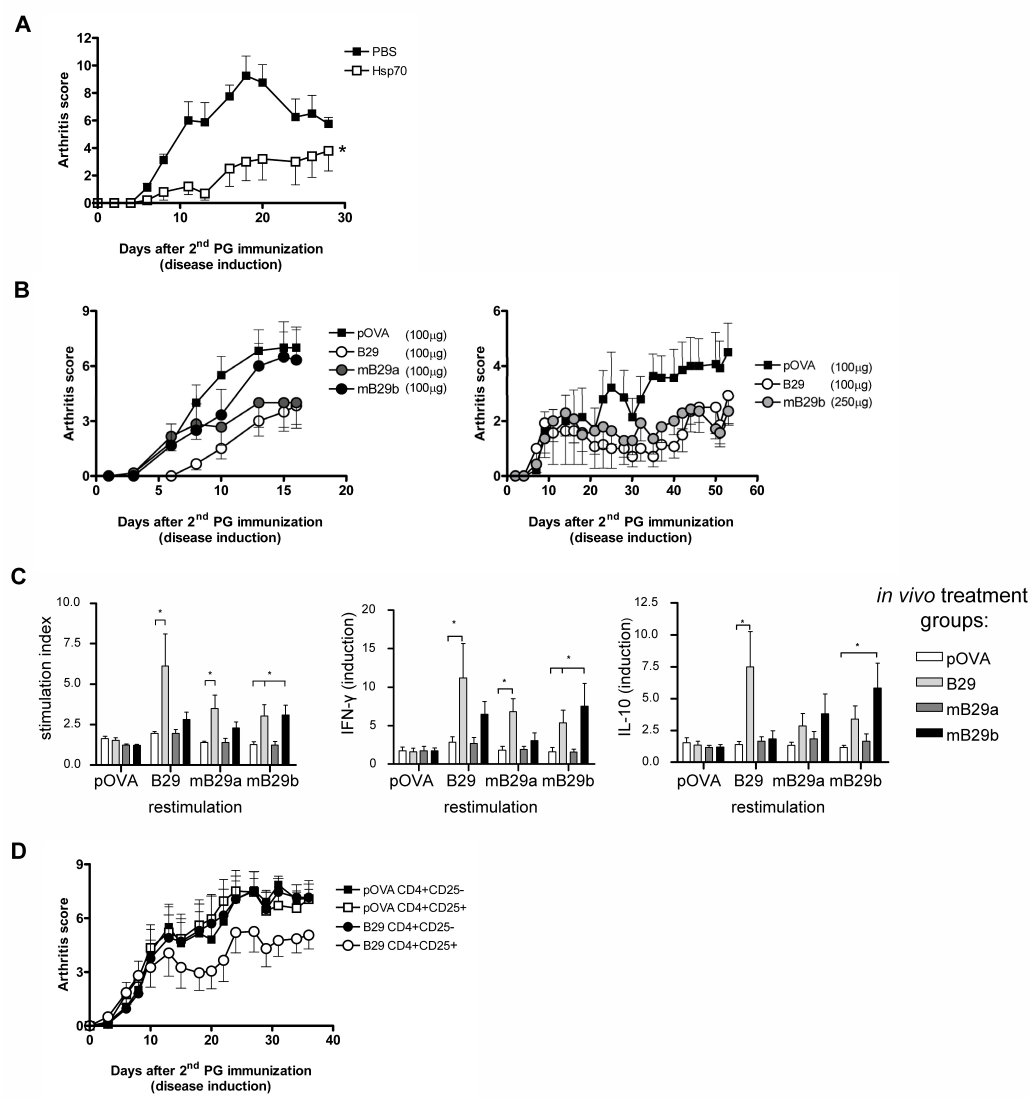

Figure 5. Nasal application of HSP70 or its peptides suppresses experimental arthritis. (A) Mean arthritis scores (± s.e.m.) after intranasal Mt Hsp70 administration. n=5 mice per treatment group. (B) Mean arthritis scores (± s.e.m.) after B29, mB29a, mB29b or control pOVA given intranasally (n=6 mice per group). (C) Proliferation and cytokine production of splenocytes from intranasally treated animals as described in (B). Proliferation in the presence of B29, mB29a, mB29b or pOVA. Peptide-specific IFN-γ and IL-10 production. presented as cytokine production in the presence of peptide relative to background production. Results are expressed as mean stimulation index (± s.e.m. n=6/group). Data are representative of two independent experiments. (D) Mean arthritis scores (± s.e.m) of recipients with PGIA after adoptive transfer. Donor mice were treated as in (B), 5*105 CD4+CD25+ or CD4+CD25- cells from spleen were transferred i.v. one day prior to disease. For (A, C and D) P values are from a two-way ANOVA followed by Bonferroni post hoc comparison * p<0.05.

cross reactive T cells that recognized the mB29a and mB29b self-homologues. In adoptive transfer studies to characterize B29-induced Tregs, we found that relatively low numbers of $CD4^+CD25^+$ cells from B29-immunized donors were able to suppress arthritis. The suppressive potential of B29-induced Tregs was further demonstrated by the fact that therapeutic transfer arrested established arthritis. Upon transfer, donor cells were found in the dLN and the joints and had an activated phenotype. Relatively few $CD4^+CD25^+$ transferred cells were located in the peripheral blood (as compared to $CD4^+CD25^-$ cells) showing that these cells actively homed to the site of inflammation.
We saw that LAG-3 was upregulated after B29-immunization (Fig S 1b), indicating LAG-3 being a marker for activation. It has been previously shown that Tregs are capable of delivering inhibitory signals via LAG-3 to MHC class II presenting cells (4, 11) . We were able to isolate $LAG\text{-}3^+$ B29-induced Tregs and using adoptive transfer, only few cells were needed to suppress disease. Therefore, it seems that the B29-specific Tregs use LAG-3 as a mechanism to suppress inflammation.
When intranasally administering B29, or its mammalian homologues mB29a and mB29b, to suppress inflammation, we observed a superior disease suppressive quality of B29 in comparison with the mammalian homologues. It is therefore possible that B29 is an example of a natural epitope acting as a relatively strongly agonistic mimetope or Altered Peptide Ligand (APL) endowing B29 with its Treg-inducing potential (35,36). For therapeutic application, we propose to apply the B29 peptide under subimmunogenic (35) conditions, for instance during anti-TNF treatment, to yield a maximum induction or conversion of T cells into antigen-specific Tregs. Alternatively, B29 may be used for maintaining tolerance subsequent to, or after discontinuation of anti-TNF treatment (37, 38) .
In conclusion, our data show that the administration of Hsp70 peptide B29 elicits potent primary antigen-specific Tregs, which suppress disease in a mouse model of rheumatoid arthritis. The transferred antigen-specific Treg population was long lived *in vivo*, expressed FoxP3 or LAG-3 and its direct presence was required for suppression.. Altogether, Hsp70-specific Tregs may well exemplify the great potential of antigen-specific immune interventions in autoimmune diseases, even without knowledge of critical disease-specific autoantigens.

Materials and Methods

Mice. Female Balb/c mice, aged 16-26 weeks (retired breeders: for PGIA) or aged 8-12 weeks (all other experiments) were purchased from Charles River. CD90.1 and FoxP3-GFP Balb/c were purchased from Jackson and bred in house. CD90.1 mice were crossed with CD90.2 mice (Charles River). CD90.1.2 offspring were used for transfer experiments. Animals were kept under standard conditions at the animal facility and all experiments were approved by the Animal Experiment Committee of Utrecht University.

Antigens, peptides and antibodies. Recombinant *Mt* Hsp70 (LIONEX Diagnostics & Therapeutics GmbH) contained less than 2.1 EU LPS/mg. Peptides for epitope mapping were prepared by automated simultaneous multiple peptide synthesis (SMPS). The SMPS set-up was developed using a standard autosampler (Gilson Medical Electronics). Briefly, standard Fmoc chemistry with in situ PyBop/NMM activation of the amino acids in a 5-fold molar excess with respect to 2 micromol reactive equivalents per peptide on

the PAL-PEG-PS resin (Perseptive Biosystems) was employed. Peptides were obtained as C-terminal amides after cleavage with 90-95% TFA/scavenger coctails. Peptides were analyzed by reversed phase HPLC and checked via electrospray mass spectrometry on an LCQ ion-trap mass spectrometer (Thermoquest). Purity of the peptides ranged between 50 to 90 %. All other peptides were obtained from GenScript Corporation (including $pOVA_{323-339}$). Peptides were analyzed by reversed-phase HPLC and checked via electrospray mass spectrometry on an LCQ ion-trap mass spectrometer. For flow cytometry antibodies were purchased from BD Bioscience: CD25-APC/ -PE (PC61), Ki-67-PE (B56), IL-10-PE (JES5-16). eBioscience: CD4-FITC/ -eFlour450 (RM4-5), FoxP3-APC/ -eFlour450/ -PE/ -PerCPCy5.5 (FJK-16), LAG-3-PE (eBioC9), CD90.1-FITC (HIS51), CD90.2-APC (53-2.1). R&D Systems: Nrp-1-APC and IL-35(p35)-APC. Biolegend: Helios-Alexa647 (22F6). For CD90.1 depletion, 250 µg anti-CD90.1 (HIS51, BD Bioscience) was given i.p.

Identification of dominant T cell epitopes of Mt Hsp70. Mice were immunized on day 0 and 14, by i.p injection of 100 µg recombinant *Mt* Hsp70 with 2 mg adjuvant DDA (Sigma). At day 28 mice spleen and LN cells were isolated and restimulated with a panel of 123 overlapping 15-mer peptides covering the complete sequence of Hsp70, followed by analysis of peptide-specific proliferation. Next, pools of identified dominant peptides were used for immunization. These pools were composed of 4-5 peptides, based on their degree of sequence identity with mouse Hsp70; Pool I contained non-conserved peptides, pool II partially conserved peptides and pool III highly conserved peptides. Immunization with 100 µl i.p. and 100 µl subcutaneous in the neck was done at 125 µg/ml of each peptide with 2 mg DDA on day 0 and day 14, followed by analysis of peptide-specific cytokine production on day 28. IFN-γ and IL-10 production upon immunization or PGIA induction was measured in culture supernatants of stimulated (20 µg/ml peptide) spleen or LN cells after 72 hours by ELISA according to manufactures protocol (BD).

MHC peptide elution. Cells were isolated from BM and cultured with GM-CSF to yield bone marrow-derived DC. MHC Class II from cultured DC was isolated as previously reported (39). Identification of the MHC Class II-presented epitopes was done as previously described (40). BioWorks 3.3.1 SP1 application tool (Thermo Scientific) was used to search the mass spectral data against a subset protein database containing the Mouse taxonomy (UniProt/Swiss-Prot protein database http://www.ebi.ac.uk).

Nasal administration of Hsp70 and peptides. Mice were treated on day -7, -5 and -3 prior to the first PG immunization with Mt Hsp70 (30 µg) or peptides (100 µg or 250 µg) in 10 µl PBS via intranasal (i.n.) application, whereas control mice received 10µl PBS alone or pOVA (100 µg). Proliferation and cytokine production of splenocytes was determined 28 days after disease induction. Proliferation was determined 72 hours later by measuring ^{3}H-thymidine incorporation. Peptide-specific IFN-γ and IL-10 production in culture supernatants was measured by ELISA according to manufacturers instructions.

***Ex vivo* restimulation and phenotypic analysis Tregs**. Mice were injected i.p and s.c. with 100 µg peptide with 2 mg DDA. Splenocytes were harvested 10 days after immunization and directly analyzed, or were cultured for 24h in the presence of 10 µg/ml peptide followed by FACS analysis. Intracellular IL-10 and p35 expression was determined by

flow cytometry after PMA/Ionomycin stimulation

Induction of proteoglycan induced arthritis (PGIA). Proteoglycan (PG) was isolated from human articular cartilage as described previously (26) Arthritis was induced with by two i.p. injections with 250-300 µg PG protein with 2 mg DDA with an interval of 21 days. Subsequently, mice were randomized among experimental groups and arthritis scores were determined in a blinded fashion by at least two independent investigators using a visual scoring system based on swelling and redness of paws as previously described (26).

Adoptive transfer. Donor mice were treated with Hsp70 or peptides as indicated above. $CD4^+CD25^{+/}$, $GFP^{+/-}$ or LAG-$3^{+/-}$ cells were isolated from spleen 10 days after the last peptide administration. Pre-enrichment for $CD4^+$ cells was performed using Dynal bead isolation (Invitrogen) followed by FACS sort (Influx, BD) with purities up to 96%. Cells were transferred via intravenous injection in 100 µl PBS.

***In vitro* suppression assay.** Cells were isolated 10 days after immunization. Irradiated splenocytes were used as APC ($2*10^5$ cells) and cultured with $CD4^+CD25^-$ cells in a ratio of 2:1. $CD4^+CD25^+$ were added in various concentrations. Cells were cultured for 72h in the presence of 5 µg/ml soluble anti-CD3 and ^{3}H-thymidine was added for an additional 18 h.

Statistical analysis. All data are presented as mean ± standard error of the mean (s.e.m.). Statistical analysis was carried out using Prism software (Graphpad Software Inc.). Comparisons between two groups were done using the Student's *t*-test (unpaired, two-tailed). Multigroup comparisons were done using one way Anova followed by a Bonferroni *post hoc* test. *P* values less than 0.05 with a 95% confidence interval were considered significant. *P <0.05, **P <0.01 and ***P < 0.001.

ACKNOWDLEGMENTS. We thank C. Keijzer, B. Margry and R. Spiering for technical assistance. K. Malone for providing scientific writing services. G. Arkesteijn for sorting, and workers at the animal facility for animal care. This work was supported by grants of IOP Genomics projects IGE3018 and IGE07004, the European Union FP7 TOLERAGE: HEALTH-F4-2008-202156 and the Dutch Arthritis Association.
W.v.E. has equity in Trajectum Pharma BV.

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Supporting Information

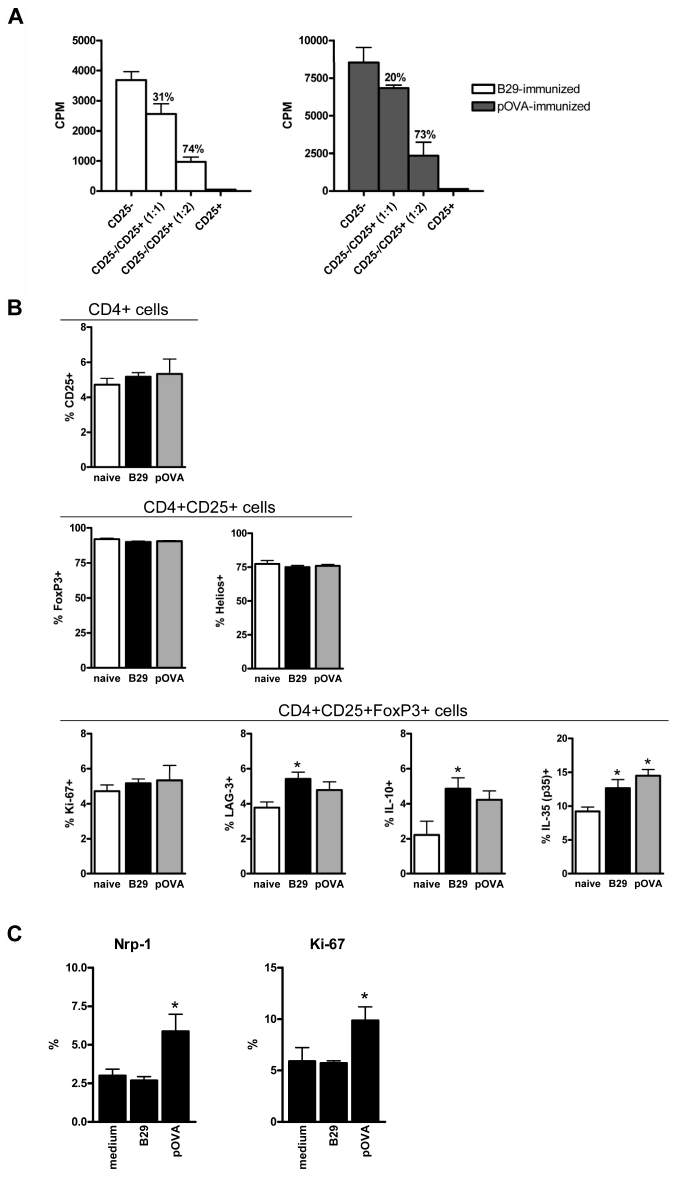

Figure S1. In vitro and phenotypic characterization CD4+CD25+ T cells from B29- or pOVA-immunized mice. (A) CD4+CD25+ and CD4+CD25- cells from B29 or pOVA immunized mice were co-cultured and stimulated by soluble anti-CD3 antibody. Data are mean of triplicate samples and figures are representative of two independent experiments. (B) Mice were immunized with B29 or pOVA and CD4+CD25+ (FoxP3+) Tregs were analyzed by flow cytometry. Expression (± s.e.m.) of markers related to Treg function were analyzed and showed enhanced expression of LAG-3, IL-10 and p35 (IL-35 subunit) in CD4+CD25+FoxP3+ cells. P values are from an unpaired two-tailed student t test in which Tregs from B29-immunized or pOVA-immunized mice were compared to Tregs from naïve mice. * $P < 0.05$, *** $P < 0.001$. Data are mean of 5-10 animals per group and data shown are representative of two independent experiments. (C) Splenocytes from pOVA-immunized mice were restimulated for 24h in the presence of B29 or pOVA and stained for Ki-67 and Nrp-1. Percentage of positive cells (± s.e.m.) is shown within the CD4+CD25+FoxP3+ population. P values are from an unpaired two-tailed Student t-test in which pOVA was compared to B29. * $P < 0.05$. Data are mean of 5-10 animals per group and are representative of two independent experiments.

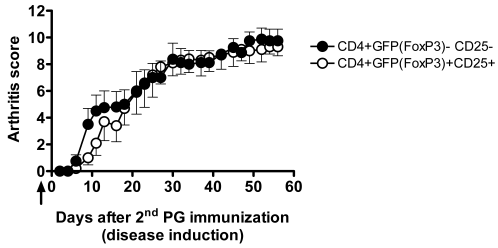

Figure S2. CD4+CD25+FoxP3+ T cells from pOVA-immunized donors are not suppressive in vivo. Mean arthritis scores (± s.e.m.) of recipients with PGIA after adoptive transfer. One day prior to the second PG immunization, animals received 3*105 CD4+ cells i.v. from pOVA-immunized FoxP3-GFP reporter donor mice (arrow). Donor CD4+ cells were selected on expression of CD25 and/or GFP (FoxP3). (CD4+GFP(FoxP3)-CD25- n=5; CD4+GFP(FoxP3)+CD25+ n=4).

Table S1. Identification of Mt-Hsp70 epitopes

Pool	Peptide name	Sequence	position
I	B18	YTAPEISARILMKLK	86-100
	B59	KPFQSVIADTGISVS	291-305
	B107	AEGGSKVPEDTLNKV	530-544
	B108	AQAASQATGAAHPGG	585-599
II	B7	EGSRTTPSIVAFARN	31-45
	B47	MQRLREAAEKAKIEL	231-245
	B67	GGKEPNKGVNPDEVV	331-345
	B69	DEVVAVGAALQAGVL	342-356
	B74	LDVTPLSLGIETKGG	366-380
III	**B29**	**VLRIVNEPTAAALAY**	**141-155**
	B34	ILVFDLGGGTFDVSL	166-180
	B89	RGIPQIEVTFDIDAN	441-455
	B90	QIEVTFDIDANGIVH	445-459

Table S1. Identification of Hsp70-derived epitopes. To identify the dominant T cell epitopes of Mt Hsp70, mice were immunized on day 0 and day 14 with Mt Hsp70 in DDA. On day 28 spleen cells were isolated and restimulated with a panel of 123 overlapping 15mer peptides covering the complete sequence of Mt Hsp70. Subsequently induced T cell responses were detected by 3H-thymidine incorporation. In multiple experiments the 13 peptides depicted, repeatedly induced proliferation upon in vitro restimulation with the peptides and were therefore selected as dominant epitopes. The peptides were divided into three pools according to the degree of sequence identity with mouse Hsp70-peptides: non-identical (pool I), moderately identical (pool II), or highly identical (pool III).

Table S2. Origin and sequence of highly conserved B29 peptides

Peptide	Protein	Origin	ID	Sequence
B29	DnaK (Hsp70)	*Mycobacterium tuberculosis*	885946	VLRIVNEPTAAALAY
mB29a*	HspA9 (GRP75)	*Mus musculus* *Homo sapiens*	15526 3313	VLR**VI**NEPTAAALAY
mB29b*	HspA1A (Hsp72)	*Mus musculus* *Homo sapiens*	193740 3303	VLRI**I**NEPTAAA**I**AY
	HspA8 (Hsc70)	*Mus musculus* *Homo sapiens*	15481 3312	VLRI**I**NEPTAAA**I**AY

Table S2. Origin and sequence of highly conserved B29 peptides. Human and mouse peptides of the same protein were completely identical. Altered residues as compared to Mycobacterium tuberculosis in bold and underlined. ID: GeneID in the NCBI Entrez Gene database (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene). * mB29a and mB29b are mammalian homologues of mycobacterial Hsp70 peptide B29.

Table S3. Hsp70 is a major contributor to the MHC-Class II ligandome (published data)

Sequence	Class II type	MHC origin	Protein Source (100% id)	Entrez Gene ID	Ref
QQYLPLPTPKVIGID	HLA–DR10 (DRB1*1001)	human	HSPA13 (23-37)	6782	1
IIANDQGNRTTPSY	I-Ak	mouse	HSPA8 (28-41) HSPA2 (29-42) HSPA1L (30-43) HSPA1A (28-41) HSPA1B (28-41)	15481 15512 15482 193740 15511	2
ITPSYVAFTPEGERL	I-Ab	mouse	HSPA5 (62-76)	14828	3
TPSYVAFTDTERLIG(DA)	HLA-DR7	human	HSPA8 (38-52) HSPA2 (39-53) HSPA1L (40-54) HSPA1A (38-52)	3312 3306 3305 3303	4
TPSYVAFTDTERLIGD	HLA-DQ2	human	as above	as above	5
DVYVGYESVELADSNPQ	HLA-DQ2	human	HSPA13 (77-93)	6782	5
DAAKNQLTSNPEN	I-Ag7	mouse	HSPA5 (79-91)	14828	6
NPTNTVFDAKRLIGRRFD	HLA-DRB1*1104	human	HSPA8 (62-79)	3312	7
QDIKFLPFKVVEKKTKPY	BoLA-DRB3*1201(in mus line)	bovine	HSPA5 (111-128)	14828	8
LNVLRIINEPTAAAIAYG (NVLRIINEPTAAAIAYG)	HLA-DRB1*0401 (in rat line)	human	HSPA8 (167-184) HSPA1A (167-184) HSPA1L (169-186) HSPA2 (168-185)	24468 24472 24963 60460	9
NVLRIINEPTAAAIAYG	HLA-DRB1*0401/DRB4*0101	human	HSPA8 (168-184) HSPA1A (168-184) HSPA1L (170-186)	3312 3303 3305	10

			HSPA2 (169-185) HSPA6 (170-186)	3306 3310	
NVLRIINEPTAAAIA	DRB1*0401/*02x/DRB5*0101	human	HSPA8 (168-184) HSPA1A (168-184) HSPA1L (170-186) HSPA2 (169-185) HSPA6 (170-186)	3312 3303 3305 3306 3310	11
NVMRIINEPTAAAIAYG	DRB1*0401/*02x/DRB5*0101	human	HSPA5 (194-210)	3309	11
VMRIINEPTAAAIAYG	HLA-DRB1*0401/DRB4*0101	human	HSPA5 (195-210)	3309	10
IINEPTAAAIAYGLD	HLA-DQ6 (B*602)	human	HSPA8 (172-186) HSPA2 (173-187) HSPA1L (174-188) HSPA1A (172-186) HSPA5 (198-212)	3312 3306 3305 3303 3309	12
FDVSILTIEDGIFE	HLA-DQ2	human	HSPA8 (205-218)	3312	5
NRMVNHFIAEFKRK	I-Ek	mouse	HSPA8 (236-249)	15481	13
RMVNHFIAEFKRKH	I-Ek	mouse	HSPA8 (236-249)	15481	14
VNHFIAEFKRKHKKD	HLA-DR11/w52	human	HSPA8 (238-252)	3312	15
XDFYTSITRAXFEE	HLA-DR11/w52	human	HSPA8 (291-304) HSPA1A (291-304) HSPA1L (293-306) HSPA2 (294-307) HSPA6 (294-306)	3312 3303 3305 3306 3310	15
EGEDFSETLTRAKFEEL	BoLA-DRB3*1201(in mus line)	bovine	HSPA5 (315-331)	14828	8
ADLFRGTLDPVEK	HLA-DQ6 (B*0604)	human	HSPA8 (307-319)	3312	12
KSINPDEAVAYG	HLA-DQ2	human	HSPA8 (361-372) HSPA1A (361-372) HSPA1L (363-374) HSPA2 (364-375) HSPA6 (363-374) HSPA8 (361-372)	3312 3303 3305 3306 3310 3312	5
TIPTKQTQTFTTYSDNQP	RT1.Bl	rat	HSPA8 (419-436) HSPA1A (419-436)	24468 24472	16
VPTKKSQIFSTASDNQPTVT	HLA-DRB1*0401/DRB4*0101	human	HSPA5 (443-462)	3309	10
GERAMTKDNNLLG	HLA-DR4Dw4	human	HSPA8 (445-457) HSPA1A (445-457) HSPA1L (447-459) HSPA2 (448-460) HSPA6 (447-459)	3312 3303 3305 3306 3310	17
GERAMTKDNNLLGKFE	HLA-DRB1*0401/DRB4*0101	human	HSPA8 (445-460) HSPA1A (445-460)	3312 3303	10
GERAMTKDNNLLGRFE	HLA-DRB1*0401/DRB4*0101	human	HSPA6 (447-462)	3310	10
ANGILNVSAVDKSTGKE	HLA-DRB*0401	human	HSPA8 (482-499)	3312	18
GILNVSAVDKSTGK	HLA-DRB*0401	human	HSPA8 (484-497)	3312	18
GILNVSAVDKSTGKE	HLA-DRB1*0401/DRB4*0101	human	HSPA8 (484-498)	3312	10
CNEIINWLDKNQ	HLA-DR4Dw10	human	HSPA8 (574-585)	3312	17
ISWLDKNQTAEKEEFE	HLA-DQ8 (transgenic in NOD)	human	HSPA8 (578-593)	15481	6
YGSGGPPPTGEEDTSEKDEL	I-Ag7	mouse	HSPA5 (636-655)	14828	6

The Hsp70 nomenclature has previously been reported (Kampinga 2009).

Table S3. Hsp70 is a major contributor to the MHC-Class II ligandome (published data). Published data of Hsp70 sequences found in MHC Class II. The sequence of B29 and its endogenous homologues were frequently eluted from different MHC Class II molecules. Peptides are listed according to sequence number (in bold peptides homologous to B29). In the boxed section B29 homologue peptides eluted from RA associated HLA-DRB1*0401.

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Table S4. MHC Class II presented Hsp70 peptides eluted from in vitro cultured murine bone marrow-derived dendritic cells (DC

Sequence	Relative abundance
VLRIVIN	4%
VLRIVINE	4%
VLRIVINEP	13%
VLRIVINEPT	1%
VLRIVINEPTA	2%
VLRIVINEPTAA	9%
VLRIVINEPTAAA	6%
VLRIVINEPTAAAL	55%
LRIVINEPTAAAL	5%
Total	100%

Table S4. MHC Class II presented Hsp70 peptides eluted from in vitro cultured murine bone marrow-derived dendritic cells (DC). Peptide-MHC complexes were isolated from bone marrow-derived DC. Subsequently, eluted peptides were analyzed by data dependent nanoscale LC/MS. Several homologues of the mB29a peptide varying in length are depicted and their relative abundance compared to all eluted mB29a variants is shown.

4

De novo induced regulatory T cells after Hsp70 peptide immunization are suppressive in experimental arthritis

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Submitted

Abstract

Targeting antigen-specific regulatory T cells (Tregs) via peptide therapy can suppress experimental autoimmune diseases. The ubiquitously expressed heat shock protein (Hsp) 70 is a suitable candidate antigen because administration of peptides from bacterial and mouse Hsp70 has been shown to induce strong immune responses and can modulate inflammatory responses via the activation or induction of Tregs. However, little is known about which subset of Tregs are activated by Hsp70 epitopes. Therefore, we set out to determine whether natural nTregs (derived from the thymus), or induced iTregs (formed in the periphery from $CD4^+CD25^-$ naïve T cells) were targeted after Hsp70-peptide immunization. We immunized mice with the previously identified Hsp70 T cell epitope B29 and investigated the formation of functional nTregs and iTregs by using the *in vitro* suppression assay and adoptive transfer therapy in mice with experimental arthritis. In order to induce Tregs *in vivo* after peptide immunization, we depleted $CD25^+$ cells prior to immunization, allowing the *in vivo* formation of Tregs from $CD4^+CD25^-$ precursors. This approach allowed us to study *in vivo* B29-induced Tregs and to compare these cells with Tregs from non-depleted immunized mice. Our results show that using this approach, immunization induced $CD4^+CD25^+$ T cells in the periphery, and these cells were suppressive *in vitro*. Additionally, transfer of B29-specific iTregs suppressed disease in a mouse model of arthritis. This study shows that immunization of mice with Hsp70 epitope B29 induces functionally suppressive iTregs from $CD4^+CD25^-$ cells.

Introduction

Several mechanisms of tolerance exist to prevent autoimmunity, excessive inflammatory responses and to maintain immune homeostasis. $CD4^+CD25^+$ regulatory T cells (Tregs) are specialized $CD4^+$ T helper cells that are of great significance to central tolerance [1]. Two main subsets of Tregs exist: natural Tregs (nTregs) or induced Tregs (iTregs) [2]. nTregs originate from the thymus as mature Tregs and are mostly directed against self-antigens [2], while iTregs are formed in the periphery from naïve $CD4^+$ T cells in response to mostly foreign antigens [3, 4]. Both subsets have been shown to suppress a variety of immune responses. However, the relative contribution of each subset is still largely unknown and might depend on specific immunological situations [2].
Targeting of antigen-specific Tregs with immunomodulatory epitopes can be used to suppress inflammatory immune responses in animal models of autoimmune diseases [5-8]. Choosing a suitable candidate epitope can be difficult for diseases for which the disease-inducing antigen is unknown, which is the case for rheumatoid arthritis. Therefore, we propose to use antigens that are constitutively expressed, and preferentially upregulated during inflammatory disease. One such antigen is heat shock protein (Hsp) 70, an evolutionary strongly conserved protein that is expressed and upregulated in the inflamed synovium [9] and which bacterial homologs have been shown to induce strong immune responses upon immunization [10].
We have previously shown that administration of Hsp70 or Hsp70-derived peptides suppresses experimental arthritis via the activation of Tregs [10, 11]. Immunization of mice with the mycobacterial Hsp70 epitope B29 generated Tregs that were cross reactive with mouse Hsp70 peptides and able to suppress established arthritis upon transfer, whereas Tregs from animals immunized with control antigen pOVA were not suppressive. These results suggest that B29-specific Tregs were activated *in vivo* by locally presented mouse B29 homologs [10].
However, it is unknown whether the administration of B29 converted naïve T cells into B29-specific iTregs, or that B29-specific nTregs were expanded. It is important to establish the contribution of Tregs subsets in suppression of disease after peptide administration in order to fine-tune peptide based therapies to optimally target Tregs in future therapies. Therefore, we setup a protocol to induce Tregs *in vivo* by first depleting $CD25^+$ Tregs with anti-CD25 antibody followed by B29 immunization. We hypothesized that if B29-specific naïve T cells existed, they could become iTregs after encounter with B29. Thus, via this approach we were able to study B29-specific iTregs.
Here, we show that depletion of $CD25^+$ cells, followed by immunization with the Hsp70 peptide B29 induced $CD4^+CD25^+$ cells that were equally well suppressive *in vitro* and *in* vivo as $CD4^+CD25^+$ cells from non-depleted immunized mice. This suggests that B29-immunization can induce antigen-specific Tregs from naïve T cells.

Materials & Methods

Mice and peptides

Female Balb/c mice were purchased from Charles River and for peptide immunization 8-12 week old mice were used. For proteoglycan induced arthritis (PGIA) experiments, retired breeders were used. Animals were kept under standard conditions at the animal facility and all experiments were approved by the Animal Experiment Committee of

Utrecht University. Peptides were purchased from GenScript Corporation (B29, mB29a, mB29b and pOVA $_{323-339}$; for details see [10]).

Immunization and depletion of CD25$^+$ cells for cell isolation, restimulation and flow cytometry

Mice were immunized with 100 µg peptide (B29, mB29a, mB29b, or pOVA) with 2 mg DDA via i.p. plus s.c. injection. Ten days after immunization, mice were sacrificed and cells were isolated from spleen as described previously [10]. For restimulation and flow cytometry, splenocytes were analyzed separately, while for adoptive transfer spleens were pooled per group. For depletion of CD25$^+$ cells, mice were given 400 µg anti-CD25 antibody (PC61, produced in house from hybridoma ATCC PC61 and purified from supernatants) in 100 µl PBS i.p. To confirm depletion of CD25$^+$ cells, blood samples were taken via the submandibular vein just prior to immunization of the mice 7 days after depletion. For adoptive transfer and the *in vitro* suppression assay, CD4$^+$ T cells were isolated from spleen 10 days after immunization with peptide using Dynal bead isolation (Invitrogen) by negatively selecting CD4$^+$ T cells, followed by FACS sort (influx, BD) to isolate CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ with purities up to 96%.

Flow cytometry and antibodies

Flow cytometry was performed with CantoII (BD) with antibodies CD4-FITC (RM4-5, eBioscience), CD4-PerCP (RM4-5, BD Bioscience), CD25-APC (PC61, BD Bioscience), CD62L-FITC (MEL-14, BD Bioscience), CD69-FITC (H1.2F3, BD Bioscience) and FoxP3-PE (FJK-16, eBioscience). For Fig 2A, whole blood cells were obtained and red blood cells were lysed with ACK (Ammonium-Chloride-Potassium) buffer and stained for CD25 and the percentage of CD25$^+$ cells was determined for the entire population of blood cells. For Fig 3, single cell suspension of splenocytes were stained for CD4 in combination with CD25, FoxP3, CD69, or CD62L.

Restimulation and suppression assay

For restimulation, splenocytes were harvested from mice immunized as described above and cultured in 96-wells plates in triplicate wells in a concentration of $2*10^5$ cells/well. Cells were restimulated with 20 µg/ml peptide for 72h, and ^{3}H-thymidine was added for another 18h. Stimulation Index (SI) was measured by dividing the counts per minute (cpm) of the stimulated conditions by the medium (unstimulated) cpm. For the *in vitro* suppression assay, CD4$^+$CD25$^-$ responder T cells ($1x10^5$) and CD4$^+$CD25$^+$ T cells ($0,5x10^5$, $1x10^5$, or $2x10^5$) were purified by FACS sort as described above and cells were co-cultured in triplicate wells with irradiated splenocytes ($2x10^5$) as APC. Cells were stimulated with 5 µg/ml soluble anti-CD3 antibody for 72h. ^{3}H-thymidine was added for an additional 18h and the percentage suppression (%supp) was calculated from cpm values obtained with responder T cells only.

Proteoglycan Induced Arthritis (PGIA) and adoptive transfer

Retired breeders were immunized twice with an interval of 21 days with 250-300 µg human proteoglycan (PG) protein with 2 mg DDA given in 200 µl PBS via i.p. injection [12]. Mice were randomly grouped and arthritis was scored in a blinded fashion using a visual scoring system based on swelling and redness as described previously [12]. For adoptive transfer, $3x10^5$ CD4$^+$CD25$^-$ or CD4$^+$CD25$^+$ cells were given i.v. in 100 µl PBS one day prior to the second PG immunization.

Statistical analysis
Data is shown as mean ± standard error of mean (s.e.m.). Statistics were done using Prism 4 (Graphpad Software Inc.). Comparisons between two groups were done with the Student's *t*-test (unpaired and two-tailed). *P* values less than 0.05 with a 95% confidence interval were considered significant, with * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Results

Model for induction of iTregs or expansion of nTregs after B29 immunization

Previously we have shown that treatment with the Hsp70 epitope B29 created a population of $CD4^+CD25^+FoxP3^+$ Tregs capable to suppress arthritis upon adoptive transfer [10]. However, peptide immunization with B29 may lead to the expansion of nTregs and/or the formation of iTregs (Fig 1). In this study we aimed to investigate this by depletion of nTregs prior to immunization with B29, to see whether this results in *de novo* formation of B29 induced iTregs. Depletion of $CD25^+$ cells *in vivo* would result in the absence of all subsets of $CD4^+CD25^+$ Tregs, leaving only $CD25^-$ naïve T cells that can differentiate into iTregs upon activation. Thus, immunization with the B29 peptide may result only in the activation of B29-specific naïve T cells, and hence induction of B29-specific CD4+CD25+ iTregs, while B29-specific nTregs will be lacking in the earlier depleted mice. Immunization of undepleted mice may result in both the activation of B29-specific nTregs and the induction of B29-specific iTregs.

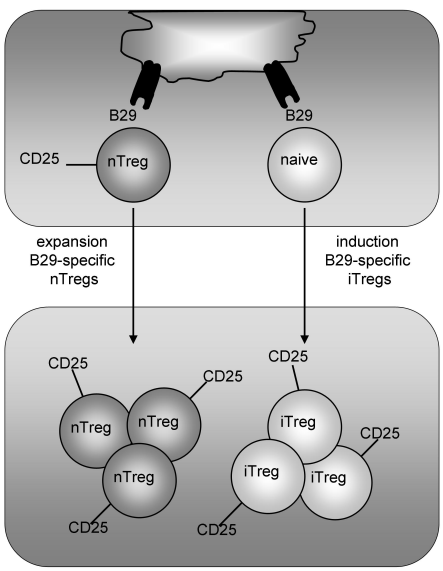

Figure 1. model for induction of iTregs or expansion of nTregs after B29 immunization. Two possible mechanisms for Hsp70 antigen specific Treg expansion could exist: recognition of B29 by already existing nTregs followed by expansion or activation of B29-specific naïve T cells that become iTregs.

B29-specific proliferation after CD25+ T cell depletion and immunization

In vitro restimulation of splenocytes from immunized mice can be used as a qualitative measure to demonstrate the induction of antigen-specific T cells. In order to determine whether depletion of CD25+ cells prior to immunization did not affect the induction of B29-specific T cells, we restimulated splenocytes from B29- immunized mice that had been pre-treated with anti-CD25 antibody. First, mice were injected with the anti-CD25 depleting antibody PC61 and at the moment of immunization with B29 or control pOVA, no CD25+ were detectable in peripheral blood (Fig 2 A). 10 days later, splenocytes obtained from immunized mice, were stimulated with B29, or its mouse homologs mB29 and mB29b. In B29 immunized mice we detected proliferation against Hsp70 peptides B29 and its homologs (Fig 2 B). Splenocytes from mice immunized with pOVA did not respond to Hsp70 peptides and responded only to pOVA. Depletion of CD25+ cells prior to immunization resulted in higher counts for all restimulation conditions (data not shown), indicating that the relative absence of CD25+ Tregs during the immunization period, had resulted in increased T cell priming. However, since the counts of peptide stimulated and unstimulated splenocytes increased relatively with the same magnitude, the SI (stimulation index) was more or less the same as the peptide-specific responses without CD25 depletion (Fig 2 B).

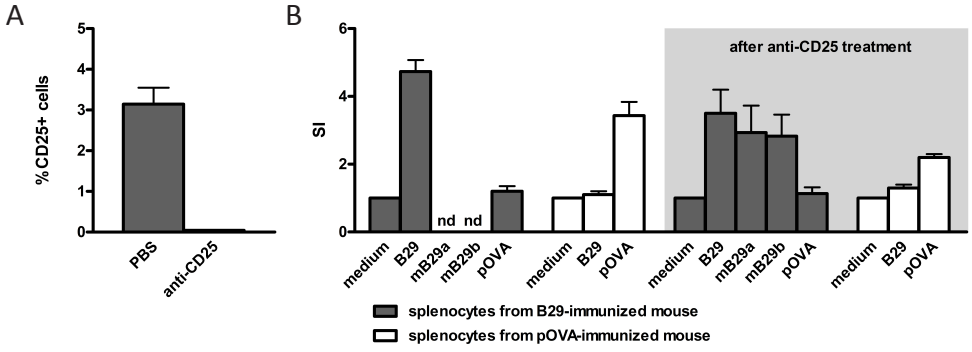

Figure 2. B29-specific T cell proliferation in mice immunized with B29 after CD25+ T cell depletion. Mice were injected with anti-CD25 depleting antibody PC61 or PBS. (A) 7 days after depletion of CD25+ cells, the presence of CD4+CD25+ or CD4+FoxP3+ cells was determined in peripheral blood directly prior to immunization from n=2-6 animals per group. Data are representative of 3 independent experiments. (B) 7 days after administration of anti-CD25, mice were immunized with Hsp70 peptide B29, or control peptide pOVA, and 10 days later splenocytes were restimulated with B29, mouse homologs mB29a or mB29b, or pOVA as a control. Results are expressed as mean SI (cpm peptide / cpm medium only) obtained from of 3-4 animals per condition and are representative of 3 independent experiments. Medium only cpm values were as follows: non-depleted mice immunized with B29: 1004 cpm, immunized with pOVA: 738 cpm; CD25+ cell depleted mice immunized with B29: 4088 cpm, immunized with pOVA: 2055 cpm.

Prior depletion of CD25$^+$ cells results in less CD4$^+$CD25$^+$ cells and CD4$^+$FoxP3$^+$ cells in subsequently immunized mice.

Subsequently, we determined the phenotype of Tregs from splenocytes of mice that had been immunized with B29 after depletion of CD25$^+$ cells and compared these with Tregs from undepleted mice immunized with B29 or pOVA. Depletion of CD25$^+$ cells prior to immunization with B29 resulted in a significant lower percentage of CD25$^+$ or FoxP3$^+$ cells in the CD4$^+$ T cell population (Fig 3). Additionally, the CD4$^+$CD25$^+$ cells that were present expressed less FoxP3 (data not shown). The percentage of CD25$^-$ and CD69$^+$ cells were not affected by CD25$^+$ cell depletion, indicating that the anti-CD25 antibody PC61 specifically targets CD25$^+$ cells.

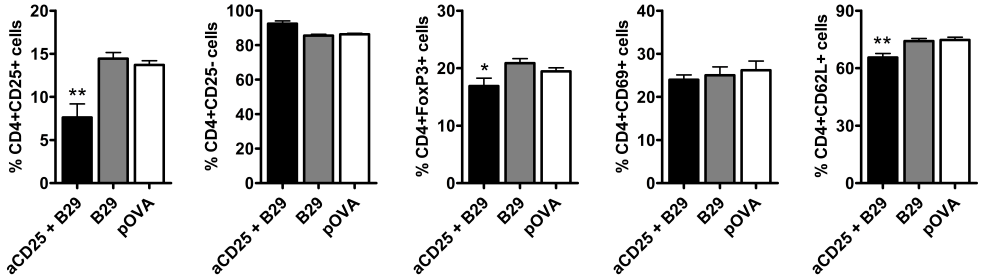

Figure 3. Prior depletion of CD25+ cells results in less CD4+CD25+ cells and CD4+FoxP3+ cells in subsequently immunized mice. Mice were injected with anti-CD25 antibody PC61 or PBS only. 7 days later, mice depleted from CD25+ cells were immunized with B29 (depicted as aCD25). Undepleted mice that received only PBS previously, were immunized with B29 or pOVA. 10 days after immunization with B29 or pOVA, mice were sacrificed and splenic CD4+ T cells were assessed for Treg or activation markers by flow cytometry. The percentages of CD4+CD25+, CD4+CD25-, CD4+FoxP3+, CD4+CD69+ and CD4+CD62L+ cells are shown. Data are mean of 8 animals per group. P values are from an unpaired two-tailed Student t-test in which aCD25 + B29 were compared to B29. * $P < 0.05$, ** $P < 0.01$.

B29-induced Tregs are suppressive *in vitro*

We hypothesized that B29 immunization converted naïve CD25$^-$ cells into suppressive CD4+CD25$^+$ cells. Therefore, we first determined whether newly formed CD4$^+$CD25$^+$ cells from B29-immunized or pOVA-immunized mice pre-treated with anti-CD25 antibody, were suppressive *in vitro*. For this, CD4$^+$CD25$^+$ T cells were isolated from splenocytes of immunized mice, either pre-treated with anti-CD25 antibody or not. As a readout of suppression, the proliferation of autologous CD4$^+$CD25$^-$ responder T cells in the presence of different numbers of CD4$^+$CD25$^+$ T cells was determined (Fig 4). The data show that CD4$^+$CD25$^+$ cells from B29-immunized mice were equally suppressive, irrespective of prior depletion of CD25$^+$ cells. This suggests that suppressive CD4$^+$CD25$^+$ cells from B29-immunized mice pre-treated with anti-CD25 antibody are formed *de novo* from CD25$^-$ cells. Immunizing depleted mice with pOVA also resulted in suppressive CD4$^+$CD25$^+$ cells, although these cells were less suppressive than B29-induced CD4$^+$CD25$^+$ cells indicating that in the pOVA-immunized mice the suppressive activity was not fully restored after the preceding depletion, and thus that immunization with B29 resulted in the *de novo* formation of potent suppressor cells.

Adoptive transfer of B29 induced Tregs reduces inflammation in a mouse model of rheumatoid arthritis

To test whether B29-induced Tregs were capable of suppressing inflammation *in vivo*, we immunized two groups of donor mice with B29, either i.p. pre-treated with anti-CD25 antibody or with PBS only. Transferring $CD4^+CD25^+$ T cells from B29-immunized donors from both, differently pre-treated groups, resulted in a similar suppression of clinical symptoms of arthritis as compared with the transfer of $CD4^+CD25^-$ control cells (Fig. 5). These results are in line with the *in vitro* suppression data (Fig. 4), indicating that B29-immunization one week after depletion of $CD25^+$ cells induces new $CD4^+CD25^+$ T cells that are suppressive. Thus both *in vitro* as *in vivo*, these B29 induced $CD4^+CD25^+$ T cells are equally suppressive as the $CD4^+CD25^+$ T cells from B29 immunized, non-depleted donors, indicating that B29 immunization in non-depleted donors leads to induction of iTregs, rather than activation of preexisting nTregs.

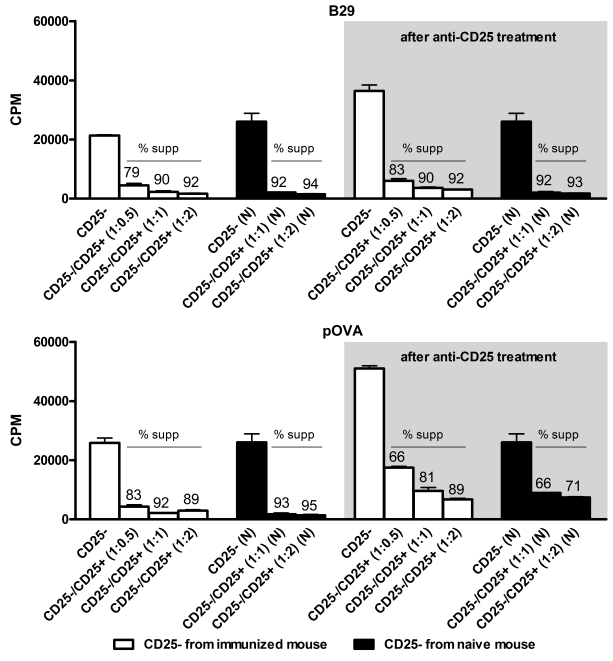

Figure 4. B29-induced Tregs are suppressive in vitro. Mice were either injected with anti-CD25 antibody to deplete CD25+ cells, or with PBS as a control. 7 days after injection, mice (n=3 per treatment) were immunized with either B29 or pOVA. 10 days later autologous CD4+CD25- responder cells and CD4+CD25+ cells were isolated and pooled for co-culture in various ratios in the presence of anti-CD3 antibody to activate the cells. As a control, CD4+CD25- responder cells from naïve donors were used to test the suppressive capacity of B29-induced Tregs or pOVA-induced Tregs on the same population of responder T cells. 3H-thymidine incorporation was determined and data are shown as triplicate samples per well. % sup. is the proliferative response of responder T cells compared to responder T cells co-cultured with Tregs. Data shown are representative for two independent experiments.

Discussion and conclusion

Utilizing Hsp peptides for the inhibition of inflammation has been shown both in animal models [11, 13] and in clinical trials with autoimmune patients [14, 15]. The immunogenic nature of Hsp [16, 17] as well as the upregulation under inflammatory conditions [9, 18, 19] make these proteins suitable candidate antigens for the suppression of autoimmune

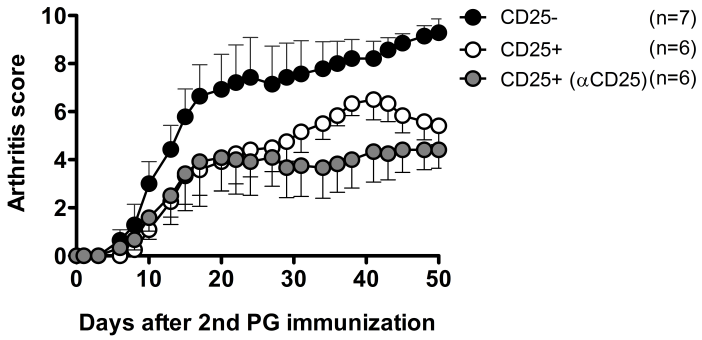

Figure 5. Adoptive transfer of B29 induced Tregs reduces inflammation in a mouse model of rheumatoid arthritis. Mean arthritis scores of recipients with PGIA after adoptive transfer of CD4+CD25+ T cells isolated from splenocytes of B29 immunized donor mice that had been pre-treated 7 days before immunization with anti-CD25 antibody or PBS only. As a control, equal numbers of CD4+CD25- cells were transferred. Recipient animals (n=6-7 mice per group) received 3x105 cells i.p. one day prior to the second PG immunization. Clinical scores were assessed over time and are depicted as the mean of the group. Data shown are representative for 2 experiments.

diseases with unknown disease-inducing antigens like rheumatoid arthritis. The peptide specific suppression after Hsp administration comes from Tregs responsive to the Hsp peptides [10]. Activating antigen-specific Tregs is crucial for optimal suppression, since antigen-specific Tregs are superior over polyclonal Tregs upon transfer [20, 21].
Recently, we showed that immunization or intranasal administration of Hsp70 peptide B29 activates Hsp-specific Tregs that suppress experimental arthritis upon adoptive transfer. Hsp-specific Tregs were suppressive in low numbers, especially when selected on lymphocyte activation gene (LAG)-3, resided in lymphoid tissues up to3 months after injection where they had an activated phenotype [10]. Thus, although the presence and suppressive activity of Hsp-specific Tregs has been shown, nothing is known about the type of subset of Treg that is activated after Hsp administration.
The Treg population can be divided into two subsets: nTregs derived from the thymus (mostly self-specific) [22] and iTregs (mostly specific against foreign peptides) [23] that are formed in the periphery from naïve T cells. It is still largely unknown what the relative contribution of nTregs and iTregs is. Some studies have addressed the contributions of both subsets to immune tolerance. For instance, adoptively transferred nTregs isolated from thymus can partially suppress autoimmunity in Foxp3 deficient mice, although complete rescue from disease only takes place in het presence of *in vitro* generated iTregs [24]. The authors suggested that is due to the fact that both subsets have different TCR repertoires that are complementary. Since Hsp peptides can either originate from self Hsp [25-28], or from bacterial Hsp in the gut [29, 30] and/or infections [31], this suggests that Hsp-specific Tregs can be both induced or natural Tregs (Fig 1). Therefore, we wanted to know whether Hsp-specific iTregs would be able to suppress experimental arthritis.
In this study we show that administration of anti-CD25 antibody PC61 resulted in the absence of $CD25^+$ cells 7 days after injection (Fig 2 A). Although we have not tested the presence of $FoxP3^+$ cells 7 days after depletion of $CD25^+$ cells in spleen, it is likely

that almost all FoxP3$^+$ cells were depleted, since in our Balb/c mice 90% of FoxP3 is expressed on CD25$^+$ cells [10], and it has been showed in previous studies that PC61 depletes FoxP3$^+$ cells [32, 33]. Subsequent immunization with the Hsp70 peptide B29 gave antigen responsive splenocytes that were responsive to the B29 peptide, or its homologs (Fig 2 B). In addition, immunization of CD25 depleted mice resulted in the presence of CD4$^+$CD25$^+$ T cells, although less than in undepleted mice that were immunized (fig 3). The CD4$^+$CD25$^+$ T cells present after immunization were tested for their suppressive function *in vitro* (Fig 4). Depletion of CD25$^+$ cells prior to immunization and *in vitro* anti-CD3 stimulation resulted in higher proliferation CD4$^+$CD25$^-$ cells, indicating that CD25$^+$ T cells give a basal immune suppression. There was no difference in the suppressive capacity of CD4$^+$CD25$^+$ cells from non-CD25 depleted B29-immunized mice compared to CD4$^+$CD25$^+$ from mice that were pre-treated with anti-CD25 before B29 immunization. This shows that B29-immunization in CD25 depleted mice results in the formation of suppressive CD4$^+$CD25$^+$ T cells. On the other hand, using the same approach for pOVA did not result in equally suppressive CD4$^+$CD25$^+$ T cells indicating that in the pOVA-immunized mice the suppressive activity was not fully restored after the previous depletion. This underlines our previous finding that the B29 epitope induces a regulatory response after immunization [10]. Next to the *in vitro* suppressive capacity, B29-induced CD4$^+$CD25$^+$ T cells were tested for their *in vivo* suppression in a mouse model for experimental arthritis. Upon adoptive transfer, the B29-induced CD4$^+$CD25$^+$ cells suppress experimental arthritis, due to cross recognition of mouse homologs of B29. These results are in line with previous work that reported that *in vitro* induced Tregs that were transferred to arthritic animals suppressed disease [10, 34]. Although in this study, CD4$^+$CD25$^+$ cells were induced *in vitro* from naïve cells, while in this study we isolated *in vivo* induced Tregs. For therapeutic purposes, it would be interesting to amplify the conversion of iTregs from naïve T via rapamycin [35, 36], IL-2 [37, 38] or anti-CD3 [39]. In the case of therapies in RA, we would suggest to use anti-inflammatory drugs, such as anti-TNFα for instance, to allow for a window of opportunity for the formation of iTregs before administration of peptide therapy.

Using the method described in this paper, we were able to convert CD4$^+$CD25$^-$ cells into antigen-specific CD4$^+$CD25$^+$ Tregs *in vivo* after immunization with Hsp70 peptide B29. However, we can not exclude that after anti-CD25 administration new CD25$^+$ cells are formed after immunization that are not B29-specific. In this model, we can not enrich for a population of nTregs specific for B29, since immunization with B29 could activate and expand B29-specific nTregs, and induce B29-specific Tregs from naïve CD4$^+$CD25$^-$ cells at the same time. There is debate whether specific phenotypic markers for Treg subsets exist to distinguish nTregs from iTregs whenever origin of the cells is unknown, for instance in *ex vivo* analysis. As a marker for nTregs, Helios was considered until later it was shown that this transcription factor identifies the activation status of Tregs, irrespectively of their origin [40]. Therefore screening for nTreg or iTregs markers can not be used to discriminate the two populations in our model. Currently, the methylation status of FoxP3 is perhaps the best marker to identify Tregs subsets [41-43]. The CD4$^+$CD25$^+$ cells formed in B29-immunized mice depleted from CD25$^+$ cells expressed FoxP3 (data not shown), thus identifying the methylation status of FoxP3 in these cells could provide additional information about to what extent these cells are iTregs.

In conclusion, in this study we show the induction of CD4$^+$CD25$^+$ Tregs via immunization with the Hsp70 epitope B29 in mice depleted of CD25$^+$ cells. We show that *de novo*

induced Tregs after Hsp70 peptide immunization are suppressive *in vitro* and suppress experimental arthritis upon adoptive transfer in similar fashion as the total Tregs population of immunized donors. This indicates that the suppression seen after adoptive transfer therapy in was due to iTregs.

Acknowledgements

This work was supported by Innovation Oriented Programme in Genomics Project Grants IGE3018 and IGE07004, European Union Grant Seventh Framework Programme TOLERAGE: HEALTH-F4-2008-202156, and the Dutch Arthritis Association.

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5

A novel TCR-transgenic mouse to study Hsp70-specific CD4$^+$ T cells

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Abstract

Immunization with the recently identified mycobacterial heat shock protein (Hsp) 70 derived peptide B29 induces antigen-specific regulatory T cells (Tregs) *in vivo* and also intranasal administration of B29 and its mouse homologues mB29a and mB29b induces anti-inflammatory responses. Generally, studying self antigen-specific Tregs is impaired by the lack of suitable tools to isolate and characterize these cells. Using T cell Receptor (TCR)-transgenic mice would be a suitable approach to study antigen-specific T cells directed at self antigens. Therefore, we isolated the TCR from T cell hybridomas generated against mB29b and produced a TCR transgenic mouse that expresses a MHC-class II restricted mB29b-specific TCR. These TCR transgenic $CD4^+$ T cells were found to cross react with the B29 epitope as identified with peptide induced proliferation and IL-2 production. Thus, we have successfully generated a novel mouse model with antigen-specific $CD4^+$ T cells that recognize both self and bacterial Hsp70 derived peptides. With this novel mouse model it will be possible to isolate self-antigen-specific T cells in general and $CD4^+CD25^+$ Tregs more specifically and characterize their functioning under conditions of inflammation. This will provide useful knowledge of the induction, activation and mode of action of Hsp70- specific Tregs, for instance during experimental arthritis.

Introduction

Heat shock protein (Hsp) 70 is a ubiquitously expressed protein and plays a role as chaperone in protein folding, either after protein synthesis or under conditions of cellular stress [1, 2]. Hsp70 is evolutionary conserved and is expressed by many species, including bacteria and vertebrates [3, 4]. This is reflected by a high homology of Hsp between species. Interestingly, Hsps are also highly immunogenic, which could be explained by the fact that Hsps are found in bacteria that surround us. However, not only Hsp derived peptides from bacteria are immunogenic, also peptides derived from self Hsp can trigger immune responses [5, 6]. Peptides derived from endogenous Hsp70 can not only be found in MHC class I molecules, but are also very frequently eluted from MHC class II [7-9]. This indicates that Hsp70 derived peptides can be recognized by immune cells in a MHC class II dependent manner. This is supported by experiments in which $CD4^+$ T cell responses against Hsp70 derived epitopes have been identified after immunization with bacterial Hsp70 [10]. Apart from presentation during cellular homeostasis, endogenous Hsp can also be presented in MHC due to upregulation during cellular stress, including inflammation [11-13]. Due to the high degree of homology of Hsp between species, cross reactive responses occur whereby foreign Hsp-peptide reactive T cells can recognize self-Hsp peptides. [10, 14-16].

Interestingly, the administration of Hsp70 can result in anti-inflammatory responses, which has been shown by the suppression of disease in animal models for chronic inflammation, due to activation of Hsp70-specific regulatory T cells (Tregs) that are cross reactive with self-epitopes of Hsp70 [4, 10]. Tregs are a subset of specialized $CD4^+$ T cells with high suppressive potential and are therefore important targets for immune modulation of inflammatory diseases [17]. Therefore, activating these cells via Hsp peptides is a growing field of interest, especially in inflammatory diseases in which the disease-inducing antigens are unknown.

Recently we have shown that B29 is a highly homologous and immunogenic peptide of Hsp70 and immunization or intranasal administration of B29 activates B29-specific Treg *in vivo* that are potent suppressors of experimental arthritis. Several tools, like T cell lines and T cell hybridomas, have been used to study Hsp-specific T cell responses in the past. For instance, T cell lines generated from *mycobacterial* Hsp60 immunized rats [14] specific for a highly conserved sequence of Hsp60 have been used to study the suppressive potential of Hsp-specific T cells. Similar results were obtained for T cell lines generated from *mycobacterial* Hsp70 immunized rats [18]. However, none of these systems allow the evaluation of primary T cell responses (*in vivo)*, the induction of particular T cell subsets like effector T cells and Tregs after administration of Hsp70, or the behavior of Hsp70-specific Tregs upon activation. Especially, antigen-specific Tregs are difficult to study since these cells comprise only a small fraction of the total T cell population and are difficult to culture and maintain *in vitro* [19].

Therefore, we aimed to generate a model to study primary Hsp70-specific $CD4^+$ T cells in more detail. Therefore, we isolated the TCR-α and TCR-β chain genes from a T cell hybridoma generated against peptide mB29b, a mouse homolog of B29. This hybridoma had a broad range of recognition of Hsp70 peptides. For instance, the mB29b-TCR hybridoma was found to cross react with B29 and mouse homolog mB29a. With the TCR-α and TCR-β chain genes we generated a TCR transgenic mouse with Hsp70 peptide-specific $CD4^+$ T cells. This allowed us to isolate and study primary (m) B29-specific $CD4^+$ T cells in more detail. We show that $CD4^+$ T cells from the mB29b-

TCR transgenic mouse undergo antigen-specific proliferation and IL-2 production after restimulation with B29 and its mouse homologs. In future studies, primary $CD4^+$ T cell responses against self and bacterial Hsp70 peptides can be investigated *in vitro* and *in vivo*. Particularly, antigen-specific $CD4^+$ regulatory T cells can be studied with this model, including activation and differentiation which is not possible with long-term T cell lines or T cell hybridomas.

Materials & Methods

Mice, peptides and antibodies

Female Balb/c mice aged 8-12 weeks were purchased from Charles River and used as cell donors for hybridomas and APC for co-culture assays. Mice strains used for the generation of the mB29b-TCR transgenic mouse were F1 of (CBA X C57BL/6) mice (Charles River). Hsp70 derived peptides (mB29a, mB29b, B29) were identified previously [10] and were obtained from GenScript Corporation. The aminoacid sequences of the peptides are shown in table 1. Anti-MHC-II (m5/114; 5 µg/ml; gift from Louis Boon from Bioceros B.V., Utrecht, The Netherlands) was used to block MHC-II-peptide TCR interactions in co-cultures.

Amino acid sequences of Hsp70 peptides used in this study

Peptide	Sequence
mB29a	VLRVINEPTAAALAY
mB29b	VLRIINEPTAAAIAY
mB29b (a1a)	DAGTIAGLNVLRIINEPTAAAIAYGLDKK
mB29b (a8)	DAGTIAGLNVLRIINEPTAAAIAYGLDRTGK
B29	VLRIVNEPTAAALAY

Table 1. The amino acid sequences of the Hsp70-derived peptides used in this study.

mB29b-TCR Hybridoma (LHEPs)

$CD4^+$ T cell hybridomas (named LHEPS) were generated in our laboratory as described previously [20]. Briefly, BALB/c mice were immunized s.c. and i.p. on day 0 and day 14 with 25 µg Hsp70 peptide mB29b (aa 169-183 of mouse Hspa1a; Entrez ID: 22891339) in 2 mg DDA. On day 28 spleen cells were isolated and restimulated with 4 µg/ml mB29b in X-vivo-15 medium (BioWhitaker) supplemented with glutamax, 100 units/ml penicillin and 100 µg/ml streptomycin. After 48 hours, viable cells were isolated using LympholyteM (Cedarlane Laboratories) and cultured for 48 hours in conditioned medium (IMDM supplemented with 10% FBS, 100 units/ ml penicillin, 100 µg/ml streptomycin, $5x10^{-5}$ M β-mercaptoethanol and 10% supernatant of ConA-activated rat splenocytes). Subsequently, spleen cells were fused with the fusion partner BW5147 as described previously [21]. The FACS Vantage (BD) was used to seed the fused cells into 96 wells plates at one cell per well. Analysis of mB29b specific activation of the obtained clones was performed by incubating hybridoma cells ($2x10^4$/well) with irradiated (10.000 rad) A20 B lymphoma cells as APC ($2x10^4$/well) loaded with Hsp70 peptides in 96 wells flat bottom plates for 48 hours and pulsed with ^{3}H-thymidine (0.4 µC/ well) for an additional 16h. mB29b responsive hybridomas were also cultured with various other irradiated (2500 rad) Hsp70 peptide loaded APC, like splenocytes ($4x10^5$/well), thymocytes ($4x10^5$/

well), and bone marrow derived DC (BMDC; differentiation described elsewhere [22] ($2x10^3$ or $2x10^4$ /well). As a positive control, hybridomas were stimulated with 2µg/ml ConA. After 48 hours co-culture (thus before adding ^{3}H-thymidine), supernatants were harvested and frozen at -20ºC, later the harvested supernatants were cultured with IL-2 responder CTLL-16 cells ($5x10^3$/well) for 24 hours and pulsed with ^{3}H-thymidine (0.4 µC/ well) for another 16h. The 5/4E8 hybridoma specific for proteoglycan (PG) derived-peptide PG70-84 [23], was used as a negative control.

Cloning of the αβ TCR

Isolation of RNA and cloning of the TCRα and TCRβ chain has been described before (11). Total RNA was isolated from the mB29b-TCR hybridoma cells by extraction with RNAzol (Invitrogen). The oligo$(dT)_{12-18}$ primer from the Superscript Reverse Transcription kit (invitrogen) was used for reverse transcription of the isolated RNA. PCR was performed using the primer for the Vα chain (Forw: TRAV7-1-Xmal: TAATCCCGGAATGTCCTTGTGTGTTTCAC, Rev; TRAJ26.01-NotI: TAATGCGGCCGCACAGTA GAC CTC AGG TCC CCC TCAC) and the Vβ chain (Forw: TRBV13-2.-1-hol: TAA TCT CGA GAA GAT GGG CTC CAG GCT CTT C; Rev: TRBJ2-7.01-SacII: TAA TCC GCG GCC TGG TCT ACT CCA AAC TAC TCC). The PCR products of the two fragments were cloned using TA overhang into the pGEM-T easy vector (Promega). The constructs were subsequently introduced into *E. coli* DH5α. The Vα and Vβ genes were cloned into pTα and pTβ cassettes obtained from C. Benoist and D. Mathis [24]. This was done after isolation the genomic DNA from the mB29b-TCR hybridoma to obtain full length DNA, including leader and intron sequences. After digestion with *Xma*I and *Not*I, the TCRα chain was cloned into the pTα cassette and after digestion with *Xho*I and *Sac*II, the TCRβ chain was cloned into the pTβ cassette and introduced to XL10 gold cells (Stratagene) by electroporation. Sequencing revealed that the isolated mB29b TCR consisted of Vα 7 and Vβ 8.2.

In vitro expression of the αβ TCR

The pTα cassette, the pTβ cassette and the pcDNA3 plasmid (containing neomycin resistance gene) were electroporated into the mouse $58\alpha^-\beta^-$ T cell hybridoma which lacks functional TCR chains [24]. Transfected cells were cloned using limiting dilution in 96 wells plates) using the FACS Vantage (BD) and cell lines were cultured in the presence of neomycin. PCR was used to validate DNA incorporation and transfected cells were tested for antigen-specificity in a similar manner as the hybridomas as described above.

Generation of the mB29b-TCR transgenic mouse

TCR transgenic mice were generated in our laboratory as described previously [25]. The pTα mB29b-TCR and the pTβ mB29b-TCR plasmids were linearized using *Sal*I (pTα) and *Knp*I (pTβ) and electrolution was used to separate prokaryotic DNA. The constructs were further purified by phenol extraction and ethanol precipitation. Via pronucleus injection equal amounts of the plasmids were introduced into fertilized eggs of F1 (CBA X C57BL/6) mice. mB29b-TCR transgenic founders were identified by PCR analysis of genomic DNA (primers are described above). Positive founders were mated with Balb/c mice and offspring was tested for peptide specificity as described below.

Measurement of antigen-specific T cell responses from mB29b-TCR mice

Blood was taken from founders and depleted from erythrocytes with ACK buffer.

Depending on cell yield after blood collection, blood cells (founder 1: $1x10^5$, founder 2: $5x10^5$) were cultured for 96 h with $1x10^6$ irradiated A20 cells as APC. Cells were stimulated in the presence of 2 µg/ml or 20 µg/ml B29 or 5 µg/ml ConA as a positive control. Founders were mated with Balb/c mice and offspring was tested for positivity of the mB29b-TCR. Splenocytes from offspring were tested for antigen-specific responses to 2 µg/ml or 20 µg/ml mB29a, mB29b or B29 peptides. Proliferation was determined by ^{3}H-thymidine incorporation during the final 16 h of culture (depicted in Fig 4 left panel and Table 4) and IL-2 production was determined by Luminex (depicted in Fig 4 right panel).

Flow cytometric analysis
Single cell suspension of splenocytes, lymph node cells, or thymocytes were made and these were stained with antibodies CD3-APC (OKT-3, BD Biosciences), CD4-V450 (RM4-5, eBioscience), CD8-V500 (RPA-T8, BD Biosciences company), or Vβ8-PE (F23.1, BD Biosciences) and incubated for 30 minutes at 4ºC. Cells were washed 3 times with PBS containing 2% FCS. Cells were acquired on the CantoII (BD) and analyzed with FlowJo 7 (Tree Star). For cell activation experiments, splenocytes from transgenic mice or littermates were cultured ($1x10^5$ cells/well) for 24h in the presence of 20 µg/ml mB29b, in which the last 4h was in the presence of 1 µg/ml Brefeldin A. Cells were stained extracellular for CD25-PerCP-Cy5.5 (PC61, BD Biosciences) and intracellular for IFN-γ-PE (XMG1.2, eBioscience) or FoxP3-V450 (FJK-16, eBioscience).

Histology
For histology, thymus, spleen, inguinal lymph nodes (iLN) and liver were isolated from mB29b-TCR positive mice, or negative littermates. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and 5 µm saggital sections were stained with hematoxylin and eosin (HE). Immunohistochemistry was performed to T cells and general proliferation in lymphoid tissues . Briefly, cryosections (5 µm) were fixed in ice-cold acetone and blocked against endogenous peroxidase with 0.3% hydrogen peroxide in methanol. Non specific staining was blocked with a 1% BSA solution and sections were incubated with primary antibodies against CD3 (BD Biosciences) or Ki-67 (BD Biosciences) Secondary staining was performed with an anti-rat HRP antibody (Millipore) and Peroxidase activity was developed using the DAB Peroxidase Substrate kit (Vector Laboratories). Sections were counterstained with hematoxylin and mounted with Aquatex (Merck, Darmstadt, Germany).

Results

MHC class II restricted recognition of Hsp70 peptides mB29a, mB29b and B29 by mB29-TCR hybridoma LHEP4

T cell hybridomas specific for Hsp70 peptide mB29b were generated by immunizing BALB/c mice with the peptide mB29b and fusing splenocytes from these mice with BW5147 cells followed by limiting dilution to obtain individual clones of the mB29b-TCR hybridomas. In order to test the TCR specificity of the generated hybridomas which we

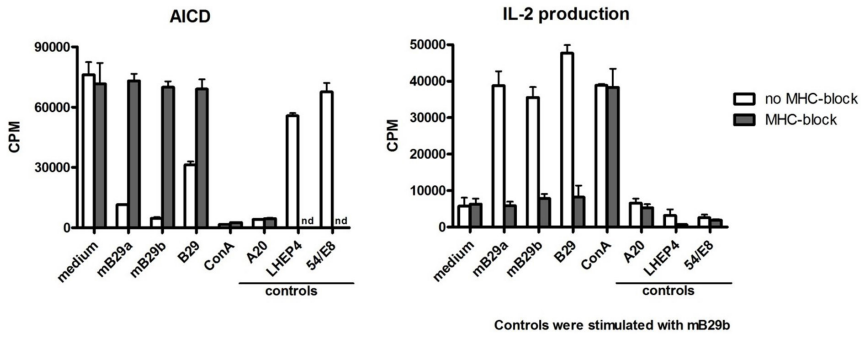

Figure 1. MHC class II restricted recognition of Hsp70 peptides mB29a, mB29b and B29 by mB29-TCR hybridoma LHEP4. Splenocytes from BALB/c mice immunized with mB29b were fused with BW5147 cells which resulted in mB29b-specific CD4+ T cell hybridomas clones which we named LHEPs. As an example, mB29-TCR hybridoma LHEP4 is depicted, which was cultured with A20 B cells as APC for 48h after which supernatants were collected to determine IL-2 production, and hybridoma cells were pulsed with 3H-thymidine for an additional 16h to determine proliferation. A20 cells were loaded 5 µg/ml with mB29a, mB29b or B29 peptides to stimulate LHEP4 activation, which was measured as activation induced cells death (AICD) (left panel), or IL-2 dependent CTLL-16 proliferation from IL-2 present in supernatants collected 48h after stimulation (right panel). As a positive control, cells were stimulated with ConA. As a negative control, LHEP4 hybridoma cells were cultured in medium only, or in the presence of mB29b without APC. Additional controls included A20 cells cultured with mB29b, or the 54/E8 hybridoma (which is specific the proteoglycan derived peptide PG70-84) cultured in the presence of mB29b peptide. 3H-thymidine incorporation is shown as mean of triplicate samples/well ± standard error of mean (s.e.m.). Data shown are representative of 3 independent experiments.

named LHEPs, the cells were cultured in the presence of peptide mB29b, or its homologs mB29a and B29 (Fig 1). Activation induced cell death (AICD) and IL-2 production [20], as measured by IL-2 dependent proliferation of the CTLL-16 cell line were measured for 7 LHEPs. Data of LHEP4 are depicted as a representative example in Figure 1.

Stimulation of LHEP4 with mB29a, mB29b and to some extend with B29 resulted in cell death, which was observed by a reduced of ^{3}H-thymidine incorporation (left panel Fig 1A). In order to determine whether recognition of Hsp70 peptides was MHC class II restricted, peptide stimulations were performed in the presence of an MHC class II blocking antibody. In this case, no AICD was seen upon stimulation with Hsp70 peptides, indicating that peptide recognition of mB29b-TCR hybridomas was indeed MHC class II restricted (Fig1, left panel). Apart from AICD, another characteristic of T cell hybridoma activation is the production of IL-2 (right panel Fig 1B). Therefore, we determined the production of bioactive IL-2 in the supernatants of the cultures described in the left panel of Fig 1. Supernatants were collected after stimulation and these supernatants were added to CTLL-16 cells. ^{3}H-thymidine incorporation of dividing CTLL-16 cells revealed IL-2 production when LHEP4 was cultured in the presence of peptides mB29a, mB29b and B29 (Fig 1, right panel). Additionally, MHC class II blocking resulted in a lack of IL-2 production confirming that peptide recognition was MHC class II dependent.

Screening of the selected mB29b-specific T cell hybridomas revealed 7 LHEPs in total that were responsive to mB29b, of which 5 were cross-reactive mB29a, and 4 that were cross-reactive to B29 (Table 2; positive responses for both AICD and IL-2 production that can be inhibited with anti-MHC class II antibody are depicted as +).

Table 2: Overview of mB29-TCR hybridomas screened for MHC class II restricted recognition of mB29b, or homologs mB29a and B29.

	mB29a	mB29b	B29
LHEP 4	+	+	+
LHEP 7	+	+	+
LHEP 8		+	
LHEP 9	+	+	
LHEP 11	+	+	+
LHEP 14		+	
LHEP 21	+	+	+

mB29-TCR hybridomas were stimulated with Hsp70 peptides mB29a, mB29b or B29 as described in Fig 1. The + symbol indicates AICD and IL-2 production upon stimulation with individual peptides of the depicted LHEPS. Peptide-specific activation of the LHEPs could be inhibited by adding blocking antibody against MHC class II, confirming MHC class II restricted recognition of the individual Hsp70 peptides. Data shown summarize 3 independent experiments.

Antigen-specific IL-2 production of mB29b-TCR hybridoma LHEP4 upon co-culture with primary antigen presenting cells

In order to investigate whether other APCs than the A20 B cell line were capable of activating the 7 responsive hybridomas depicted in Table 2, the LHEPs were cultured in the presence of bone marrow-derived dendritic cells (BMDC), irradiated splenocytes, or irritated thymocytes and stimulated with the Hsp70 peptides. IL-2 dependent proliferation of CTLL-16 cells was most profound when cells were cultured in the presence of supernatants of LHEP4 co-cultured with BMDC indicating that BMDC induced high levels of activation in LHEP4 (Fig 2 A). Supernatants from co-cultured LHEP4 cells with irradiated splenocytes and thymocytes in the presence of the different B29 homologous peptides gave similar CTLL-16 proliferation profiles, although lower compared to proliferation in the presence of supernatant from BMDC cultures. In all cases, LHEP4 produced IL-2 upon stimulation with mB29b, however, cross recognition of mB29a and B29 was also observed after stimulation with primary APC.

We next determined a dose response of LHEP4 to the Hsp70 peptides, as well as recognition of specific length extension variants of mB29b. For this, LHEP4 was co-cultured with irradiated splenocytes in the presence of the extended hspa8 (= Hsc70) or hspa1a (=Hsp72 see [26]) variants of the mB29b peptide. CTLL-16 proliferation induced by IL-2 production from LHEP4 indicated that these length variants could be recognized (Fig 2 B). Although IL-2 production decreased in cultures in which LHEP4 was stimulated in low amounts of mB29b or B29, CTLL-16 proliferation remained detectable, indicating that LHEP4 responds sensitively to presented Hsp70 peptides (Fig 2B).

Apart from LHEP4, the previously selected T cell hybridomas (Table 2) were screened for the recognition of Hsp70 peptide (length variants) as well (Table 3). All LHEPS responded to the mB29b peptide when presented by primary APC. As was previously seen (Table 2), not all LHEPS were cross-reactive to other peptides, including the hspa8 or hspa1a length variants of mB29b. These results indicate that the mB29b-TCR hybridomas recognize Hsp70 peptides presented by APC from various sources. Additionally, different length variants of mB29b can be recognized, showing a broad range of recognition for some of the generated hybridomas.

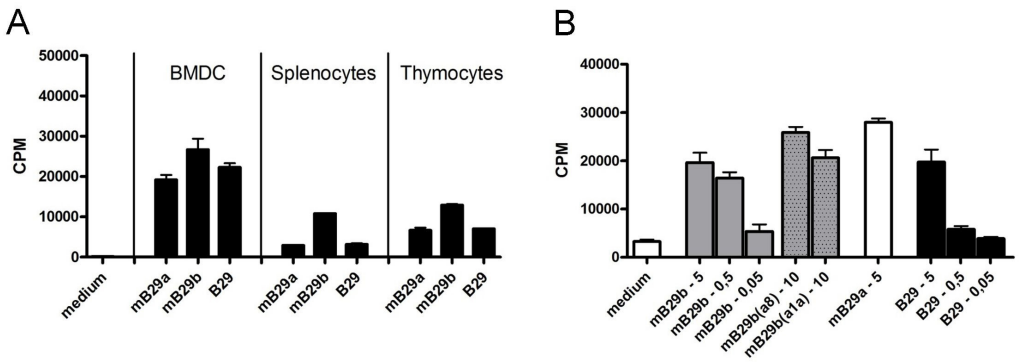

Figure 2. Antigen-specific IL-2 production of mB29b-TCR hybridoma LHEP4 upon co-culture with primary antigen presenting cells. (A) mB29b-TCR hybridoma LHEP4 was co-cultured with BMDC, irradiated splenocytes or irradiated thymocytes which were loaded with 5 µg/ml Hsp70 peptides mB29a, mB29b or B29 for 48h, after which supernatants were collected. Supernatants were added to CTLL-16 cells and IL-2 dependent proliferation was determined after 24h by pulsing CTLL-16 cells with 3H-thymidine for an additional 16h. (B) LHEP4 was co-cultured with irradiated splenocytes as described in (A) with different concentrations of peptides ranging from 5 µg/ml, 0,5 µg/ml to 0,05 µg/ml, as well as 10 µg/ml length variants of mB29b (hspa8=Hsc70 or hspa1a=Hsp72 [26]).CTLL-16 cell cultures were supplemented with supernatants from these stimulations to determine IL-2 dependent proliferation of CTLL-16 cells. As a control, co-cultured LHEP4 cells were unstimulated, or stimulated with ConA (data not shown). 3H-thymidine incorporation is shown as mean of triplicate samples/well ± standard error of mean (s.e.m.). Data shown are representative of 3 independent experiments.

Table 3. Overview of Hsp70 peptide recognition by mB29b-TCR hybridomas upon co-culture with primary APC

	mB29a	mB29b	mB29b(a8)	mB29b(a1a)	B29
LHEP 4	+	+	+	+	+
LHEP 7	+	+	+	+	+
LHEP 8		+	+		+
LHEP 9	+	+	+/-		
LHEP 11	+	+	+	+	+
LHEP 14	+	+	+/-		
LHEP 21	+	+	+	+	+

mB29-TCR hybridomas were stimulated with Hsp70 peptides mB29a, mB29b or B29, as well as length variants of mB29b (hspa8=Hsc70 or hspa1a=Hsp72 [26] as described in Fig 2. The + symbol indicates AICD and IL-2 production upon co-culture with supernatants from peptide-stimulated LHEPS. LHEP9 and LHEP14 did not respond to peptide stimulation with all APC tested, and recognition is therefore depicted as +/-. Data shown summarize 3 independent experiments.

Cloning of the TCRα and TCRβ chain from mB29b-TCR hybridoma LHEP4 into TCR$^-$ cells results in Hsp70 peptide-specific transfectants

Based on the specificity and the strong cross reactive responses of LHEP4, the TCRα and TCRβ chain of this mB29b-TCR hybridoma were cloned into TCR expression vectors which were transfected into TCR$^-$ cells. The transfected cell line was co-cultured with BMDC or irradiated A20 B cells which were loaded with Hsp70 peptides to confirm the antigen-specificity of the transfectant. Results showed a weak but dose dependent response to mB29b and B29, but not to mB29a (Fig 3). Irradiated thymocytes and splenocytes were also used as APC, and gave similar proliferative responses (data not shown). These data confirmed that the TCRα and TCRβ chain were successfully cloned into TCR expression vectors and therefore we transferred the TCRα and TCRβ chain constructs to mouse oocytes via intranuclear injection.

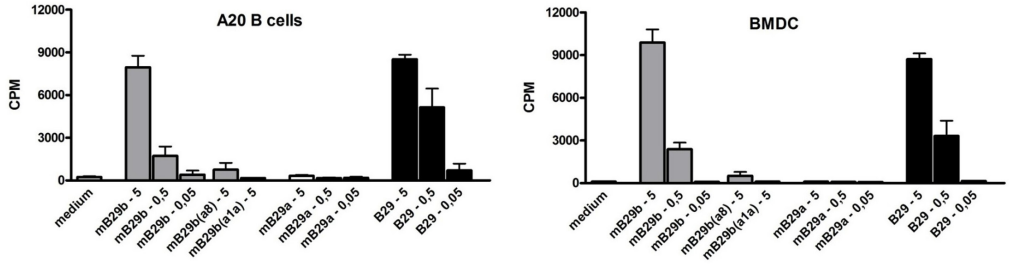

Figure 3. Cloning of the TCRα and TCRβ chain from mB29b-TCR hybridoma LHEP4 into TCR- cells results in Hsp70 peptide-specific transfectants. The TCRα and TCRβ chain from the mB29b-TCR hybridoma LHEP4 were cloned into pTα and pTβ cassettes and were electroporated into the mouse 58α-β- T cell hybridoma which lacks functional TCR chains [24]. Transfectants were cloned and the results of the stimulation of 1is given as an example. The transfectant was co-cultured with BMDC or irradiated A20 B cells which were loaded with 5 μg/ml, 0,5 μg/ml or 0,05 μg/ml Hsp70 peptides 48h, after which supernatants were collected. Cells were stimulated with Hsp70 peptides mB29a, mB29b or B29, as well as length variants of mB29b (a1a or a8). Supernatants were added to CTLL-16 cells and IL-2 dependent proliferation was determined after 24h by pulsing CTLL-16 cells with 3H-thymidine for an additional 16h. As a control, co-cultured LHEP4 cells were unstimulated, or stimulated with ConA (data not shown). 3H-thymidine incorporation is shown as mean of triplicate samples/well ± standard error of mean (s.e.m.). Data shown are representative of 2 independent experiments.

Antigen recognition of cells from mB29b-TCR transgenic mouse

After intranuclear injection of the TCRα and TCRβ expression vectors, several pups were born that had incorporated the constructs, of which two founders were positive for both constructs with PCR (data not shown). Peripheral blood lymphocytes (PBLs) from the two founders were stimulated with B29 in a co-culture with irradiated splenocytes. PBLs from one founder proliferated and produced IL-2 in response to B29 stimulation (Fig 4). Next, the positive founder was mated with Balb/c mice and F1 mice were screened for the expression of the TCRα and TCRβ chain and splenocytes from mB29b-TCR positive offspring were tested for antigen specificity and compared to negative littermates. We observed responses to mB29b, B29 and mB29b length variant mB29b(a8) (Table

3), while mB29b-TCR negative littermates showed no response to any of the peptides tested (data not shown). These data show that we successfully generated mB29b-TCR transgenic mice with cells with a functional TCR that recognized Hsp70 peptides.

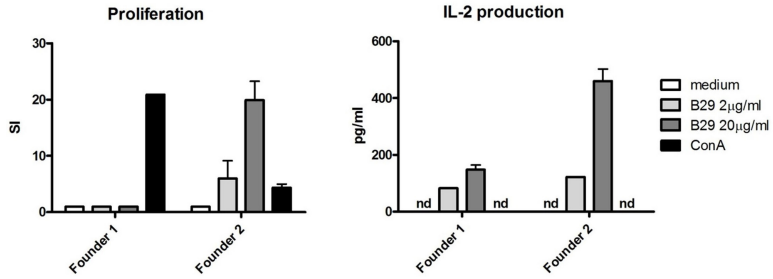

Figure 4. antigen recognition of cells from mB29b-TCR transgenic mouse. PBLs from two founders positive for the TCRα and TCRβ chain were cultured for 96h with irradiated A20 B cells and stimulated with 2 µg/ml or 20 µg/ml B29, or 5 µg/ml ConA as a positive control. Proliferation was determined by 3H-thymidine incorporation during the final 16h of culture and IL-2 production was determined by Luminex. Data are from 1 experiment.

Table 4. Overview of Hsp70 peptide recognition by founders mB29b-TCR transgenic mouse

Peptide	
mB29a	
mB29b	+
mB29b(a1a)	+
mB29b(a8)	+
B29	+

Splenocytes from mB29b-TCR positive offspring of the founder mated with Balb/c mice were stimulated with mB29a, mB29b, B29 or length variants of mB29b (a1a or a8) and proliferation was determined by 3H-thymidine incorporation. Positive response (+ symbol) indicate 3H-thymidine incorporation upon stimulation. Data shown summarize 4 independent experiments.

Flow cytrometric analysis of mB29-TCR transgenic mouse tissues

Next, we examined the presence of $CD3^+$, $CD4^+$, $CD8^+$ and $V\beta8^+$ T cells in thymus of the mB29b-TCR transgenic mouse. The transgenic mouse had a different distribution of $CD4^+$ and $CD8^+$ cells compared to littermates, as well as an increased number of $V\beta8^+$ T cells in the thymus (Fig 5A), probably due to the prearranged TCR specificity. In the spleen, there was also a difference in $CD4^+$ T cell and $CD8^+$ T cell distribution, as well as an increased number of $V\beta8^+$ T cells (Fig 5 B). We observed a similar distribution and percentage of T cells in the LN (data not shown). The difference in $CD4^+$ and $CD8^+$ T cell distribution compared to non-transgenic littermates is due to the transgenic background of the mB29b-TCR mouse, in which formation of T cells is altered.

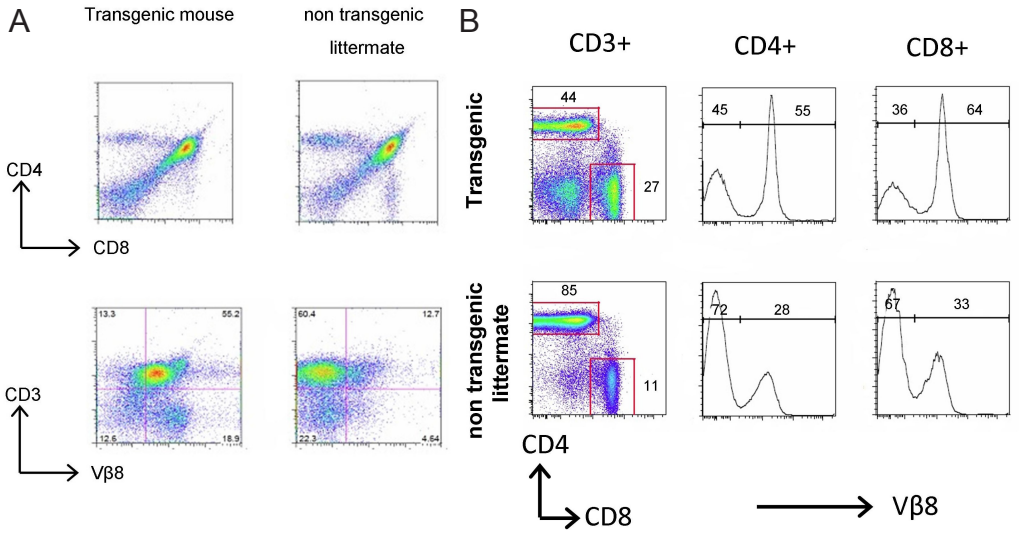

Figure 5. Flow cytrometric analysis of mB29-TCR transgenic mouse tissues. (A) Single cell suspension were made from thymus of mB29b-TCR transgenic mice or negative littermates. Cells were stained for the expression of CD3, CD4, CD8 and Vβ8. Live cells were gated on the forward scatter (FSC) and side scatter (SSC) and the percentage of CD4+ and CD8+ cells of the live cells are depicted in the upper panel of Fig 5 A. In the lower panel of Fig 5 A the percentage of CD3+ and Vβ8+ of all live cells are shown. (B) Distribution of CD4+ and CD8+ cells in the live gate (as described above) of CD3+ cells (left row of graphs. Histograms of CD4+ cells (middle row of graphs) and CD8+ cells (right row of graphs) of total CD3+Vβ8+ cells are depicted.

mB29b-TCR transgenic mouse shows no pathology in histology

In order to investigate cell distribution and activation, tissue sections from thymus, spleen, inguinal lymph nodes (iLN), and liver were made and stained for HE or CD3 (Fig 6). Based on the HE stained tissue slides, no apparent changes are observed in iLN, spleen, thymic or liver (data not shown) tissue architecture (Fig 6 A). In addition the distribution of $CD3^+$ T cell in the different lymphoid tissues was comparable between the TCR transgenic mouse and the littermate. There was no difference in proliferative activity, as based on the Ki-67 expression in the tissues examined (data not shown). Overall, histological analysis revealed no major differences in T cell distribution, activation and proliferation between the founder and control littermates in lymphoid organs, suggesting no development of gross pathology.

Discussion and Conclusion

Investigating Hsp-specific T cell responses *in vivo* and *in vitro* is of great interest to examine the effect of Hsp administration on T cell activation and differentiation. Although there are Hsp-specific cell lines that have been generated after long *in vitro* culture [14, 27],

as well as Hsp-specific T cell hybridomas [20], these cells lack the properties of primary T cells like the ability to differentiate from naïve cell to effector T cell or Tregs and can therefore only be used for qualitative analysis of Hsp recognition. Several studies have shown that the immuno-modulatory effect of Hsp administration (being immunization, intranasal administration or oral administration) is due to the activation of Hsp-specific Treg [10, 16, 28]. However, little is still know about the function of Hsp-specific Tregs. For instance, it is especially difficult to study Tregs *in vitro*, since these cells require more than peptide stimulation alone (e.g growth factors like IL-2 and/or TGF-β) for their expansion and differentiation [29, 30], in comparison to immortalized T cell lines. Eventually, one would like to study primary antigen-specific T cells, however these are quite a rare population of the total population of T cells. Therefore, TCR-transgenic mice are valuable tools to obtain larger quantities of antigen-specific T cells. Therefore we set out to generate a TCR transgenic mouse with Hsp70 peptide-specific T cells to establish a tool to study the activation, differentiation and suppressive capacity of Hsp-specific T cells.

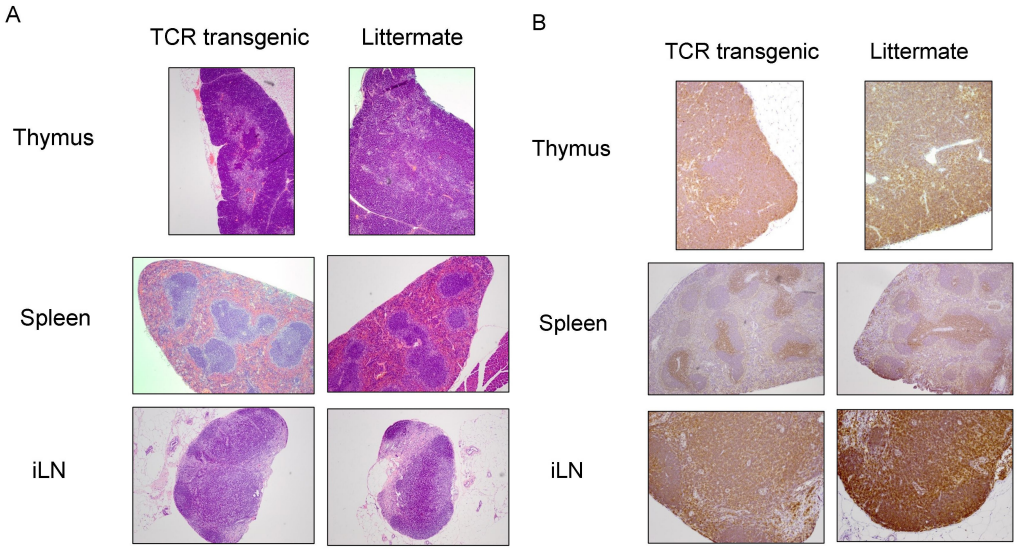

Figure 6. mB29b-TCR transgenic mouse shows no pathology in histology. (A) Thymus, spleen, inguinal lymph nodes (iLN) and liver were isolated from mB29b-TCR positive mice, or negative littermates. Tissues were fixed in formalin, embedded in paraffin and sections were stained with hematoxylin and eosin (HE). Sections shown were magnified 4x. (B) Immunohistochemistry was performed to T cells and general proliferation in lymphoid tissues. Cryosections were fixed in ice-cold acetone and blocked against endogenous peroxidas. Tissue sections were incubated with primary antibody against CD3. Secondary staining was performed with an anti-rat HRP antibody followed DAB Peroxidase Substrate to determine Peroxidase activity. Then, sections were counterstained with hematoxylin. Pictures show thymus and iLN in a 10x magnification and spleen in a 4x magnification.

In this study, we have successfully cloned the functional TCR from a mB29b-TCR hybridoma with specificity for Hsp70 peptide mB29b. Due to the homology of Hsp, this hybridoma can recognize self Hsp peptides mB29a and mB29b, and the recently identified foreign Hsp peptide B29. Recognition was MHC class II dependent and all APCs tested were capable of activating the mB29b-TCR hybridoma (Fig 1 and Fig 2). Next, the TCRα and TCRβ chain were isolated and cloned into TCR expression vectors which were electroporated into TCR^- T cells, which showed peptide-specific activation upon electroporation of the construct (Fig 3). Next, the TCRα and TCRβ chain were injected intranuclear into mouse oocytes. Two founders were born that carried both vectors. PBL from one mB29b-TCR transgenic founder showed mB29b-specifc activation, as well as cross recognition to Hsp70 peptide B29 (Fig 4). The founder was mated with Balb/c mice and offspring was screened for bearing the mB29b-TCR. Splenocytes were stimulated with Hsp peptides and showed similar peptide recognition as the founder did (Table 4). FACS analysis revealed that the distribution of $CD4^+$ and $CD8^+$ T cells in thymus and spleen of the mB29b-TCR transgenic were different to that of littermates, which is normal for a TCR transgenic [31], since these mice have selective development of αβTCR T cells in the thymus (Fig 5). The distribution of TCR transgenic cells could be seen in histology of the thymus (Fig 6). Although some minor differences is tissue distribution of T cells were observed in tissue sections, the amount of proliferation cells is not altered in lymphoid tissues from the mB29b-TCR transgenic compared to littermates as was shown in histology. These data indicate not only that tissue morphology is normal in the mB29b-TCR transgenic mice, but also that the self-specific T cells, in this case cross-reactive Hsp-specific T cells are not highly activated *in vivo* and no spontaneous autoimmune disease was observed in these young TCR transgenic mouse.
With this TCR transgenic mouse, we can now study the effects of cellular stress (in particular inflammation) and Hsp70 vaccination on Hsp70-specific T cells and Tregs. Additionally, we can now study the effects of cross-reactive responses of self-Hsp-specific T cells to foreign Hsp *in vivo*.

Acknowledgements

This work was supported by Innovation Oriented Programme in Genomics Project Grants IGE3018 and IGE07004, European Union Grant Seventh Framework Programme TOLERAGE: HEALTH-F4-2008-202156, and the Dutch Arthritis Association.

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6

Summarizing discussion

Hsp upregulation and recognition under various conditions

The Hsp family consists of several family members (Hsp10, 40, 60, 70, 90, and 100) that are categorized on molecular weight. Hsp act as chaperones that help the folding of newly formed proteins or misfolded proteins [1, 2]. Hsp are not only ubiquitously expressed, but some members of the Hsp family are upregulated under various conditions [1]. Cellular stress like hypoxia, toxic chemicals and heat can compromise the three dimensional structure of intracellular proteins. Therefore, cells upregulate Hsp during stress in order to rescue protein conformation and survive the stressful event. Hsp are evolutionary conserved and are expressed in all living organisms from prokaryotes to eukaryotes [3]. Although Hsp are self-antigens and immune responses against self are tightly regulated, Hsps are quite immunogenic. This immunogenicity could come from the Hsp expression in bacteria [4] and because of the homologous nature of Hsp, cross reactive responses occur whereby cells directed against foreign Hsp can cross-recognize self Hsp and visa versa [5-7], which was also demonstrated in **chapter 3** and **chapter 4**. The sources for Hsp, being self or foreign, are diverse and APC can potentially acquire Hsp via several routes. Once taken up, Hsp can be degraded and Hsp-derived peptides can subsequently be loaded into MHC molecules to be presented to T cells (Fig 1).

Ubiquitous expression in steady state

As previously mentioned, Hsp help the folding of newly synthesized proteins and are therefore essential for cellular homeostasis. Endogenous proteins are continuously being degraded and presented in MHC Class I molecules in all nucleated cells. Via mechanisms like autophagy, endogenous antigens can be presented via MHC class II molecules to $CD4^+$ T cells [8] and an overview of Hsp peptides eluted from MHC class II molecules is given in table 1 of **chapter 2**. Also, some of the Hsp70 family members are involved in chaperone-mediated autophagy [9]. And Hsp70-derived peptides have been eluted from MHC-II [10, 11], showing that Hsp70 peptides are abundantly presented to $CD4^+$ T cells.

Upregulation of self Hsp

Cellular stress induces the upregulation of self Hsp [12], for instance a raised body temperature due to fever induces Hsp expression in cells [13]. It has been shown in numerous studies that inflammation is a stressor of cells and cells in areas of inflammation express self-Hsp [14-18], and that self-Hsp can activate Hsp-specific T cells [19-21], but also B cells [20]. In addition to production of Hsp for their own cellular homeostasis, bacteria can also induce the upregulation of self-Hsp70 in gut endothelial cells [22, 23], showing that the sources for upregulated Hsp are abundant and distributed throughout the body. The upregulation of endogenous Hsp can be boosted by co-inducers [24]. For instance, exposing DC to thermal stress induces Hsp70 expression, which can be boosted by the recently identified co-inducer carvacrol and leads to the formation of tolerogenic DC that can suppress experimental arthritis [25]. Next to expression of Hsp-derived peptides from endogenous Hsp, extracellular Hsp can be secreted from stressed, damaged, or necrotic cells and can be found in the bloodstream during inflammation [26-28], as a consequence of infection or autoimmunity. However, self-Hsp is also secreted during homeostasis [29, 30]. As with foreign Hsp, these proteins can bind to extracellular receptors on APC, thereby activating or inhibiting their function [31, 32].

Also, extracellular self Hsp can be taken up from the cell's environment and loaded into MHC molecules and be presented to T cells. For instance, Hsp from synovial fluid in RA patients is presented by DC [17].

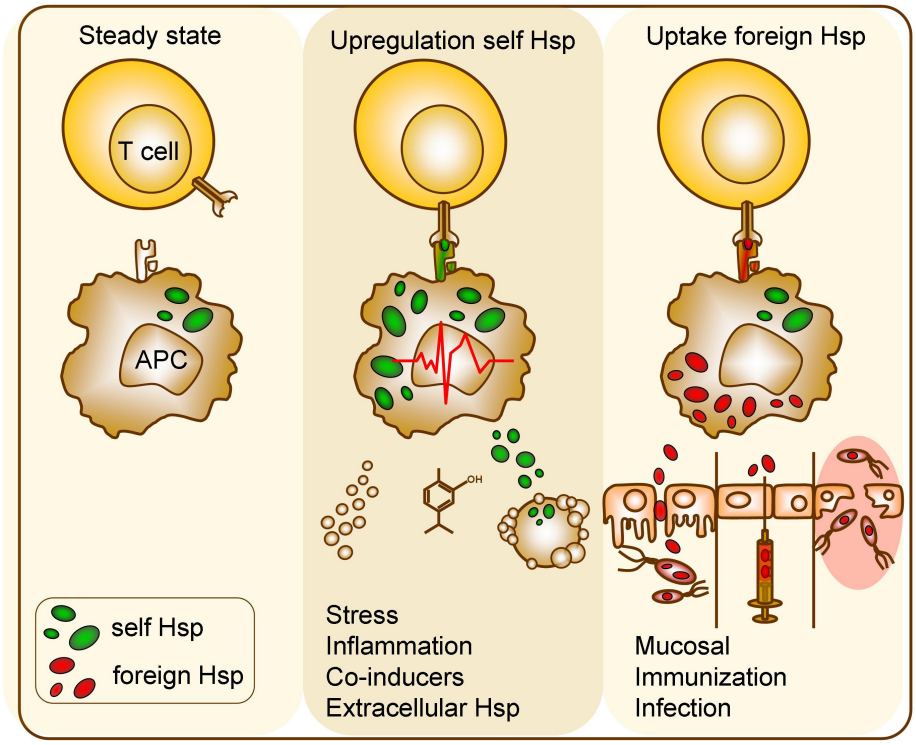

Figure 1. Sources or mechanisms for APC to acquire self or foreign Hsp. Self Hsp (depicted in green) are expressed under homeostatic conditions to allow proper cell function (left panel). Additionally, self Hsp can be upregulated as a consequence of cellular stress, inflammatory mediators like cytokines, co-inducers, or can be obtained from extracellular self Hsp (middle panel). Finally, foreign Hsp (depicted in red) can be acquired from mucosal areas, via immunization, or as a consequence of infection (right panel). Due to the high homology of Hsp peptides, T cells are able to recognize both self and foreign Hsp.

Uptake of foreign Hsp

Foreign Hsps produced by bacteria, for instance in the gut [33] or peritoneal cavity after infection [34], can be secreted and several groups have reported binding of bacterial Hsp to various receptors on monocytes and DC. For instance it has been suggested that extracellular Hsp70 can bind to Toll-like receptor (TLR) molecules TLR2 [35], TLR4 [35, 36] and it has been shown that endocytosis of foreign Hsp is necessary for TLR4 activation [37]. CD14, which is part of the receptor complex for TLR4 activation, has also been shown to bind Hsp [38, 39]. Other molecules that have been reported to bind Hsp are CD40 [40], CD91 [41], CCR5 [42], lectin-like oxidised low-density lipoprotein receptor (LOX)-1 [43] and SREC-1 [42]. In the case of TLR activation, it needs to be taken in consideration that bacterial or recombinant Hsp can be contaminated with other

bacterial products that can activate TLRs [44]. Additionally, since Hsp interact with a variety of other molecules, it can not be excluded that binding to any one of the mentioned receptors is directly to Hsp, or a molecule in complex with Hsp [45]. Nevertheless, many studies have been performed with highly purified Hsp and considerable controls, which suggests that a variety of extracellular proteins is capable of detecting extracellular Hsp. Ligation of Hsp to extracellular receptors not only activates cells, stimulation of monocytes with Hsp70 BiP for instance has shown to deactivate these cells and induced Tregs [46] and Hsp70 induces tolerogenic DC [47, 48]. These data show that Hsp70 can promote or inhibit activation of immune cells, which depends on the setting in which cells come into contact with Hsp70 protein, or Hsp70 peptides. For instance, Wang et al. have identified stimulating and inhibitory epitopes of Hsp70 that modulate cytokine production and maturation of DC [49]. In conclusion, APC like monocytes and DC have various receptors that allow for the recognition or uptake of extracellular foreign and self Hsp. Next to this, self Hsp can be upregulated under stress or inflammation. These Hsp can eventually be presented to T cells via MHC molecules.

Regulation or activation is determined by self or non-self of epitopes

Hsp can regulate or activate immune cells dependent on several factors like the presence of other (inhibitory or activating) signals, but also the foreign or endogenous nature of the Hsp. Hsp-specific T cells are often raised against foreign Hsp for instance during infection or immunization (in experimental settings, and illustrated in **chapter 3**) [6, 34, 50, 51], but due to the high homology between Hsp from eukaryotes and prokaryotes some Hsp-specific T cells are cross reactive to self-Hsp epitopes [5, 19, 52]. Therefore, it is interesting to note that self and non-self epitopes from Hsp can determine regulation or activation of cells, which is proposed in the model depicted in Figure 2. For example, immunization with Hsp60 peptide from bacterial Hsp60, that is homologues to rat Hsp60 suppresses experimental arthritis, while non-homologous peptides do not [53]. Additionally, only cell lines that were cross-reactive to the self-homologous peptide [53] suppressed experimental arthritis upon adoptive transfer, while a cell line reactive to non-homologous Hsp60 peptide did not inhibit disease [5]. Although cross-recognition can come with a price, as was shown in a study in which Hsp-specific Tregs were induced after *Listeria* infection that made rats more susceptible for infection [50], these Hsp-specific Tregs were cross-reactive and capable of suppressing experimental arthritis in rats upon adoptive transfer [34, 50]. In addition, in **chapter 3** and **chapter 4** we have shown that Hsp-specific regulatory T cells that were raised against mycobacterial Hp70 peptide B29 after immunization suppressed experimental arthritis upon adoptive transfer [6]. Cross-reaction of B29-specific Tregs was likely due to locally expressed homologs of the B29 epitope and was supported by the localization of activated donor Treg in lymphoid tissue and joints as described in **chapter 3** [6].
An explanation for activation of T cells by non-self epitopes may be that these cells only recognize non-self and are therefore a welcome addition in the response against pathogens, without risking autoimmunity. Cross-reactive T cells that were raised against foreign antigens, but also recognize self antigens can be harmful when activated, unless these cells are suppressive. Therefore, cross-reactive T cells that are formed after immunization or intranasal administration with Hsp, or Hsp-derived peptides are

induced to become Tregs which was shown in **chapter 4** with Hsp70 peptide B29 and we show in this chapter that B29-induced Tregs can be potent inhibitors of inflammation. The induction or activation of Hsp-specific Tregs after Hsp administration has also been shown by others [6, 19, 52].

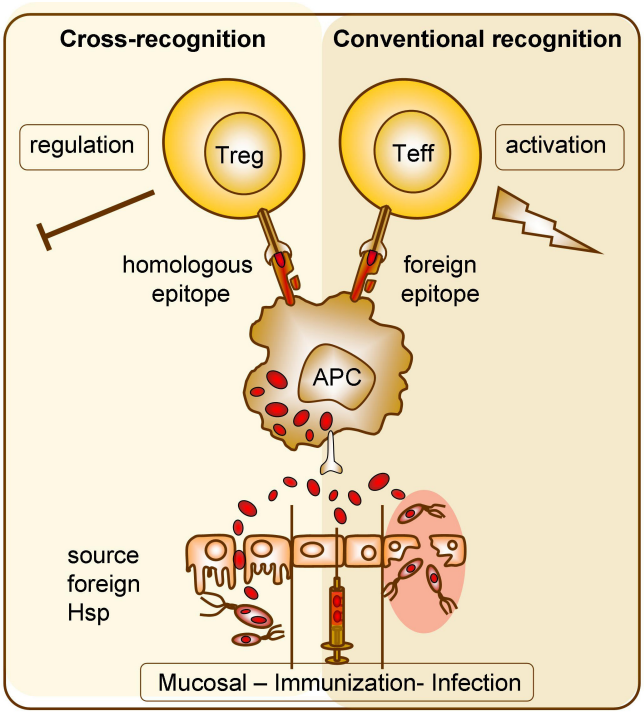

Figure 2. Cross-recognition of self and non-self Hsp can lead to inhibition of inflammation. This model proposes that effector T cells (Teff) that recognize foreign Hsp epitopes presented by antigen presenting cells (APC) are activated in order to combat pathogens (right panel), while self Hsp-specific T cells (Tregs) that are cross-reactive to homologous foreign Hsp peptides are regulators that can efficiently suppress inflammation (left panel). The source of foreign Hsp can be diverse, because bacteria reside in mucosal areas or invade during infection. In experimental settings immunization is often used as a method to deliver Hsp.

Mechanisms of suppression by Hsp-specific Tregs

Hsp proteins and Hsp-derived peptides are immunogenic and can elicit strong immune responses that can be either pro or anti-inflammatory. There is no question that Hsp can promote the activation of several immune cells like APC [17, 40, 49], T cells [20, 53, 54] and B cells [20], and thereby promote inflammation in various pathological conditions [55-57]. It should be noted that effector responses are easier to observe than anti-inflammatory responses, since the latter require more difficult *in vitro* suppression assays, or adoptive transfer with highly purified Tregs The balance between induction of immune tolerance and other immune responses depends on several factors. They

include the route of administration and the local environment in which the antigen is recognized, the use of adjuvants, the amount of antigen and even which epitopes are used for administration [49, 53, 58-61]. Depending on the above mentioned factors, Hsp administration can induce anti-inflammatory responses via the activation or induction of Hsp-specific Tregs. In fact, one of the most notable marker for Tregs, the transcription factor FoxP3, has been detected in Hsp-specific Tregs [6, 62-65]. In **chapter 3** we demonstrate that FoxP3 expression is essential for suppression of experimental arthritis after adoptive transfer of B29-specific Tregs, because Hsp-induced cells lacking FoxP3 do not suppress arthritis. The following paragraph will focus on the identification and function of Hsp-specific Tregs, since these cells have great potential for the suppression of inflammation, and are of particular interest for conditions of inflammation in which the disease inducing antigen is unknown.
Several anti-inflammatory molecules (either secreted or cell bound) have been identified on cells after activation with Hsp. However, identification of markers on T cells alone is insufficient to confirm the suppressive capacity. The presence of markers in combination with Treg-associated proteins like CD25 and FoxP3 make it more likely that the molecule is expressed by Hsp-specific Tregs, and not Hsp-specific effector T cells. Even better are experiments like the *in vitro* suppression assay, or adoptive cell transfer to inhibit inflammation, possibly in combination with deletion of specific genes that code for the identified marker in order to evaluate the role of the specific molecule in Hsp-induced suppression. There are studies that have used these particular assays and have shown that Hsp activation leads to Hsp-driven suppression by activation of Hsp-specific Tregs [66, 67]. The Treg marker identified in these studies have been summarized in Fig 3 and were categorized under three known mechanisms by which activated Tregs suppress inflammation, which will be discussed in further detail.

1. Anti-inflammatory and T helper subset-specific cytokines

Tregs can secrete a variety of anti-inflammatory cytokines like IL-10, IL-35 or TGF-β that suppress other cells [68]. Additionally, some diseases are driven by specific T helper (Th) responses and deviation from this response by the induction of opposing Th subsets can thereby suppress ongoing inflammation. For instance IL-4 producing Th2 cells can suppress Th1 driven arthritis [60, 69, 70]. In fact, IL-4 production by Hsp-specific Tregs has been reported [60, 71-73] and has also been associated with reduced inflammation after Hsp administration [60]. The secretion of IL-10 by Hsp-responsive T cells has also been shown in **chapter 3** and in other studies [6, 50, 61-63, 65, 71-75], and was even necessary for Hsp-induced suppression of experimental arthritis, because IL-10 k.o. mice remained susceptible for arthritis induction despite Hsp70 administration [51]. Finally, the inhibitory cytokine TGF-β has also been identified as a product of Hsp-specific Treg activation [50, 74-77]. Blocking TGF-β function *in vitro* with blocking antibodies hampered Hsp-induced suppression [75], while adoptive transfer of TGF-β producing Hsp-specific Tregs suppressed experimental arthritis [77], indicating a mechanistic role of this suppressive cytokine for Hsp-specific Tregs. Overall, many reports have shown production of anti-inflammatory or immune-deviating cytokines of Tregs in response to Hsp, allowing Hsp-specific Tregs to suppress target cells in a cell contact-independent manner.

2. Metabolic disruption

Tregs express high quantities of the IL-2 alpha chain CD25 [78], because these cells, like other cells, use IL-2 for survival and differentiation. It has been shown that Tregs consume so much IL-2 that they are considered as IL-2 sink [79], depriving other cells from this growth factor and thereby disrupting their survival [80]. CD25 expression has been identified on Hsp-responsive Tregs in numerous studies [6, 33, 46, 61, 62, 64, 65, 75, 81-84], and therefore depriving other cells from IL-2 might be a mechanism by which Hsp-specific Tregs control their environment. In **chapter 3** and **chapter 4** we illustrate that CD25 expression is essential for *in vivo* suppression of B29-specific Tregs after adoptive transfer, since $CD4^{+}CD25^{-}$ Hsp-specific cells do not suppress experimental arthritis.

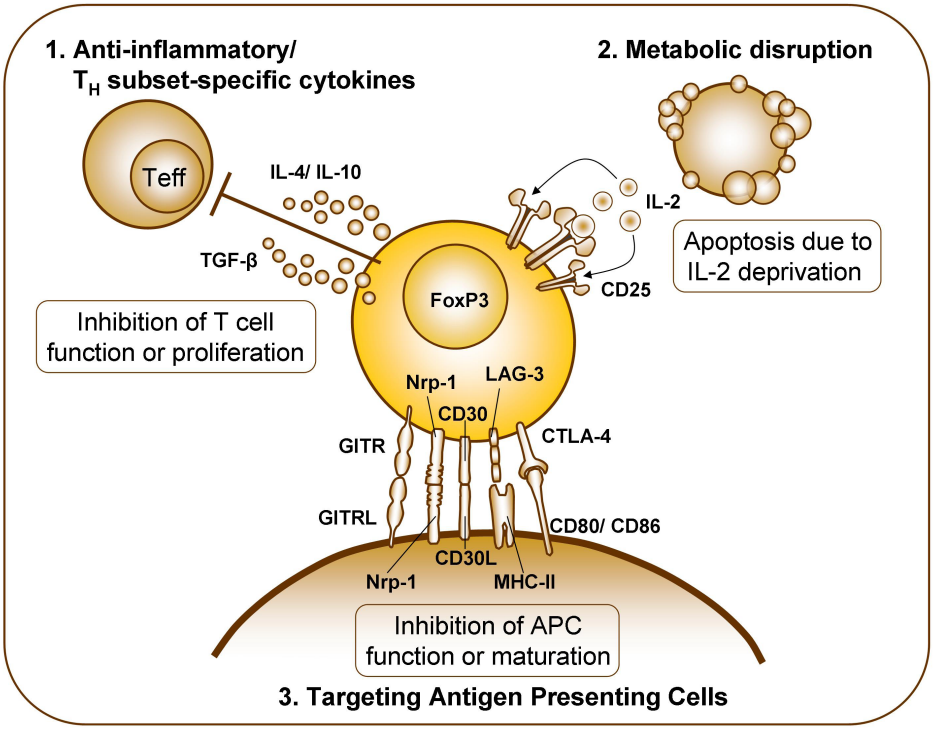

Figure 3. Treg associated markers identified on Hsp-specific Tregs. Hsp-specific Tregs can secrete anti-inflammatory cytokines like IL-10 and TGF-β, as well as IL_4 that can deviate immune responses by inhibiting effector T cell (Teff) function or proliferation. Additionally, Hsp-specific Tregs express CD25 that binds IL-2 and can thereby disrupt the metabolism of other cells by depriving them from this survival factor which can result in apoptosis. Finally, antigen presenting cells (APC) can be inhibited in function or maturation by being targeted by Hsp-specific Tregs via cell surface markers..

For this figure, molecules were included if they identified on Hsp-specific CD4+CD25+(FoxP3+) cells. Or, of they were required by Hsp-specific CD4+CD25+(FoxP3+) Tregs to be suppressive. Or, if the molecule was essential for Hsp-mediated suppression as tested in k.o. mice or by blocking antibodies.

3. Targeting of antigen presenting cells (APC)

One other mechanism by which Tregs can exert immune regulation is by suppressing APC activation and function. Several surface markers have been identified on Hsp-specific Tregs that are known to inhibit APC function and act as co-stimulatory molecules. Glucocorticoid induced TNFR (GITR) and CD30 play a role in cell activation, survival or apoptosis and interact with their ligands GITRL and CD30L [85-88]. CD30 can be used to detect suppressive Hsp-specific Tregs [63] and blocking GITR inhibited Hsp-specific suppression of Tregs [89]. Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a co-stimulatory molecule with a high affinity for CD80 and CD86 on APC and is expressed on Tregs [90]. It negatively regulates APC function and has been identified on suppressive T cells in response to Hsp. Neuropilin-1 (Nrp1) which plays a role in cell migration and survival is considered a Tregs marker [91] and was identified on Hsp-responsive T cells [6]. Lymphocyte activation gene-3 (LAG-3) is a molecule homologous to CD4 and binds MHC-class II molecules and is upregulated after activation [92, 93]. Ligation to MHC class II impairs APC maturation [68]. In **chapter 3** LAG-3 has been identified on Hsp-specific Tregs and isolated LAG-3$^+$ Hsp-specific Tregs were able to suppress experimental arthritis in very low numbers [6]. All these cell surface molecules allow Hsp-specific Tregs to interact with APC and to downregulate their function, explaining how activated Hsp-specific Tregs suppress inflammation. In conclusion, Hsp specific Tregs can use an array of suppressive molecules to inhibit other immune cells. Using Hsp-specific T cells from the mB29b-TCR transgenic mouse described in **chapter 6**, will allow us to further investigate the role of Hsp-specific Tregs under conditions of stress, inflammation or after adoptive transfer of these antigen-specific cells.

Are Hsp-specific Tregs induced or natural Tregs?

The Treg population can be divided into two subsets: natural Tregs (nTregs), that are formed in the thymus as a mature and differentiated subset of Th cells, and induced Tregs (iTregs) that differentiated from naïve T cells after exposure to antigen in combination with suitable growth factors (for instance IL-2 and TGF-β) [94-97]. There a currently no subset specific markers that can be used to distinguish nTregs from iTregs *ex vivo*. However, several studies have addressed the function of both subsets after isolation of nTregs from thymus or induction of iTregs *in vitro* and *in vivo* (for instance, *in vivo* induction of Hsp-specific Tregs is described in **chapter 4**) [98, 99]. In general it is believed that nTregs and iTregs share mechanisms of suppression and that these subsets compliment each other because their TCR specificity is non-overlapping, with nTregs being specific for self-antigens and iTregs being specific for foreign antigens [95, 97, 98, 100]. Considering their suppressive capacity, iTregs induced *in vitro* are considered less potent suppressors of inflammation than iTregs induced *in vivo*, which could have to do with the stability of FoxP3 expression, which regulates Treg function [101-103]. The oral or intranasal administration, as well as immunization, of Hsp is therefore a suitable approach to induce Hsp-specific Tregs.

In general it is believed that iTregs are more suppressive than nTregs, but this can be due to the fact that it is far easier to induce antigen-specific iTregs as a pure population of cells, whereas nTregs have already been differentiated and are isolated as a polyclonal population from the thymus. By using TCR transgenic mice it is possible to obtain antigen-specific nTregs as was done by Haribhai et al. [98]. In this study, efficient

suppression of chronic inflammation was achieved by iTregs and not nTregs. Therefore it will be interesting to test the suppressive capacity of mB29b-specific nTregs isolated from the mB29b-TCR transgenic mouse generated in **chapter 5**.
It has not been established yet to which subset Hsp-specific Tregs belong. In theory this could be both, because if nTregs are more often self antigen-specific these should be able to recognize self Hsp, while iTregs are more often directed against foreign antigens and would therefore be specific against foreign Hsp. Thus, Hsp-specific Tregs are likely to be both nTregs or iTregs. Hsp administration can therefore expand already existing Hsp-specific nTregs, or induce Tregs from Hsp-specific naïve T cells, allowing both subsets to intervene in inflammation.

Hsp-Therapy: The induction or expansion of Hsp-specific Tregs

The administration of Hsp-derived peptides has shown great therapeutic potential in animal studies and in clinical trials [58, 61, 65, 104, 105]. The mechanism of suppression by Hsp therapy is thought to come from the activation and induction of Hsp-specific Tregs [3], which has been confirmed by adoptive transfer studies using Hsp-specific Tregs as described in **chapter 3** and **chapter 4** [6, 71] Currently, the transfer of Tregs to patients with autoimmune diseases is heavily studied and several clinical trials are being conducted to investigate the safety and efficacy of Treg transfers in the clinic. In line with this development, we would like to suggest several strategies for the induction or expansion of Hsp-specific Tregs for the treatment of autoimmune diseases. Strategies that target Hsp-specific Tregs can be divided in those that occur *in vivo*, after the oral or intranasal administration or immunization of Hsp (encapsulated in particles, or in combination with anti-inflammatory drugs), or to those that expand/induce antigen-specific Tregs ex vivo, in which the Hsp-specific Tregs are given back to the patient at a later time point. The approaches to utilize Hsp-specific Tregs for clinical therapy are summarized in figure 4.

In vivo induction or expansion Hsp-specific Tregs
Administering Hsps to expand/induce Tregs *in vivo* might be an achievable strategy, especially since various routes of administration have already been tested in animal models or in clinical trials. One approach could be the intranasal administration of Hsps. The idea behind this technique is that Tregs are abundantly present in mucosal areas and can be induced locally when they encounter antigens [106]. Previous studies have shown that intranasal Hsp or Hsp-derived peptides can suppress experimental arthritis as was shown in **chapter 3**, as well as in other studies [6, 52, 107-109]. In order to enhance the delivery of Hsp peptides, these can be put in nanoparticles to enhance delivery and allow for the reduction of antigen needed to suppress inflammation [110]. Besides intranasal delivery, Hsp antigens can be given orally, which has been shown to reduce inflammation in patients with RA due to induced Tregs [60, 111]. One interesting approach was the oral administration of a recombinant *Lactococcus lactis* strain producing Hsp, inducing Tregs that suppressed experimental multiple sclerosis [112]. Immunization has been widely used in the experimental setting to suppress inflammation in animals models for autoimmunity [5, 21, 51, 58, 73, 113]. These studies show that the activation and induction of Hsp-specific Tregs *in vivo* is a powerful method to dampen inflammation. Further studying the effects of Hsp administration on

Hsp-specific T cells should be considered by using TCR-transgenic T cells cross-reactive for self and foreign Hsp70 as described in **chapter 5**.

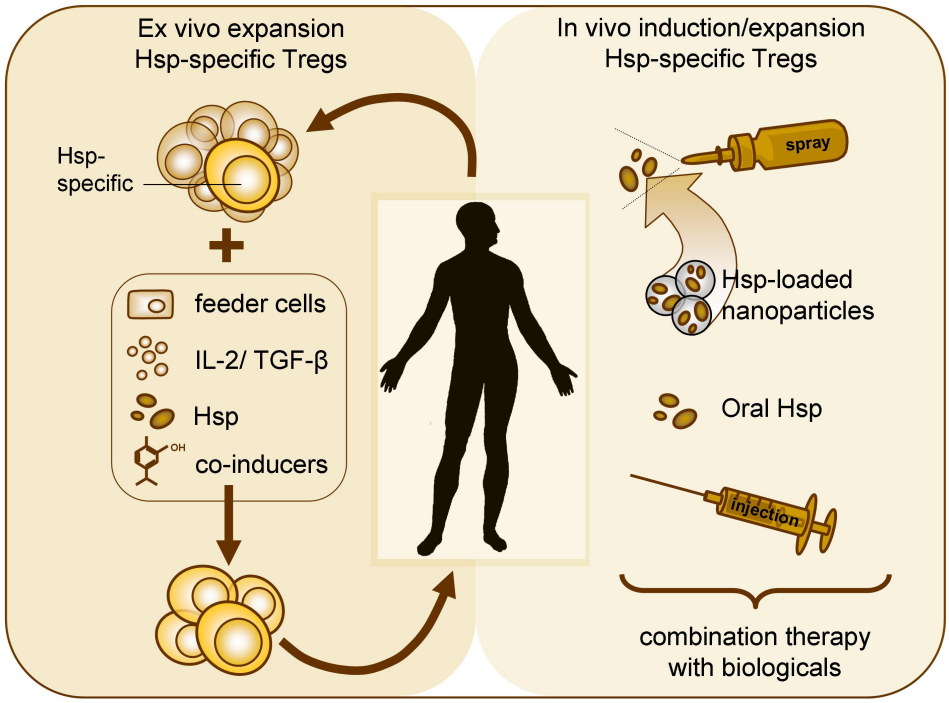

Figure 4. Proposed strategies for the induction or expansion of Hsp-specific Tregs for therapy. Hsp-specific Tregs can be induced or expanded in vivo by administration of Hsp intranasally (possibly encapsulated in nanoparticles), orally, or via immunization. Combining Hsp therapy with biologicals that neutralize inflammation is expected to enhance efficacy of Hsp therapy (right panel). Ex vivo expansion and reinfusion of Hsp-specific Tregs is another proposed strategy to suppress inflammation. For this, isolated Hsp-specific T cells are expanded and induced via the presentation of Hsp-derived peptides by feeder cells. Cell survival and differentiation is supported by the addition of IL-2 and TGF-β. Additionally, endogenous Hsp levels of feeder cells can be increased by culturing cells in the presence of co-inducers like carvacrol (left panel).

To further enhance the efficacy of Hsp administration, we propose to give Hsp in combination with anti-inflammatory drugs (like anti-TNFα therapy) so that their suppressive capacity will be enhanced [114-116]. Studies have shown that Treg suppression can be inhibited under inflammatory conditions, such is the case in RA. Therefore, therapy with anti-inflammatory drugs in combination with Hsp administration could be considered. In this case, short-term general immuno suppression will give a window of opportunity in which peptide therapy can be administered in order to allow the expansion/induction of antigen-specific Tregs. Several biologicals that specifically target mediators of inflammation are now in the clinic, or are in clinical trials [117], with anti-TNFα being a potent inhibitory antibody that neutralizes the pro-inflammatory cytokine TNFα thereby suppressing RA [115, 116]. Also, the effect on TNFα on Tregs

has been studied in detail [114, 115], therefore we will discuss anti-TNFα antibodies as potential candidate for combination therapy together with Hsps. One study has already shown promising data in which arthritic mice were given low-dose of anti-TNFα in combination with intranasal administration of [118].

Ex vivo expansion and transfer of Hsp-specific Tregs

Adoptive transfer therapies to suppress autoimmunity in animal models, even with Hsp-reactive T cells, was already used many years ago [5, 113]. However, its was not until recently that transferring Tregs into patients is considered a feasible therapy [119, 120]. The current strategy for Treg transfers involves the isolation of polyclonal Tregs followed by activation protocols to expand these cells in order to obtain sufficient numbers of cells to suppress inflammation [121]. Instead of using polyclonal Tregs, we would consider the use of antigen-specific Tregs, since it has been demonstrated that these are cells are more potent suppressors than activated polyclonal Tregs in animal models for autoimmune diseases [122-124]. Hsps are very suitable antigens, (as was already argued in **chapter 1**), because in the first place these proteins are ubiquitously expressed, but secondly these proteins are upregulated during inflammation. When using self Hsp-specific Tregs for adoptive transfer therapy, these cells will be activated *in vivo* and suppress inflammation in a comparable manner as we have observed in our mouse model for experimental arthritis described in **chapter 3** and **chapter 4**. By using Hsp as antigens, we can now suppress autoimmune diseases, for which the disease inducing antigens are unknown.

In order to obtain sufficient numbers of Hsp-specific Tregs, these cells will need to be expanded in vitro. Either naïve $CD4^{+}CD25^{-}$ cells could be isolated from peripheral blood in order to induce Tregs, or $CD4^{+}CD25^{high}CD127^{low}$ Treg could be used and further expanded (Fig 4). Conditions for *in vitro* expansion of Tregs should favor the induction, survival and expansion of Hsp-specific Tregs. First, feeder cells (APC) are needed to present Hsp-derived peptides in MHC class II molecules in order to activate Hsp-specific $CD4^{+}$ T cells. Secondly, survival factors like IL-2 are indispensible for the survival and expansion of Tregs [125, 126], while TGF-b is important for the induction of Tregs [127-129]. Alternatively, feeders cells could be heat shocked and cultured with co-inducers, like carvacrol [24], in order to boost endogenous Hsp expression [25], allowing additional loading of self-Hsp into MHC class II molecules. In fact, it has already been shown that T cells can respond to upregulated Hsp from heat-stressed APC [5, 24, 130].

One sufficient numbers of Hsp-specific Tregs are generated these need to be assessed for the expression of Treg associated markers like CD25 and FoxP3. Additionally, the cells can be functionally tested for their *in vitro* suppressive activity. This could be done by using the suppression assays used in **chapter 3** and **chapter 4**. The antigen specificity of the cells can be determined by restimulation with Hsp peptide after which the cells are analyzed for the expression of activation markers Ki-67, Nrp-1 and IL-10 by flow cytometry as described in **chapter 3**. The question remains how many cells are required to sustain inflammation upon transfer. By using antigen-specific Tregs we believe that far less Tregs are needed for reinfusion. We have shown in **chapter 3** that it is possible to suppress established disease in mice with Hsp-specific Tregs. And also by selecting on LAG-3, as little as 4.000 cells were required for suppression of experimental arthritis in a mouse, indicating that suppressing autoimmunity by Treg transfer is feasible.

After adoptive transfer, Hsp-specific Tregs need to remain stable in phenotype in order to combat inflammation. Therefore, it is much better to use antigen-specific Tregs with

specificity for target antigens found at the site of inflammation. It is not certain that the polyclonal Tregs will survive for so long. As a matter of fact, in **chapter 3** we show that B29-specific Tregs express CD25 and FoxP3 up to 50 days after transfer. Also, the transferred cells live longer and have a more activated phenotype after adoptive transfer compared to transferred naïve Tregs or irrelevant-antigen-specific Tregs (pOVA) did. There is a concern that general immuno suppression can lead to opportunistic infections while treating autoimmune diseases. However, by using antigen-specific Tregs activated by Hsp, inflammated tissues can be specifically targeted due to locally upregulated Hsp expression. Rezende et al. have shown for instance that oral administration of Hsp65 produced by *Lactocuccus lactis* induced tolerance to experimental multiple sclerosis (MS), but did not decrease immune responses against *salmonella typhimurium* [112]. This indicates that Hsp-specific tolerance should not be considered as general immuno suppression, but rather as a tailor-made regulation of chronic inflammation due to the inducible properties of Hsp.

In conclusion, we believe that it will be possible to induce and expand Hsp-specific Tregs either *in vivo* by administrating Hsp via a suitable route, or *ex vivo* by expansion cultures followed by reinfusion back into patients. We will have to await the first results from the clinical trials performed with polyclonal Tregs, but when these are successful the next step will be to implement antigen-specific Tregs. In this regard, the utilization of Hsp-specific Tregs has great potential given the results obtained from mouse studies, and given the inducible properties of Hsp that allow for local expression in inflamed tissues.

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List of abbreviations

AA	adjuvant arthritis
AD	atopic dermatitis
APC	antigen presenting cell
CIA	collagen-induced arthritis
CPM	counts per minute
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DMARDs	disease-modifying anti-rheumatic drugs
DTH	delayed-type hypersensitivity reaction
EAE	Experimental Autoimmune Encephalomyelitis
FACS	Fluorescence-activated cell sorting
FoxP3	forkhead box P3
GITR	glucocorticoid-induced TNFR family related gene
Hsp	Heat shock protein
HS APC	heat-shocked antigen presenting cells
IFN-γ	interferon gamma
IL	interleukin
iTreg	induced Treg
JIA	juvenile idiopathic arthritis (human)
LAG-3	Lymphocyte-activation gene 3
LOX-1	lectin-like oxidised low-density lipoprotein receptor-1
MHC	major histocompatibility complex
MS	multiple sclerosis
Nrp-1	Neuropilin-1
nTreg	natural Treg
PGIA	proteoglycan-induced arthritis (mouse)
RA	rheumatoid arthritis (human)
SEM	standard error of mean
TCR	T cell receptor
Teff	effector T cell
TGF-β	transforming growth factor beta
Th cell	T helper cell
TLR	Toll-like receptor
TNFα	tumor necrosis factor alpha
Treg	regulatory T cell

Nederlandse samenvatting

Het immuunsysteem

Het immuunsysteem bestaat uit een verzameling van gespecialiseerde witte bloedcellen die samen een verdediging vormen tegen indringers zoals virussen, bacteriën en schimmels. Maar ook tegen ongewenste eigen cellen, zoals tumoren. Voor een goede verdediging tegen ongewenste indringers communiceren de cellen van het immuunsysteem met elkaar om zo tot een goede gemeenschappelijke respons te komen.

Eén groep cellen uit het immuunsysteem bestaat uit T cellen. Deze cellen worden gevormd in de thymus, een klier waarin voorloper cellen uitrijpen tot T cellen. Er bestaan verschillende typen T cellen die allen een specifieke functie beoefenen tijdens immuunresponsen.

Onderdrukking van ongewenste immuunresponsen

Alhoewel een sterke immuunrespons tegen indringers gewenst is om pathogenen succesvol te bestrijden, vindt er tijdens een respons schade plaats aan de gastheer. Dus naast het bestrijden van indringers is het ook van belang schade te minimaliseren. Daarnaast zijn er situaties waarin het immuunsysteem geactiveerd wordt, terwijl er geen direct gevaar bestaat voor besmetting. Je kunt hierbij denken aan allergische reacties zoals hooikoorts of auto-immuunziekten zoals reuma. Bij reuma vind er een immuun respons plaats in de gewrichten van de patiënt. Dit zorgt voor veel pijn en schade aan de gewrichten, wat zelfs kan leiden tot vergroeiingen of afbraak van kraakbeen.

In het geval van een immuun respons is het dus nodig dat deze goed wordt gecontroleerd en niet uit de hand loopt. Daarom bezit het immuunsysteem ook T cellen die immuun responsen reguleren. Deze T cellen worden regulatoire T cellen genoemd en kunnen adequaat andere cellen onderdrukken om zodoende de immuun respons in goede banen te leiden en schade te minimaliseren.

In het geval van reuma zijn er enkele aanwijzingen dat de regulatoire T cellen van de patiënt soms niet goed functioneren. Er zijn ook aanwijzingen dat de andere cellen van het immuunsysteem zodanig geactiveerd zijn, dat deze cellen niet willen luisteren naar de onderdrukking door de regulatoire T cellen. Als het gaat om therapie voor reuma is het daarom zinvol om er voor te zorgen dat enerzijds de regulatoire T cellen beter gaan werken en anderzijds dat immuun cellen tot rust worden gebracht zodat ze beter te onderdrukken zijn door de regulatoire T cellen. Ondanks dat huidige therapieën voor veel reuma patiënten goed werken, is niet iedereen gebaat bij de medicijnen of hebben patiënten soms last van bijwerkingen. Vandaar dat nieuw onderzoek nodig is om therapieën te verbeteren. In dit geval hebben wij onderzocht of het mogelijk is om de regulatoire T cellen sterk te activeren, zodat ze ongewenste immuunreacties kunnen onderdrukken.

Regulatoire T cellen geactiveerd door Heat Shock Eiwitten

Omdat regulatoire T cellen in staat zijn om ontstekingsreacties te onderdrukken, zijn deze cellen bijzonder interessante doelwitten voor therapieën bij ziektes zoals reuma. Het is echter nodig om deze cellen op de juiste manier te activeren door middel van peptiden. De keuze van het peptide is hierbij van groot belang. Peptiden van heat shock eiwitten zijn hiervoor uitermate geschikt, omdat deze peptiden goed in staat zijn om immuun cellen te activeren (waaronder regulatoire T cellen) en van nature worden aangemaakt op de plaats van ontsteking. Hierdoor kunnen regulatoire T cellen lokaal worden geactiveerd, waarna de andere immuun cellen die verantwoordelijk zijn voor de ontsteking worden uitgeschakeld.

Het was al eerder bekend dat door toediening van Heat shock eiwitten reuma bij proefdieren zoals ratten en muizen kon worden onderdrukt. Tevens is aangetoond dat dieren na behandeling meer regulatoire T cellen hadden dan voor de behandeling. In het onderzoek beschreven in dit proefschrift hebben we verder gekeken naar de mogelijkheden van regulatoire T cel activatie door middel van Heat Shock Eiwitten.

Dit proefschrift

In dit proefschrift wordt beschreven hoe inflammatoire immuun responsen in auto-imuunziekten zoals rheumatische arthrtitis onderdrukt kunnen worden door regulatoire T cellen met specificiteit voor heat shock eiwitten (Hsp) te activeren. Het doel van het onderzoek was het gebruiken van peptiden van heat shock eiwitten om chronische onstekingsreacties te remmen en om uit te zoeken hoe regulatoire T cellen werken na activatie met de peptiden.

In het review beschreven in **hoofdstuk 2** wordt een overzicht gegeven van het onderzoek dat eerder is gepubliceerd over immuun regulatie door regulatoire T cellen en heat shock eiwitten. In dit hoofdstuk gaan we verder in op de werking van regulatoire T cel activatie via Heat Shock Eiwitten.

In **hoofdstuk 3** beschrijven we de identificatie van Hsp70 peptide B29 en zijn homologen, die na nasale toediening chronische inflammatie onderdrukten in muizen met artritis. In dit hoofdstuk zijn we verder ingegaan op het mechanisme hoe Heat Shock Eiwitten antigen-specifieke regulatoire T cellen activeren. Immunizatie van muizen met het B29 peptide activeerde CD4+CD25+ regulatoire T cellen die we geïsoleerd hebben en vervolgens ingespoten hebben bij muizen met rheumatische arthritis. De ingespoten cellen waren in staat om ziekte bij muizen te onderdrukken. We hebben eerst aangetoond dat deze cellen langdurig in leven blijven en ook hebben we gevonden dat de directe aanwezigheid van de Hsp-geactiveerde regulatoire T cellen nodig is om de ziekte te blijven onderdrukken. Daarnaast hebben we de isolatie van de regulatoire T cellen uit donor muizen geoptimaliseerd door de cellen te selecteren op de expressie van LAG-3, waardoor we nog minder cellen nodig hadden om artritis te onderdrukken in ontvanger dieren.

In **hoofdstuk 4** gingen we dieper in op de biologie van Hsp-specifieke regulatoire T cellen. Regulatoire T cellen kunnen verdeeld worden in twee typen: natuurlijke regulatoire T cellen en geïnduceerde regulatoire T cellen. Ieder type heeft zijn eigen inbreng in de immuun regulatie. Het is daarom belangrijk te weten welk type regulatoire T cel door Heat Shock Eiwitten wordt geactiveerd. We hebben in dit hoofdstuk aangetoond dat het mogelijk is om regulatoire T cellen te induceren na immunizatie met Hsp peptide B29. Deze geïnduceerde regulatoire T cellen waren suppressief zowel *in vitro*, als *in vivo*.

In **hoofdstuk 5** is een nieuwe T cell receptor (TCR) transgene muis beschreven. Om antigeen-specifieke immuunresponsen van T cellen te bestuderen hebben we in hoofdstuk 3 en 4 gebruik gemaakt van T cellen uit geïmmuniseerde dieren. Deze cellen zijn echter geactiveerd door de immunizatie met het Hsp peptide. Om naive antigeen-specifieke cellen te bestuderen hebben we een TCR transgene muis gegenereerd. De CD4+ T cellen van deze muis zijn reactief tegen Hsp peptiden

B29 en mB29b. In de toekomst zullen de kenmerken van deze cellen verder worden onderzocht. Daarnaast zullen we de functie van de regulatoire T cellen van deze muis verder onderzoeken.

Conclusie

Het onderzoek beschreven in dit proefschrift laat zien dat het gebruik van peptiden van Heat Shock Eiwitten regulatoire T cellen activeert, die in staat zijn om chronische ontstekingsreacties te onderdrukken. We hebben aangetoond dat toediening van peptiden van Heat Shock Eiwitten artritis in muizen kan onderdrukken en we hebben laten zien dat het inspuiten van peptide-specifieke regulatoire T cellen als cellulaire therapie kan worden gebruikt. Verder zijn we ingegaan op de functie van de ingespoten regulatoire T cellen. Zo hebben we aangetoond dat de cellen na injectie zich verspreiden in de ontvangermuis en lang blijven leven. Tevens hebben we aangetoond dat de cellen CD25 en FoxP3 nodig hebben om onderdrukkend te zijn. Tot slot hebben we een transgene muis gemaakt die ons in staat stelt om Hsp-specifieke cellen verder te bestuderen.

Uiteindelijk willen we Heat Shock Eiwitten en peptide-specifieke regulatoire T cellen gebruiken om reuma in mensen te kunnen behandelen, echter er moet nog veel onderzoek gedaan worden voordat dat zover is.

Dankwoord

Zo, de experimenten waren gedaan en alleen het proefschrift moest nog geschreven worden… En 1,5 jaar later is het dan eindelijk zo ver! Het schrijven van dit proefschrift is ongetwijfeld de grootste uitdaging geweest voor mij als promovendus. Het viel niet mee om mijn innerlijke strijd te overwinnen, maar toch ben ik ongelofelijk blij met het uiteindelijke resultaat, want uiteindelijk heb ik met veel plezier aan mijn proefschrift gewerkt.

In het lab staan en experimenten doen, dat wilde ik al toen ik klein was (ik had zelfs mijn eigen microscoop). Maar ook in het schrijven van artikelen heb ik veel plezier gekregen, net zoals het bezoeken van congressen en het bespreken van (soms spannende, vaker nog mislukte) experimenten. Promoveren en het maken van een proefschrift is zeker geen one-man show. Ik heb een grote groep mensen te bedanken, zonder wie dit proefschrift niet tot stand zou zijn gekomen.

Ten eerste, Willem “kunnen-we-niet-iets-met?” van Eden, mijn promotor. Altijd opportunistisch en overal de kansen in zien. Ik heb veel geleerd van je manier van mensen benaderen. Nee heb je, ja kun je krijgen. Daarnaast geef jij onderzoekers de vrijheid om onderzoeker te zijn en in vrijheid te werken. Niets was te gek en ik heb veel mogelijkheden gekregen om het onderzoek te presenteren in binnen en buitenland (als beginnende AIO meteen naar een Tolerage meeting in Seefeld blijft voor mij een mooie herinnering). Bedankt voor de fijne samenwerking en de begeleiding die ik heb gekregen.

Ook ben ik dank verschuldigd aan mijn co-promotor Ruurd van der Zee. Bedankt voor de (soms hoog oplopende) discussies, waar ik veel van heb geleerd. Het bespreken van data tijdens de dinsdag ochtend werkbespreking (waarom heb je die controle niet meegenomen?) heb ik altijd kunnen waarderen. Net zoals de nagezonden artikelen (schijnbaar nodig om je punt te maken…). In de laatste maanden van het project is mij duidelijk geworden dat je geweldig goed kunt schrijven (en zeer snel reviewd), dit was een goede impuls voor het werk!

In het bijzonder wil ik Femke Broere bedanken, mijn andere co-promotor en dagelijkse begeleidster. Laat ik maar met de deur in huis vallen: zonder jou, Femke, had ik niet kunnen promoveren. Het mag geen geheim zijn dat promoveren met veel valkuilen komt (valkuilen uit de omgeving en die van de promovendus zelf). Jij hebt mij behoedt voor veel valkuilen, of me juist in valkuilen laten vallen om daarvan te leren. Mijn beste herinneringen zijn die van onze wekelijkse werkbesprekingen waarna ik altijd met een plan naar buiten liep en soms zelfs dacht dat ik iets briljants had bedacht terwijl jij toch echt dat zaadje had geplant. Daarnaast heb je me in het begin veel geholpen met het muizenwerk, waardoor ik uiteindelijk alle technieken zelf beheerste (intraveneuze injecties met kostbare cellen na 14 uur isoleren kan ik nu gewoon!). Bedankt voor je

betrokkenheid en deskundigheid, ik denk dat jij soms meer stress van het proefschrift hebt gehad dan ik ☺.

Naast mijn promotor en co-promotoren wil ik de analisten bedanken. Ik herinner me de legendarische woorden van Willem tijdens mijn eerste functioneringsgesprek: "iedereen kan promoveren, desnoods zetten we er een paar analisten op om het boekje bij elkaar te pipetteren". Dit is voor een gedeelte zeker van toepassing geweest. Zonder de hulp van Alice, Josée, Peter en Aad was het eindresultaat niet zo mooi geweest als het nu is. Daarnaast kon ik het onbehouwen advies om vage experimenten gewoon niet te doen (want ze vergeten dat toch weer) erg waarderen. Als promovendus heb je niet veel aan alleen een intellectuele begeleiding. Je moet daarnaast ook praktisch wordt geïnstrueerd en worden bijgestaan door de mensen die al jaren op het lab staan. Naast de analisten wil ik ook Ger en Ineke bedanken. Het isoleren van CD4+(FITC)CD25+(APC) cellen was misschien geen rocket-science, maar het moest wel goed gebeuren en bij jou was dat in goede handen Ger. En die antigen-specific suppression assay die er zo goed uit leek te zien, maar die veranderde in een "depression-assay", daar heb je toch veel werk aan gehad Ineke. Ook wil ik de medewerkers van het GDL bedanken voor de zorg voor de muizen, want die kamers stonden soms best vol met kooien.

Dan zijn er ook nog andere promovendi binnen de afdeling geweest die een aanzienlijke bijdrage hebben geleverd aan dit boekje. Graag zou ik mijn mede-AIO's willen bedanken voor de hulp met experimenten en discussies (of het zeiken over onze begeleiders, want zeiken is sommigen van ons wel toevertrouwd, ik zal geen namen noemen…). In het bijzonder zou ik Rachel, Chantal en Bram willen bedanken, omdat zij mij geholpen hebben met mijn (soms idioot grote) experimenten. Zonder jullie hulp had het allemaal met minder muizen, samples, of controles gemoeten, en daar hou ik gewoon niet van… Lotte, bedankt voor al het voorwerk dat je verricht had, hierdoor hebben we uiteindelijk een heel mooi verhaal kunnen schrijven onder andere in PNAS!

Dan is er nog zitkamer W440, waar ik 4 jaar met veel plezier een goede internet connectie had (voor nieuws en roddel sites, youtube, hyves en facebook, voetbal kampioenschappen en de Tour de France), maar waar ik natuurlijk ook gewoon uit het raam kon kijken of met mijn kamergenoten kon kletsen. Daarnaast is het uitwerken van data en kijken naar online filmpjes gewoon leuker met anderen en dan neemt een zitkamer na 4 jaar toch wel een bijzondere plaats in. Het verloop in de wetenschap is groot en er zijn veel mensen voorbij gekomen in deze kamer gedurende 4 jaar: Janine, Miriam, Janneke, Eveline (bedankt voor de InDesign template), Marij (mede-organisator van de leukste PhD get-together ooit), Natascha (Kabouter Wesley ☺), Daphne, Cornelis, Reina, Andrea. Ik wil jullie allemaal bedanken voor de gezelligheid en voor de steun die ik heb gekregen bij mijn werk.

Daarnaast wil ik de rest van de afdeling Immunologie bedanken voor de gezelligheid bij de lunches en uitjes en voor de goede vragen en fijne discussies tijdens werkbesprekingen.

My dear Tolerage colleagues, many thanks for the nice meetings with helpful discussions and pleasant get togethers. I will never forget the one time I tried to order a cappuccino in Paris… and was turned down…

Ik wil ook graag de studenten bedanken die een bijdrage hebben geleverd aan mijn onderzoek, zij het met experimenten, of met het doen van literatuurstudies: Ilona, Cindy en Prince. Daarnaast heb ik niet stil gezeten als het gaat om het vertegenwoordigen van mede AIO's. Daarom wil ik ook de leden van PrOUt en de Vet-PhD Council bedanken voor de interessante en gezellige bijeenkomsten.

Voordat ik begon met promoveren heb ik tijdens mijn studie aan de HLO veel geleerd van mijn stages, daarvoor wil ik Gijs, Johan, Jeroen en Renate hartelijk danken. Ook de docenten van de HLO ben ik dankbaar voor het bijbrengen van de nodige pipetteer vaardigheden. Oud-studenten Jean-Paul, Richarda en Greetje, het is altijd leuk om met jullie af te spreken. JP, tegelijk gingen we naar Utrecht om een Master te volgen, nadat we jarenlang de mensen in Etten-Leur hadden verveeld. Als oud-studiegenoot en goede vriend vind ik het een eer dat jij mijn paranimf bent. Ik wil graag ook mijn stagebegeleiders bedanken tijdens mijn Master studie: Liana, Jos, Machteld en Leonie (bedankt voor de Londense lunches).

Veel dank aan mijn familie en vrienden. Jullie hebben waarschijnlijk geen idee in wat voor rat-race je zit als AIO. Maar daarin schuilt juist de kracht van je dierbaren, even uit het wetenschapswereldje zijn en meedoen met de realiteit. Want tijdens het klussen heb je nu eenmaal meer aan schilder skills dan aan pipetteervaardigheden, nietwaar UBN? Christian, bedankt dat je als grote broer achter me staat als paranimf tijdens mijn promotie.

Pa, het is jammer dat je mijn AIO-schap niet hebt meegemaakt. Maar ik koester de herinneringen; altijd was je geïnteresseerd in mijn studie en stages. Waarschijnlijk snapte je er helemaal niets van wat ik deed, maar je enthousiasme was bewonderenswaardig. Vandaar dat ik mijn proefschrift aan jou opdraag. Jij bent de reden dat ik niet zenuwachtig overkom tijdens presentaties, want dan beeld ik me in dat jij tussen de mensen zit, trots te zijn… Moeders en Herman, voor jullie geldt hetzelfde. Altijd vol lof over "de jongens". Vol trots vertellen over mijn studie en werk aan familie en vrienden. Herman, wat is het toch jammer dat je mijn verdediging niet kunt meemaken.

En dan tot slot: Myra! Vlak voor mijn promotietraject heb ik je leren kennen. Ondanks dat ik je probeerde voor te bereiden op het werk van een wetenschapper (soms lange dagen en onregelmatige werktijden, altijd met het vak bezig, zelfs tijdens vakanties), was de praktijk toch de beste leerschool. Ik vind het bijzonder dat jij veel beter aan anderen (in de "echte wereld") kunt uitleggen wat ik onderzoek en hoe dat gaat. Zoals ik in mijn promotie onderzoek groeide als onderzoeker, zo groeien wij ook steeds verder. Wat hebben we al een hoop mee gemaakt, en hopelijk komen er nog veel mooie dingen bij. It's better together!

Curriculum Vitae

Martijn van Herwijnen werd op 3 mei 1983 geboren in 's-Hertogenbosch. In 2000 haalde hij het HAVO diploma aan het Marklandcollege in Oudenbosch. In datzelfde jaar begon hij aan de opleiding Biologie en Medisch Laboratoriumonderzoek in Etten-Leur van de Avans Hogeschool. Zijn stages volgde hij aan de Universiteit Leiden en het Erasmus MC en in 2005 behaalde hij zijn Bachelor diploma. Daarna vervolgde hij zijn studie met de Master Immunity & Infection aan de Universiteit Utrecht. Met wederom twee stages, ditmaal aan de Universiteit Utrecht en King's College in Londen. Hij studeerde af in 2008 en sinds april van datzelfde jaar was hij werkzaam als Assistent in Opleiding (AIO) op de afdeling Infectieziekten en Immunologie aan de Universiteit Utrecht, waar hij onderzoek deed naar regulatoire T cellen in reumatische artritis. Dit onderzoek, onder begeleiding van Dr. Femke Broere, Dr. Ruurd van der Zee en Prof. Dr. Willem van Eden, staat beschreven in dit proefschrift.

List of publications

De novo induced regulatory T cells after Hsp70 peptide immunization are suppressive in experimental arthritis
Martijn J. C. van Herwijnen, Ruurd van der Zee, Willem van Eden, Femke Broere
Submitted

Heat shock proteins can be targets of inflammation controlling regulatory T cells
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you know a little about everything
and by the end
you know a lot about nothing

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