Regulatory T cells in health and disease

Putting the pieces together

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Regulatoire T cellen in gezondheid en ziekte

(met een samenvatting in het Nederlands)

Proefschrift

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The lymphocyte perspective

Again another circle
and still I haven't found it.
They tell you that you know it when..
so there's no doubt about that.
I am not sure however whether
it really is a good thing;
to recognize your antigen,
or rather die from searching.

Mette Bakker, 2010.

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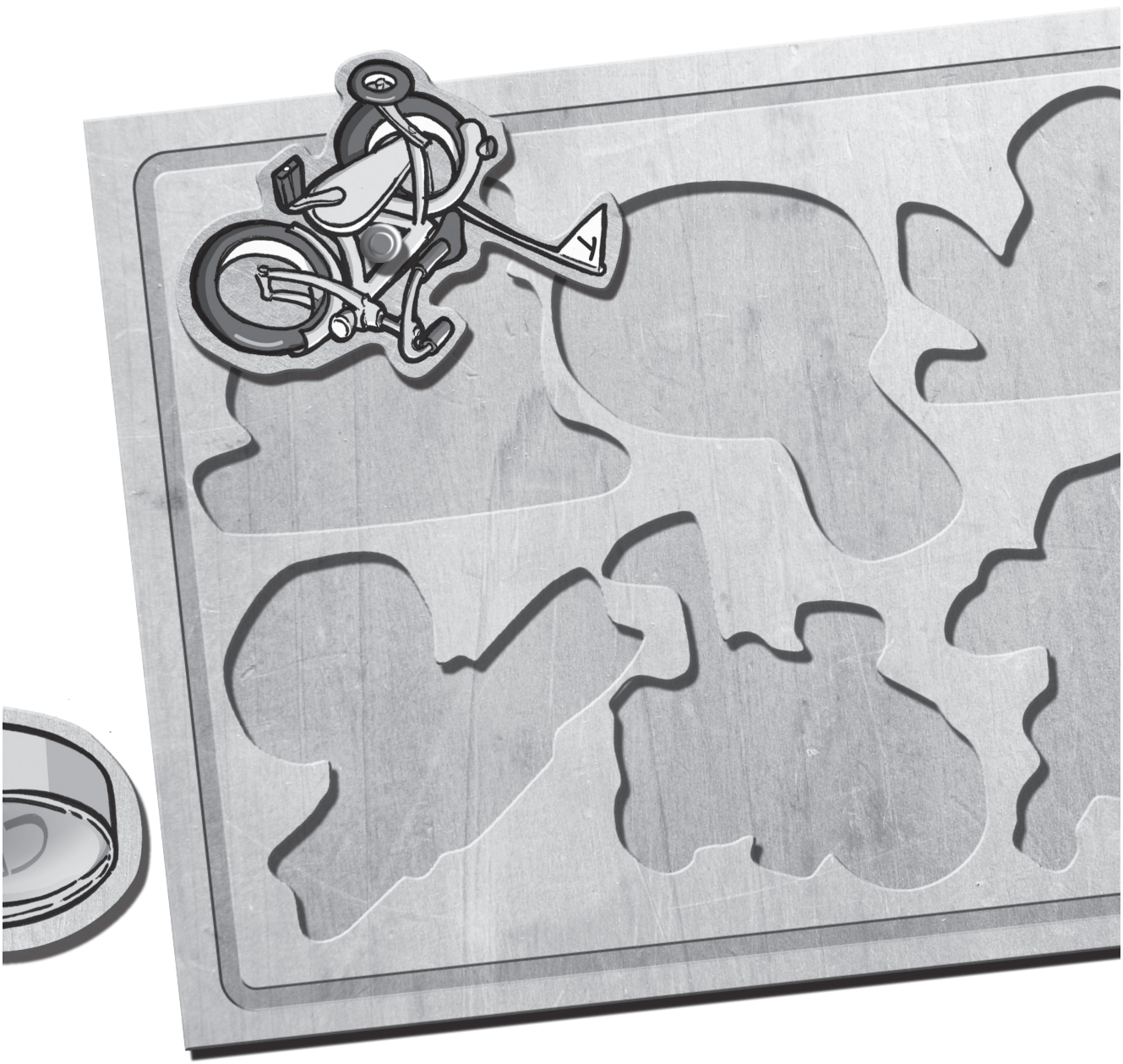

General Introduction

T cells: important players in immune responses

The immune system is the central defense mechanism against invading pathogens, such as viruses, bacteria and parasites. Furthermore, it is crucial in eliminating malignant tumor cells. When pathological microorganisms enter the body through epithelial surfaces or the mucosa, this first invasion is countered by innate immune mechanisms. The innate immune response utilizes several components, for example antimicrobial peptides, the complement system, mucus, and immune cells such as macrophages, neutrophils and NK cells. Later, a second defense mechanism can be applied; the adaptive immune system, employing immune cells displaying a wide range of specific receptors, each recognizing specific peptides. Key players in the adaptive immune response are B cells and T cells. T cells express the T cell receptor (TCR) by which they recognize peptides presented by antigen presenting cells (APC); cells able to process antigens and display them on a major histocompatibility compex (MHC) molecule (in humans called human leukocyte antigen; HLA) (1). The TCR differs between T cells due to random rearrangements of the variable part of the receptor (2). Therefore, T cells can recognize a wide variety of peptide–MHC combinations. T cells can be divided into two major classes, distinguished by expression of the cell-surface proteins CD4 and CD8, both co-receptors of the TCR. $CD8^+$ T cells recognize peptides presented in MHCI and $CD4^+$ T cells recognize peptides presented in MHCII (3). This thesis will focus on $CD4^+$ T cells, recognizing peptides in MHCII molecules, which are mostly presented by dendritic cells (DC), but also by B cells and macrophages.

T cells: many subtypes to create the appropriate immune response

$CD4^+$ T cells are key players in the adaptive immune system. They direct activation of other immune cells, such as B cells, which upon activation produce antibodies, or macrophages that phagocyte bacteria. Various subtypes of $CD4^+$ T cells exist, of which some have only recently been identified **(Figure 1)**. T helper 1 (Th1) cells can produce IFNγ and are able to activate

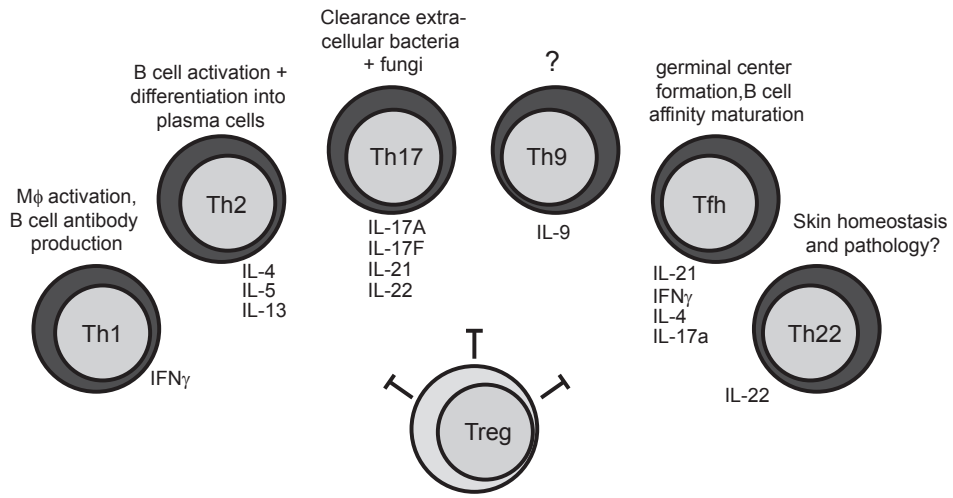

Figure 1. $CD4^+$ T cell subtypes. Established effector T cell lineages are T helper 1 (Th1), Th2 , and Th17 cells. Recently proposed lineages are Th9, Tfh (T follicular helper cells), and Th22. Depicted are the cytokines produced by each lineage, and their immunological function(s). Regulatory T cells (Treg) are the master regulators of all helper T cells, since they can suppress effector T cell activity. Mϕ = macrophage, ?=under discussion.

macrophages. Furthermore, they promote production of antibodies by B cells. Second, Th2 cells produce high levels of IL-4, IL-5 and IL-13 and are involved in initial B cell activation and differentiation into antibody-secreting plasma cells (4). The recently discovered Th17 cells, produce IL-17A, IL-17F (5, 6), IL-21 and IL-22 (7), and promote clearing of extra-cellular bacteria and fungi. Finally, Th9 (producing IL-9, function still under discussion) (8, 9), T follicular helper cells (Tfh, producing IL-21, and sometimes IL-4, IFNγ and IL-17, and promote formation of germinal centers and immunoglobulin class switching and affinity maturation in B cells (10), and Th22 (producing IL-22, memory T cells expressing skin-homing receptors CCR10 and CCR4 (11, 12), and involved in skin pathogenesis in a murine model (13)) were recently identified (8-10), and more subtypes may follow in the future. However, whether these cells are actually separate lineages of helper T cells remains unknown, since their cytokine profiles are not unique to these T helper cell lineages (8-10). Thus, each T helper cell subtype specifically contributes to the host defense system and together they provide protection from a wide range of pathogens. T helper cells are from now on addressed as effector T cells. Effector T cell subsets can regulate each other in order to drive immune responses towards one particular effector T cell type response; for instance, IL-4 and IFNγ inhibit production of IL-17 (5, 14). However, one important subset of $CD4^+$ T cells regulates all effector T cells: the regulatory T cells (Treg). Treg can suppress effector T cell action, independent of T helper subtype, and thereby direct inflammatory responses.

Regulatory T cells: key regulators of immune balance

Due to strict selection processes in the thymus (central tolerance) most T cells recognize foreign antigens, so they can recognize and elicit a response towards invading pathogens. Occasionally, some effector T cells recognizing self-antigens 'escape' from the thymus. To prevent unwanted responses of these effector T cells towards self-antigens, tolerance has to be maintained in the periphery. One important mechanism of peripheral tolerance is provided by regulatory T cells (Treg). Most Treg recognize self antigens (15), however, upon recognition of these antigens in the host tissues, they respond tolerogenic and inhibit effector T cell responses.

In 1970, Gershon and Kondo were the first to demonstrate in a mouse model that T cells (in this case a subset of $CD8^+$ T cells) can actually inhibit responses to foreign antigen (16). However, a role for $CD4^+$ T cells in controlling autoimmunity was only reported many years later. Newborn mice were thymectomized during the first 3 days after birth and were demonstrated to develop severe autoimmunity due to a lack of $CD4^+$ T cells expressing IL-2 receptor α (CD25). When $CD4^+CD25^+$ T cells were infused into these mice they inhibited development of autoimmunity (17). These findings provided the first clue to the presence and immune suppressive capacity of CD25 expressing Treg in autoimmune disease.

Treg not only play an important role in suppressing immune responses targeted against self-tissues, but also prevent responses towards non-infectious antigens encountered on epithelial surfaces; for instance, food in the gut and pollen in the airways. Altogether, regulatory T cells can prevent both autoimmune responses (towards self-tissues, mostly caused by Th1 cells and Th17 cells), and allergic responses (towards non-infectious external antigens, mostly caused by Th2 cells), and thus are considered to be keepers of immune balance.

Subtypes of regulatory T cells

Several subtypes of $CD4^+$ Treg have been described **(Table 1)**. In addition to peripherally induced regulatory T1 (Tr1) cells (18), and T helper 3 (Th3) cells (19), there are the Forkhead-box P3 (FOXP3) expressing Treg. This thesis will focus on this specific population of Treg; expressing FOXP3 and high levels of CD25.

Table 1. Treg subtypes

Name	Origin	Markers	Suppressive mechanism
Tr1	Induced in periphery	High IL-10, low IFNγ	IL-10
Th3	Induced in mucosa (gut)	TGFβ	TGFβ
FOXP3$^+$ Treg	Thymus (nTreg),	FOXP3, CD25, low CD127, HLA-DR, CTLA-4, GITR, CD27	Cell contact, cytokine production
	Induced in periphery (iTreg)		

FOXP3: essential regulator of Treg function

The first clue that FOXP3 was an important T cell regulatory protein came from mice with a spontaneous X-linked mutation, called scurfy mice (20). These mice suffered from scaliness of the skin and spontaneously developed splenomegaly, hepatomegaly, and lymphocyte infiltration in several organs, leading to death within 3 weeks (21). Further experiments showed that transfer of T cells from scurfy mice to immunodeficient mice caused similar disease symptoms, while immunocompetent mice receiving scurfy T cells remained clinically normal (22). Years later the gene responsible for scurfy disease was identified to be Foxp3, in which a 2 basepare insertion led to a truncated Foxp3 protein product (23). Scurfy mice had hyper-responsive effector T cells and no suppressive Treg, and therefore developed severe autoimmune and allergic disease (24). Similar symptoms were observed in a group of patients suffering from IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked (25). IPEX is a fatal disorder marked by both autoimmune symptoms, and allergic responses. In these patients mutations in FOXP3, the human ortholog for murine Foxp3, were identified by sequencing (26, 27). Consistently, in these patients Treg were not functional, while effector T cells were hyperactive (28).
The importance of FOXP3 expression for Treg function was further established by experiments in which ectopic expression of FOXP3 in murine CD4+CD25- T cells was demonstrated to induce a suppressive phenotype, suggesting that FOXP3 expression was sufficient for the development of functional Treg (29, 30). Thus, in both human and mice, FOXP3 is an essential transcription factor for Treg function, and thereby for suppression of autoimmunity and allergy.

Treg in Mice and Men

Many differences have been described between the murine and human immune system (31). However, both FOXP3 deficient mice and humans have similar disease symptoms; in both species there seems a clear correlation between autoimmune disease and a decrease of Treg frequency or function. A majority of research regarding Treg function in vivo has for obvious reasons been performed in transgenic mouse models. Especially the development of FOXP3-GFP reporter mice, enabling identification of cells based on FOXP3 expression, and not only surface marker expression, has shed some new light on the development and function of FOXP3$^+$ T cells (32). However, not all findings may directly be translated from the mouse to the human immune system.
In humans it is impossible to isolate FOXP3$^+$ Treg for functional analysis, since these cells can only be selected based on intracellular staining of T cells. Therefore, surface markers are required to distinguish Treg from effector T cells. The first marker identified on human Treg was CD25 (IL-2 Receptor α) (33), and a combination of CD25$^+$ and CD127^{low} (IL-7 Receptor α) expression is often used to isolate Treg from human peripheral blood. Most of CD25$^+$CD127^{low} T cells in peripheral blood of healthy volunteers express FOXP3 (86.6% average) (34). Additional markers expressed on FOXP3$^+$ Treg include CTLA-4 (Cytotoxic T Lymphocyte Antigen 4) (35), GITR (Glucocorticoid-induced TNFR related protein) (36), HLA-DR (part of MHCII) (37), CD27 (38) and CD62L (39). Even though none of these markers is sufficient for pure selection of FOXP3$^+$ cells, a clear correlation

between FOXP3 and these markers is apparent. However, many of these markers are also upregulated in activated T cells, making it difficult to distinguish human Treg from activated effector T cells.
Until recently, analysis of human Treg function was only performed in vitro, which cannot be directly compared to in vivo murine models (see below). Furthermore, the working mechanism of human Treg remains unclear, and ways to manipulate human Treg in order to increase frequency or function have not been thoroughly investigated.

Treg in autoimmune disease

The relevance of $CD4^+CD25^+FOXP3^+$ Regulatory T cells (Treg) in prevention of autoimmune diseases, such as Rheumatoid Arthritis (RA), Type 1 Diabetes, inflammatory bowel disease (IBD) and multiple sclerosis (MS), has been extensively demonstrated; transfer of Treg into mice diminishes development of these diseases (40-44).
In human autoimmune diseases, Treg frequency and function have been mostly studied in peripheral blood with variable results. For example, for systemic lupus erythematodes (SLE) patients, peripheral blood has been analyzed for expression of Treg markers. In a majority of these studies a decreased level of Treg was found, compared to healthy controls and inversely correlating with disease activity. However, some studies showed unaltered or even increased levels of Treg in SLE patients. It is difficult to compare these studies, since in some cases CD25 is used as Treg marker, and in other cases FOXP3 (reviewed in (45). Similar observations have been made when analyzing patients with Type 1 diabetes and studies remain contradictory.
In patients with arthritis, it is possible to separately analyze Treg from inflammatory sites (the synovial fluid) and Treg from peripheral blood, therefore providing a more accurate representation of Treg frequencies at the site of inflammation, compared to the periphery. In both juvenile idiopathic arthritis (JIA) and RA patients FOXP3 levels in T cells from peripheral blood were normal compared to healthy controls (46, 47). Synovial fluid T cells from JIA patients expressed higher levels of FOXP3, compared to autologous peripheral blood T cells, suggesting that sufficient numbers of Treg were present at the site of inflammation(38, 46). However, patients with a relatively mild disease course had higher levels of FOXP3 mRNA in T cells from both synovial fluid and in peripheral blood, compared to patients suffering from more severe disease, while percentages of $CD25^{high}$ expressing T cells were higher in peripheral blood and equal in synovial fluid (46). This suggests an inverse correlation between the presence of $FOXP3^+$ Treg in the synovial fluid and disease severity.
Additional studies have described suppressive function of Treg from patients with autoimmune disease. Peripheral blood $CD25^{high}$ Treg from MS patients were present in similar numbers to controls, but displayed decreased in vitro suppressive function compared to healthy controls (48). Similarly, $CD25^+CD127^{low}$ Treg from peripheral blood from SLE patients showed less suppression (49), and Treg from peripheral blood from RA patients showed either equal suppression by $CD25^+$ (50) or $CD25^{hi}$ (51) Treg, or decreased suppression by $CD25^{hi}$(52, 53) or $CD25^+CD127^{low}$ (47) Treg, compared to healthy controls (reviewed in (54). Taken together, increasing Treg frequency or Treg function could provide an important strategy for treatment of T cell mediated autoimmune diseases.

Treg targeted intervention in the clinic

Several clinical trials have been aimed at modulating the Treg population, and thereby allowing restoration of immune balance. In completed trials, diabetes mellitus type 1 patients treated with a humanized non-activating anti-CD3 monoclonal antibody had increased residual β-cell function and clinical improvement (55-57). In mouse models for type 1 diabetes, it was demonstrated that this improvement is likely due to an anti-CD3 mediated induction of Treg, which suppress autoimmunity via TGFβ secretion (58, 59). Furthermore, in ongoing clinical trials for haematopoietic stem cell transplantation in leukemia patients, infusion of donor-derived

Treg is being tested to determine whether it can prevent or reduce Graft versus Host Disease (GvHD) (NCT01050764 and NCT00725062, www.clinicaltrials.gov).
Such strategies need to be tested with extreme caution, particularly after the disastrous outcome of a recent trial with anti-CD28 (TGN1412, a superagonistic monoclonal antibody). CD28 is a co-receptor for the T cell receptor (TCR) complex, present on all T cells, and interacts with CD80/CD86 on APC. The TCR requires additional signaling through CD28 to generate optimal effector T cell activation (60). In several murine models and in vitro, treatment with superagonistic anti-CD28 antibody specifically expanded Treg, and not effector T cells and this was accompanied by high IL-10 production (61-63). Furthermore, intravenous administration of anti-CD28 superagonist to rats suffering from experimental autoimmune encephalitis (EAE) protected animals from EAE. In line with this, Treg isolated from anti-CD28 treated rats upon adopted transfer protected recipients from EAE (61). A Similar increase of Treg was demonstrated in rhesus and cynomolgus macaques (64), however, it is not clear why Treg in these animal models were more sensitive to this antibody than effector T cells.
In contrast, in humans the anti-CD28 superagonist activated all T cells, initiating a systemic cytokine storm within 90 minutes in all participants, resulting in multi-organ failure (65). After this disastrous clinical trial, no contaminations in the anti-CD28 batch or manufacturing or administration errors were found (64). However, it was demonstrated that a small adjustment of in vitro presentation of the anti-CD28 superagonist (air dried on wells) induced release of high amounts of inflammatory cytokines by human PBMC and endothelial cells, but not by macaque cells (66). Furthermore, in human, but not in macaque T cells, the anti-CD28 superagonist induced an extremely sustained Ca^{2+} signal resulting in rapid de novo synthesis of inflammatory cytokines (67). This difference in inflammatory response is likely due to a 4 amino acid difference between the species in the extra-cellular domain of CD28 at the binding site of anti-CD28 (64, 66). Furthermore, in a rat model anti-CD28 superagonist first induced redistribution of T cells to secondary lymphoid organs, resulting in lymphopenia, which was also observed in the participants of the clinical trial, but in rats was accompanied by only mildly elevated levels of inflammatory cytokines (68). The cytokine storm in both rats and mice was probably never fully elicited due to a rapid regulatory T cell induction and response upon administration of the anti-CD28 superagonist (68, 69). Taken together, extensive in vitro and in vivo analysis should be performed in order to exclude all risks of such treatment strategies.

Working mechanisms of Treg

Treg are able to suppress effector T cell activation through several different mechanisms. In general, Treg can either kill effector T cells, or provide a signal in order to prevent effector T cell activation (reviewed in (70-72). Since the mechanism of Treg suppression could provide a target for therapeutic intervention, it is important to understand these suppressive mechanisms for human Treg **(Figure 2)**.

Cytokine secretion

Cytokine signaling is involved in all immune processes, and some cytokines have been associated with Treg mediated suppression **(Figure 2A)**.

TGFβ is secreted by Treg, or present as a membrane-bound protein on the cell surface of Treg (73). Interaction of TGFβ with cognate receptors on activated T cells can lead to suppression of inflammatory responses (74, 75). In addition, several studies have described that TGFβ can induce FOXP3 expression in T cells (76, 77), leading to effector T cell inactivation or conversion to Treg.

IL-10 is another important immunosuppressive cytokine secreted by Treg. In experimental models of Inflammatory Bowel disease and hepatitis, Treg producing IL-10 are essential for preventing disease (78, 79). Furthermore, in an experimental model for arthritis (proteoglycan induced arthritis) infusion of transgenic IL-10 producing T cells suppresses disease (80).

Another suppressive cytokine secreted by Treg in mice is IL-35. Treg lacking IL-35 were unable to suppress inflammatory bowel disease, and ectopic expression of IL-35 in naïve T cells induced suppressive function (81). However, in a study investigating human Treg in vitro, IL-35 was not secreted by Treg (82), thus its relevance for human Treg mediated suppression remains unknown.

Interaction with other immune cells

Interaction of effector T cells with dendritic cells (DC). is required to initiate effector T cell responses. Treg can suppress effector T cell activation through interaction or modulation of DC. Firstly, Treg can bind to MHCII-peptide complexes on the DC, and thereby block interaction of effector T cells with DC (83). Secondly, interaction of Treg with DC can inhibit DC activity. For instance, CTLA-4 a T cell co-receptor highly expressed on Treg, interacts with CD80 and CD86 on DC and prevents DC maturation, rendering the DC unable to stimulate effector T cells (84). Furthermore, this stimulates expression of indoleamine 2,3-dioxygenase (IDO), which induces catabolism of tryptophan into pro-apoptotic metabolites, resulting in suppression of effector T cell activation (85, 86).

Treg can also directly interact with receptors on effector T cells and suppress activation. Signaling through PD-1 (Programmed Death 1), expressed on activated human T cells, inhibits effector T cell proliferation. Consequently, in vitro blockade of this signaling pathway prevents human Treg mediated suppression (33). This suggests that PD-Ligand 1 on Treg is able to signal through PD-1 on effector T cells, and thereby blocks proliferation **(Figure 2B)**.

Granzyme B mediated apoptosis of effector T cells

Besides suppressing responses of effector T cells either through cell-cell interaction or cytokine production, Treg can also kill target cells. Some Treg produce high levels of granzyme B and/or perforin and by release of these factors could induce rapid apoptosis of the effector T cell **(Figure 2C)**. Murine activated Treg expressing granzymes and/or perforin have been demonstrated to prevent skin graft rejection (87) and tumor clearance (88), and in vitro were able to kill effector T cells (89) and B cells (90). Expression of granzyme B has only been demonstrated in activated human T cells, which were able to kill several human cell lines in a perforin dependent manner (91). However, whether human $FOXP3^+$ Treg utilize this mechanism to suppress effector T cells remains unclear.

Depletion of IL-2 by Treg

An alternative method of killing effector T cells is by deprivation of essential survival factors, such as IL-2. Over the last few years it has been established that IL-2 is also important for Treg function and survival (92, 93), being required for Treg expansion FOXP3 expression. However, FOXP3 also suppresses IL-2 expression, by directly repressing the IL-2 promoter (94, 95). As a result, Treg do not produce IL-2 themselves, and a role for Treg as a 'sink' for IL-2 has been proposed. Since Treg express higher levels of IL-2Rα (CD25), they may have an advantage over effector T cells, depriving effector T cells from IL-2 (96). In line with this, in mice Treg have been described to consume IL-2 and thereby induce cell death in the effector T cell population (97, 98) **(Figure 2D)**. Consistently, administration of exogenous IL-2 and other IL-2Rγ-chain binding cytokines, such as IL-7, reversed Treg-mediated cytokine deprivation, and prevented effector T cell death (97).

Increasing cAMP in effector T cells

An increased level of the second messenger cyclic Adenosine Mono Phosphate (cAMP) is associated with inhibition of of cytokine production, including IL-2 and IFNγ by either blockade of nuclear factor-κB (NFκB) activity (99), or activation of the transcriptional repressor inducible cAMP early repressor (ICER) (72, 100). Treg can increase cAMP in effector T cells through two independent mechanisms. They can directly transport cAMP to the effector T cell through gap junctions (101). Furthermore, Treg express the endonucleotidases CD39 and CD73, which convert ATP into AMP and adenosine. Adenosine binds to the adenosine receptor A_{2a} on effector T cells, and signaling through this receptor leads to an increase of intracellular cAMP in both murine and human cells (102-105). This results in suppression of effector T cell proliferation and cytokine production. **(Figure 2E)**

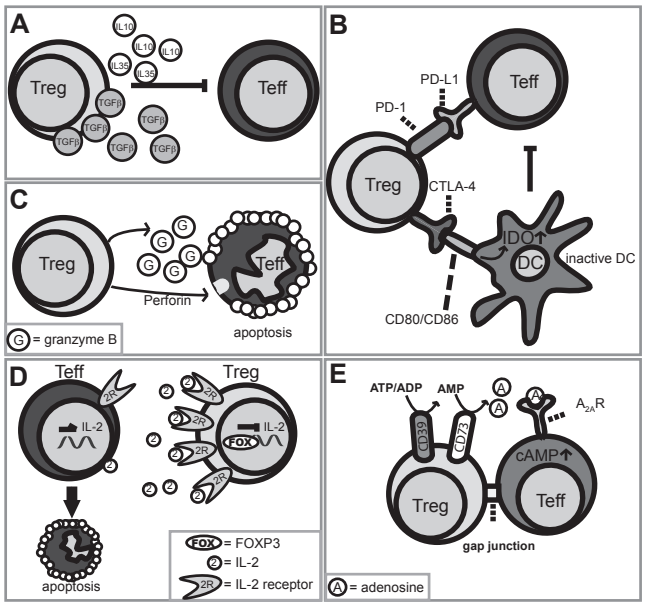

Figure 2. Mechanisms of suppression by Treg. (**A**) Treg can produce suppressive cytokines, which are either membrane bound (TGFβ), or secreted (IL-10, IL-35, TGFβ) and inhibit effector T cells (Teff). (**B**) Suppression of effector T cells through interaction of Treg with DC via CTLA-4 interaction with CD80/CD86 inactivates DC and induces DC production of the immune suppressor IDO. Furthermore, interaction of PD-1 on Treg with PD-L1 on Teff inhibits Teff activation. (**C**) Treg induce apoptosis of Teff by secretion of granzyme B and perforin. (**D**) Active Teff produce IL-2. FOXP3 inhibits IL-2 transcription in Treg. T cells require IL-2 for survival and express high levels of IL-2 receptor, compared to Teff. Therefore, Treg deprive Teff from IL-2, resulting in Teff apoptosis. (**E**) Treg elevate cAMP levels in Teff by either direct transfer of cAMP via gap junctions, or by processing of ATP into adenosine by CD39 and CD73. Adenosine signals via the adenosine receptor A2AR, resulting in increased cAMP.

Increasing Treg population numbers

In order to increase Treg function or numbers, several approaches are possible. To increase Treg numbers, new Treg could be induced, or existing Treg could be expanded. A distinction in the Treg population can be made between natural Treg (nTreg), which are derived from the thymus, and peripherally induced Treg (iTreg). Several approaches have been used to expand (106, 107) or induce (108) Treg, all of which utilize stimulation of the T cell receptor and co-receptor CD28. It has been shown for both human and mouse Treg that IL-2 is critical for FOXP3 expression, survival, proliferation, and suppressive function (92-94, 109, 110). Furthermore, TGFβ is required for induction and maintenance of FOXP3 expression (95, 111). Numbers of nTreg are limited, thus $CD4^+CD25^-$ T cells, which are more easily accessible in large numbers, are a more convenient source to increase the Treg population by Treg induction. However, although expanded nTreg seem to be suppressive in in vitro assays, the suppressive function of the iTreg in humans has been highly debated. In humans it appears that iTreg do not consistently function as true suppressive cells in vitro (77, 112), probably due to a loss of FOXP3. Notably, after repeated stimulation of nTreg, FOXP3 expression, and thus nTreg function, can also be lost (113). Thus, FOXP3 expression in itself is an insufficient marker for functional Treg since sustained expression of FOXP3 is required.

Analysis of human Treg suppressive function.

Thus far, expression of marker proteins is not always sufficient to discriminate functional Treg. The only way to investigate suppressive function is direct analysis of Treg inhibition of effector T cell activation in functional assays. Currently, most published data on Treg functionality have been obtained from in vitro suppression assays. These assays are based on proliferation of activated effector T cells cultured alone, compared to effector T cells in co-culture with Treg. Proliferation is either visualized by CFSE dilution in labeled effector T cells (114), or by ^3H thymidine incorporation (33, 115). CFSE labeling enables separate analysis of Treg and effector T cells, whereas ^3H thymidine is incorporated in all proliferating cells. In some cases, in order to establish whether Treg inhibit effector T cell cytokine production and secretion, cells are also analyzed for intracellular expression of cytokines (only possible when using CFSE labeling) (114), or cytokines are measured in culture supernatants (33). Usually, several ratios of cells in co-cultures are compared, since suppression is dose-dependent (46, 114), and duration of the cultures varies from 3-7 days (33, 46, 114, 115).

While in vitro assays are often used to test human Treg function, important drawbacks must be taken into account. Firstly, experimental setup can result in a differential outcome (116). Upon high stimulation effector T cells are too activated to be suppressed (33). Since the strength of in vitro stimulation strongly depends on the concentration of anti-CD3 (33), it is difficult to compare results between different studies. Secondly, human Treg behave very different in vivo, making it hard to translate in vitro results to the in vivo situation. This is highlighted by the observation that in vivo human Treg proliferate more rapidly than effector T cells (117), but in vitro Treg are anergic. This anergy can only be overcome by administration of high levels of IL-2 in addition to T cell receptor stimulation. However, this also abrogates suppression of effector T cells in vitro (118). Furthermore, the suppressive effect of the Treg has been mostly analyzed after 3-6 days, when effector proliferation is clearly visible in cultures with effector T cells only. However, the suppressive effect is already exerted in the first day of culture (119). When Treg are added later to the culture of activated effector T cells, proliferation is not suppressed. Thus, Treg and T effector cells have to be in co-culture directly upon activation (119), which is again hard to translate to the in vivo situation where effector T cells and Treg interactions may not occur within one day.

Recently several in vivo model systems have become available for assessment of human Treg function. For instance, a mouse model for xenogeneic (x)–GvHD has been developed, in which

injection of human PBMC in RAG2$^{-/-}$γ-chain$^{-/-}$ mice induced acute x-GvHD. Upon co-injection of freshly isolated CD4^{+}CD25^{+} T cells, x-GvHD development was suppressed (120) **(Figure 3)**. More recently in an independent study using a comparable model, expanded nTreg were able to suppress GvHD (121). These models enable analysis of in vivo function of human Treg, and are thus important for the translation of in vitro results.

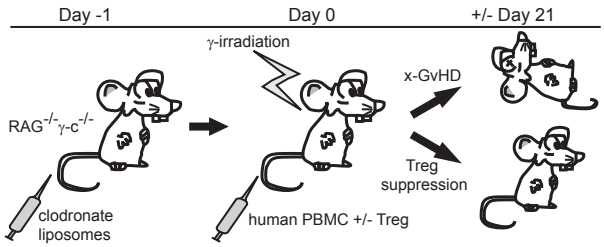

Figure 3. In vivo Treg function assay: humanized mouse model for x-GvHD. Clodronate containing cytotoxic liposomes were intravenously (i.v.) administrated to RAG$^{-/-}$ γ-chain$^{-/-}$ mice, killing phagocytes upon phagocytosis. The next day (Day 0), mice received 350 cGy γ-irradiation, and human PBMC alone, or with Treg are injected i.v.. When not suppressed by Treg, the human PBMC attack the murine tissues, resulting in severe xenogeneic GvHD (x-GvHD), usually peaking and therefore lethal, after 21 days.

Heat Shock Protein 60: a self-antigen able to influence Treg

As previously suggested, increasing numbers of Treg can be achieved by either induction of new Treg or expansion of existing Treg. For in vivo Treg induction or expansion, it would be preferable that these Treg recognize an antigen that is highly expressed at inflammatory sites and only at the time of inflammation or tissue damage. This is important, since Treg action should be restricted when and where needed, independent of the antigen to which effector responses are targeted. One example of a protein, that is recognized by T cells and is expressed upon inflammation and cell stress, is heat shock protein 60 (HSP60). Thus, HSP60 fulfils two non-redundant criteria for antigen-specific Treg based immunotherapy, namely selective expression at sites of inflammation and immunogenicity.

Previous studies have suggested that heat shock proteins (HSPs) are targets for antigen-specific Treg based immunotherapy in human inflammatory disease(122). HSPs are evolutionary strongly conserved proteins present in all eukaryotic and prokaryotic cells. They are expressed constitutively, and are upregulated under conditions of cellular stress, such as ultraviolet radiation, infection and malignancy (123-125). Besides being potent activators of the innate immune system (126, 127), HSPs have important immune regulatory effects. The human 60-kDa heat shock protein (HSP60) molecule can inhibit T cell migration (128) and inhibit the secretion of pro-inflammatory cytokines by activated T cells (129). This anti-inflammatory effect may be mediated by a direct binding of HSP60 to Toll-like receptor 2 (TLR2) on intrinsic Treg, resulting in enhanced Treg function (130).

Extensive studies in animal models, as well as Juvenile Idiopathic Arthritis (JIA) patients (131-133), have provided evidence that specific T cell responses against HSPs, especially HSP60, are tolerogenic (134). In models of experimental arthritis, including adjuvant arthritis (AA), pre-immunization with HSP65 (the rat homologue for HSP60) protects animals from arthritis (135-137). This protection is mediated by self-HSP-specific T cells, which are capable of suppressing inflammation (135, 138). Furthermore, in arthritis patients specific T cell responses towards endogenously produced HSP60, which is abundantly expressed in the synovial lining cells of

the patients (139), are associated with a good prognosis (132, 140). Thus, numbers of HSP60 specific T cells correlate with suppression of arthritis, and initiate tolerogenic responses in both patients and mice. This suggests that the T cells recognizing HSP60 are actually Treg, or that HSP60 through an alternative mechanism, for example through interaction with TLR2 receptors on Treg, promotes Treg function (141).

CD30: a surface marker for Treg

As previously discussed, it is difficult to distinguish functional Treg from activated T effector cells. In cells stimulated in vitro with an antigen, there are few differences in cell surface marker expression, since activated T cells similarly to Treg downregulate CD127 and increase expression of CD25, GITR, CTLA-4 and HLA-DR.

In JIA patients the response of T cells to HSP60 has been correlated with a beneficial outcome of disease, and characterized by a high production of IL-10 and expression of the cell surface receptor CD30 (142). CD30 is a member of the TNF receptor (TNFR) family, and in mouse models expression of CD30 on Treg has been demonstrated to be important for Treg function. Already early in the course of GvHD, Treg showed an increased CD30 expression, and DC exhibited increased CD30 ligand (CD30L) expression. Moreover, blocking of CD30-CD30L interaction, or infusion of $CD30^{-/-}$ Treg abrogated suppression of GvHD (143). In a model for graft rejection, CD30-CD30L interaction was also required for Treg mediated suppression, possibly by CD30-CD30L induced apoptosis in $CD8^{+}$ effector T cells (144). Furthermore, CD30 expression on T cells correlated with the production of Th2 cytokines and IL-10, but also with high expression of CD25. A suppressive role for CD30-expressing $CD4^{+}$ T cells in the regulation of human autoimmune diseases has also been suggested, since $CD30^{+}$ T cells produce Th2 cytokines and could thereby suppress Th1 mediated diseases, such as Rheumatoid Arthritis (145). Furthermore, in vitro studies utilizing Hodgkin's Reed-Sternberg tumor cells, typically expressing high levels of surface CD30, showed that CD30 either as membranous protein or as plate-bound chimeric protein, inhibited T cell activation in vitro (146). Taken together, CD30 expressing T cells may represent a population of T cells able to suppress immune responses, and CD30 could be a potential surface marker discriminating Treg from activated effector T cells.

Modulation of Treg function by epigenetic regulation of FOXP3

Since Foxp3 is an essential transcription factor for maintenance of immune homeostasis, its activity must be tightly and specifically regulated. In humans it seems that induced FOXP3 expressing T cells do not consistently function as suppressor cells in vitro (77, 112), which may be due to transient expression of FOXP3, suggesting that regulatory mechanisms of FOXP3 expression are involved.

DNA is organized in nucleosomes, consisting of 156 basepairs of DNA organized around an octamer of histones. Epigenetic processes affect gene expression without altering the sequences of bases in the DNA. Examples are methylation of cytosines in CpG motifs in the DNA, and post-translational histone modifications, changing the structure of the nucleosomes (147, 148). Post-translational acetylation of histones alters histone charge, reducing interaction between histones and DNA (149), and demethylation of CpG motifs, often clustered in CpG-rich regions in promoters, results in the relaxation of chromatin (148), both establishing a higher accessibility of the DNA for transcription factors. In Treg the FOXP3 promoter has been demonstrated to be less methylated (150-152) and stronger associated with acetylated histones (151) than in effector T cells, suggesting that the FOXP3 promoter is more accessible in Treg. Furthermore, demethylation of a non-coding region in the FOXP3 locus called Treg-cell-specific

demethylated region (TSDR) stabilizes expression of FOXP3 resulting in stable human and murine Treg function (12,59,60). In line with this, drug-mediated DNA demethylation in effector T cells induced stable FOXP3 expression and Treg phenotype (60). Thus, demethylation of the TSDR distinguishes transiently FOXP3 expressing induced Treg from stable FOXP3$^+$ Treg (148). In murine Treg, three demethylated conserved non-coding DNA elements were recently defined in the FOXP3 locus, all involved in separate facets of FOXP3 regulation; one region established FOXP3 expression in TGFβ induced Treg, another in dividing Treg, and the last in thymic Treg (153). These findings show that demethylation of different regions in the FOXP3 locus regulates FOXP3 expression, each in a specific fashion.
Histone acetylation has also been demonstrated to regulate FOXP3 expression. In addition to the association of acetylated histones to the FOXP3 promoter in Treg, acetylated histones have been shown to accumulate at the TSDR in mice (154). Furthermore, administration of histone deacetylase (HDAC) inhibitors to human Treg, stabilized FOXP3 expression (155). Thus both demethylation of the FOXP3 locus and histone acetylation regulate expression of FOXP3. Recently, in murine models post-translational acetylation of the FOXP3 protein has been suggested to be involved in regulation of FOXP3 expression and function, and was related to suppression of colitis (156), arthritis (157) and graft rejection (156). In order to unravel the molecular mechanisms of FOXP3 regulation by protein acetylation, further investigation is required.

Juvenile dermatomyositis: are Treg involved?

Juvenile dermatomyositis (JDM) is a systemic inflammatory disorder caused by inflammation of the microvasculature of skeletal muscles, skin and other organs. In most patients this results in muscle weakness, abnormalities in nailfold capillaries, and a characteristic rash, consisting of heliotrope eyelid discoloration and Gottron's papules (pink elevated patches) at extensor surfaces of finger joints (**Figure 4)**. JDM is a rare disease with an incidence rate of 3.2 per million, occurring mostly in females. Patients are under 17 years of age, and the median age at diagnosis is 7 (158). The cause is still unknown, however there are suggestions for genetic predisposition. JDM is strongly associated with HLA subtypes, such as HLA—DRB1 0301 (159, 160) , HLA-B8 (161), HLA-DQA1 0501 ((162), and HLA-DRB1 1401 (163). Furthermore, TNFα and IL-1 cytokine polymorphisms have been identified to be risk factors for JDM development (160). Environmental factors such as bacterial and viral infection could contribute to the pathogenesis of JDM (164). Upper respiratory and gastrointestinal illness, three months before JDM onset, were mostly observed (165). However, in case control studies it has been demonstrated that there was no difference between JDM patients and age-matched controls in antibody titres and bacterial or viral PCR-based detection in blood and muscle tissue (166, 167).
Mostly JDM disease is relapsing, and effective treatment can establish long term, drug-free remission. However, chronic or uncontrolled JDM has a poor clinical outcome (168). Currently, JDM treatment is mostly based on the use of general immune suppressants, such as corticosteroids, methotrexate (MTX), and in some cases anti-TNFα (169). While early aggressive immune suppressive treatment with, for example, methylprednisolone pulses followed by MTX treatment is highly effective in establishing early remission of disease (168), such approaches result in aspecific immune suppression, leading to an increased risk of infection and severe side effects, such as growth retardation(170), osteoporosis (171), steroid-induced diabetes (172), hypertension, cataract (173) and muscle weakness (174), without actually curing disease. Therefore, more specific treatment strategies are needed.
In muscle biopsies of JDM patients, mostly T cells, in particular CD4$^+$ T cells(175), were found, with occasionally a few B cells within the T cell clusters, and also myeloid cells were present, although usually less than T cells (176). These findings suggest a pathogenic role for these T cells at the site of inflammation, and support the idea that Treg may be able to suppress JDM tissue inflammation. However, the presence of FOXP3 expressing Treg has not been studied

yet in either peripheral blood or muscle tissue of these patients. In order to establish whether Treg could have beneficial properties in JDM, it should first be investigated whether they play a role in this particular autoimmune disease.

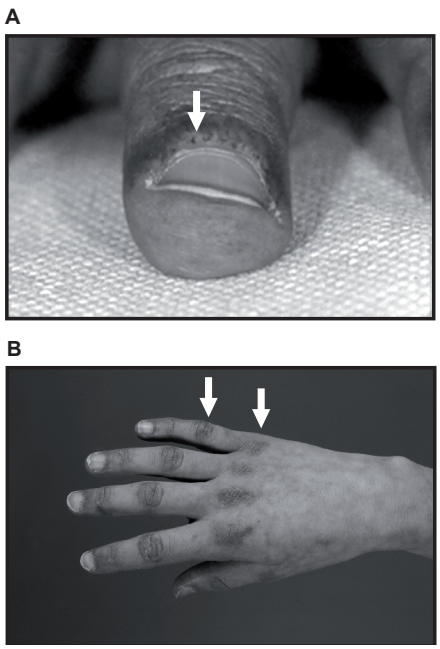

Figure 4. JDM disease characteristics. In JDM patients inflammation of the microvasculature results in typical disease symptoms such as **(A)** abnormalities in nailfold capillaries, and **(B)** Gottron's papules on extensor surfaces of finger joints, indicated by arrows.

Aim and outline of the thesis

In order to find potential intervention strategies for human autoimmune diseases, it is first important to understand how Treg phenotype and suppressive function should be analyzed. Firstly, a unique surface marker distinguishing Treg is lacking. Therefore, we extensively analyzed the phenotype of human Treg. We investigated CD30, since it seemed to be required for Treg function in murine models for GvHD and graft rejection, but also in vitro plate-bound CD30 suppressed human effector T cells. In **Chapter 4** we investigated whether CD30 is a marker for human FOXP3$^+$Treg.

Secondly, in vitro assays for testing Treg suppressive function are often difficult to interpret, since in vitro properties of Treg do not always reflect those of in vivo; in vitro Treg are anergic, and therefore, a proper experimental model for investigation of human Treg function in vivo is required. In **Chapter 3**, we used an x-GvHD mouse model (120) to translate in vitro human Treg suppressive function to an in vivo situation.

Treg are widely investigated, but not all murine Treg properties can be directly translated to human Treg. The mechanism of human Treg mediated suppression is still not completely clear. While several mechanisms have been shown in human Treg, apoptosis induction in effector T cells through Treg consumption of IL-2 (97) has not been validated for human Treg yet. In **Chapter 2** we investigated whether this mechanism is important for human Treg mediated suppression, to establish whether intervention strategies should be aimed at this particular Treg feature.

A more obvious way of promoting suppression of autoimmunity, would be by increasing Treg frequency and/or function. Since nTreg numbers are very low, Treg frequency could be more readily increased by induction of new Treg (iTreg). In order to obtain large numbers of iTreg, polyclonal TCR stimulation would be most efficient. Since these T cells are polyclonally stimulated, it is very important to carefully monitor FOXP3 expression and iTreg suppressive function, compared to nTreg. In **Chapter 3** we aimed to determine whether iTreg are safe for therapeutic intervention, by comparing in vitro and in vivo FOXP3 expression and suppressive capacity of both iTreg and nTreg.

HSP60 appears to induce tolerogenic T cell responses in both animal models (134, 135) and patients with arthritis (131, 132). In **Chapter 7** we review the immuno-modulatory properties and therapeutic potential of HSP60 in juvenile idiopathic arthritis. Stimulation of T cells with HSP60 could provide an opportunity for inducing or expanding Treg. However, it remains unclear precisely how HSP60 influences Treg. In **Chapter 4**, the potential of self-antigen human HSP60 for Treg based immunotherapy was explored.

Furthermore, as previously discussed, control of FOXP3 expression in Treg is very important, since sustained FOXP3 expression is required for Treg function. Thus, investigation of mechanisms regulating FOXP3 expression could lead to important intervention strategies. Post-translational acetylation of FOXP3 protein promotes Treg function in murine models, but the molecular mechanisms are still unknown. In **Chapter 5,** we aimed to establish the molecular mechanism by which acetylation of FOXP3 influences FOXP3 expression and, thereby, Treg suppressive function.

In **Chapter 6** we investigated whether Treg are involved in juvenile dermatomyositis pathogenesis, in order to establish whether Treg targeted intervention could be a potential treatment strategy. To this end, we investigated Treg numbers and suppressive function in peripheral blood, and Treg numbers in inflamed muscle tissue.

Taken together, in this thesis we established an overview of human Treg function and phenotype, and investigated opportunities to increase Treg frequency and function. We not only translated findings from mouse to human, but also from in vitro to in vivo, and from protein modification to cell function. This way, we aimed to identify proper tools for human Treg investigation and to evaluate Treg mediated intervention strategies for safe and efficient Treg-based therapy for autoimmune disease.

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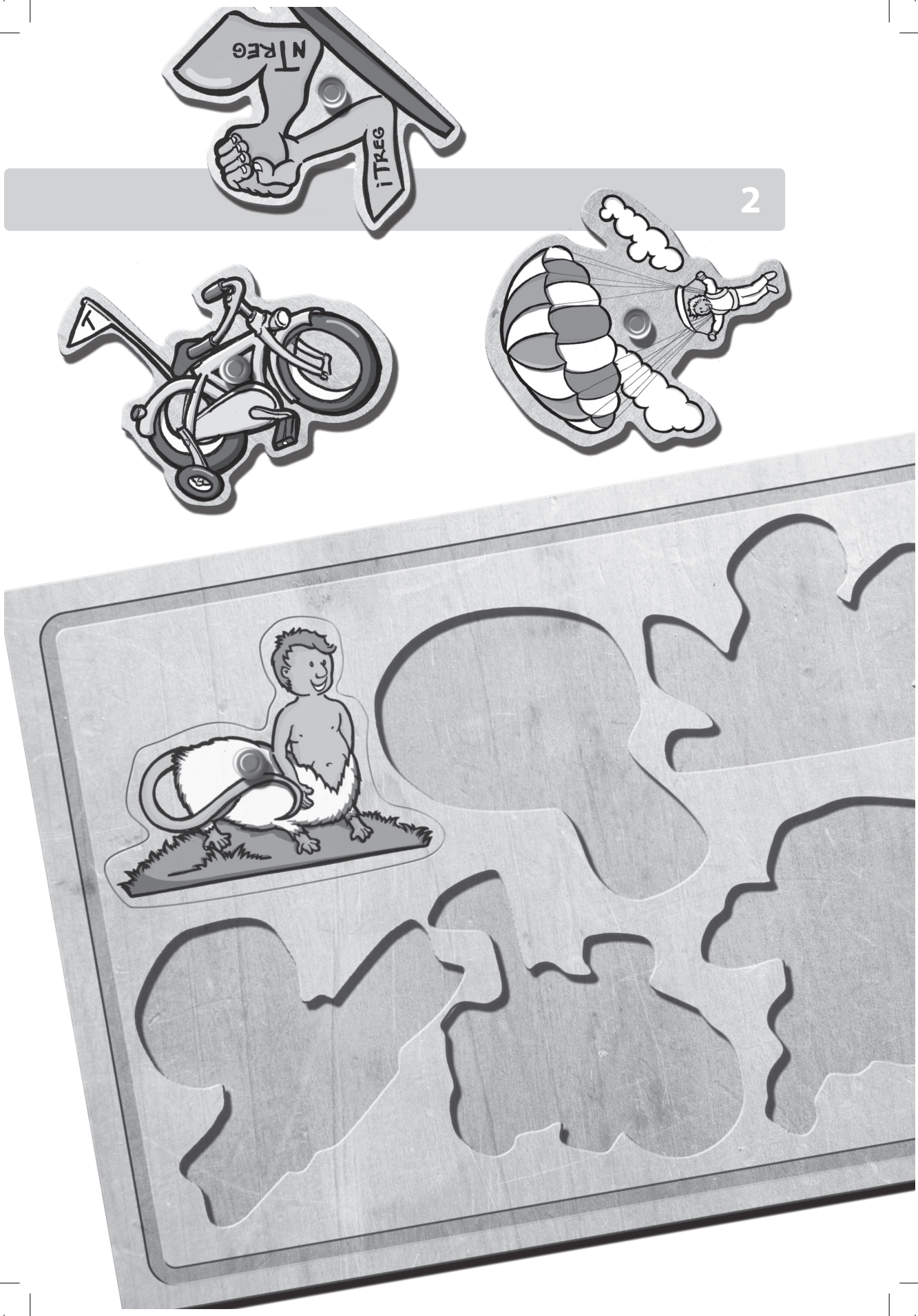
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iTREG
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Human regulatory T cell suppressive function is independent of apoptosis induction in activated effector T cells

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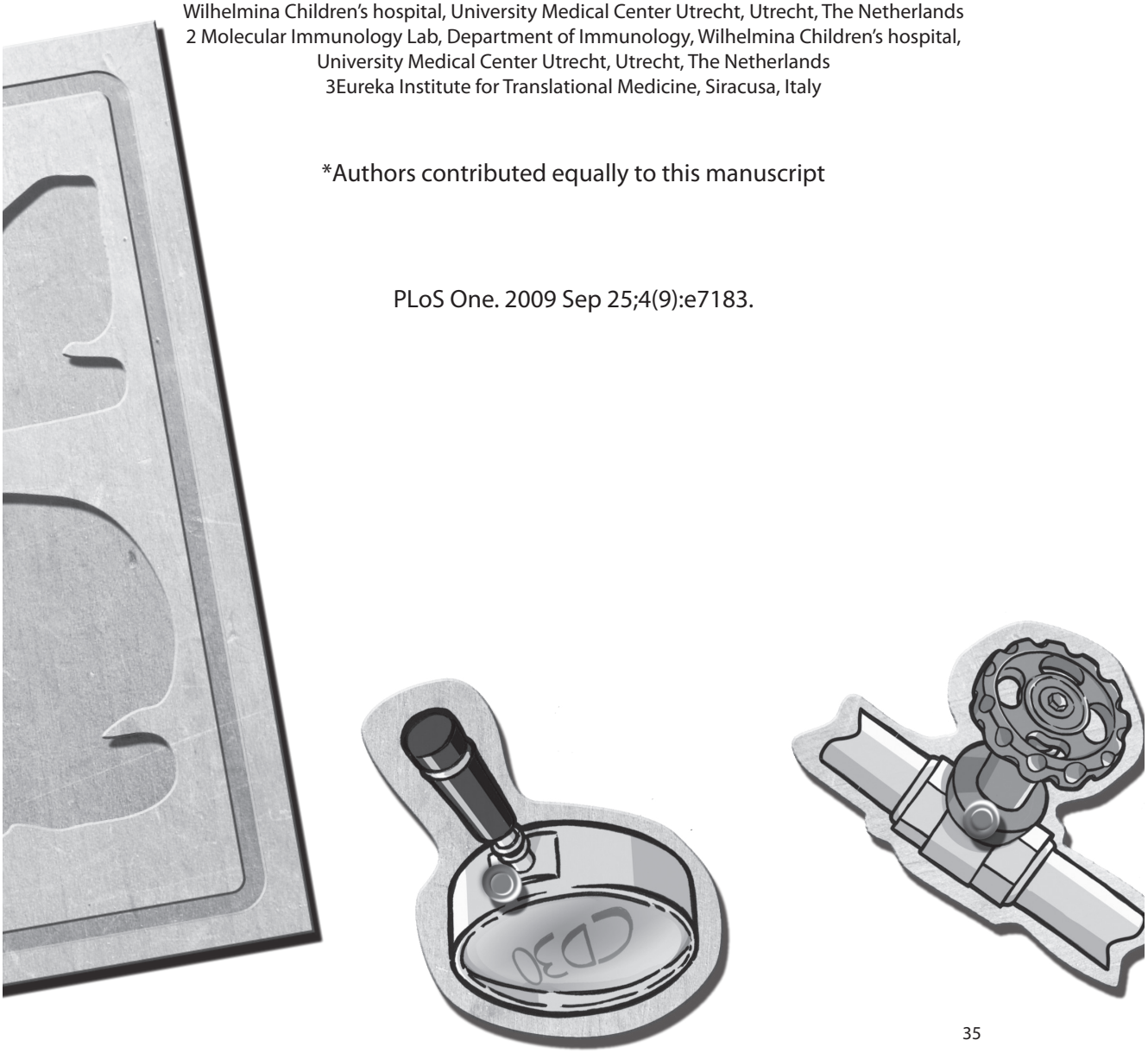


Abstract

CD4$^+$CD25$^+$FOXP3$^+$ Regulatory T cells (Treg) play a central role in the immune balance to prevent autoimmune disease. One outstanding question is how Tregs suppress effector immune responses in human. Experiments in mice demonstrated that Treg restrict effector T cell (Teff) responses by deprivation of the growth factor IL-2 through Treg consumption, resulting in apoptosis of Teff. In this study we investigated the relevance of Teff apoptosis induction to human Treg function. To this end, we studied naturally occurring Treg (nTreg) from peripheral blood of healthy donors, and, to investigate Treg function in inflammation *in vivo,* Treg from synovial fluid of Juvenile Idiopathic Arthritis (JIA) patients (SF-Treg). Both nTreg and SF-Treg suppress Teff proliferation and cytokine production efficiently as predicted. However, in contrast with murine Treg, neither nTreg nor SF-Treg induce apoptosis in Teff. Furthermore, exogenously supplied IL-2 and IL-7 reverse suppression, but do not influence apoptosis of Teff. Our functional data here support that Treg are excellent clinical targets to counteract autoimmune diseases. For optimal functional outcome in human clinical trials, future work should focus on the ability of Treg to suppress proliferation and cytokine production of Teff, rather than induction of Teff apoptosis.

Introduction

$CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) are of critical importance for the maintenance of immune homeostasis, as numerous experimental mouse models for autoimmune diseases correlate the presence of functional Tregs with amelioration of disease severity (1, 2). In humans Treg also play an important role in the immune balance, as patients lacking functional Treg, due to loss-of-function mutations in the transcription factor FOXP3, suffer from severe generalized autoimmune disease; immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) (3, 4). In addition, in human autoimmune diseases, like Juvenile Idiopathic Arthritis (JIA), negative correlations are found between the presence of regulatory T cells and disease severity (5). Therefore, Treg are considered an important therapeutic target for a large range of human immune mediated diseases, and ongoing clinical trials attempt to modulate the population of Treg, and thereby restore immune balance. For example, in diabetes mellitus type 1, patients were treated with anti-CD3 antibodies in order to enhance Treg function, which resulted in clinical improvement and increased residual-cell function (6, 7). Moreover, in a clinical trial for cord blood transplantation in patients suffering from haematological cancer, infusion of donor-derived Treg is tested to prevent or reduce Graft versus Host Disease (GvHD) (NCT00602693, www.clinicaltrials.gov).

Despite these potentially far-reaching applications of Tregs in humans, questions remain with regard to the underlying mechanisms of Treg action, particularly in humans. Tregs may suppress effector cells either through cell-cell contact, the production of suppressive cytokines, and/or through the consumption of cytokines and growth factors such as IL-2 (8). It is clear that IL-2 in many aspects is crucial for Treg function (9, 10). For one, it is required for Treg expansion, and regulates FOXP3 expression (11, 12), and it is also indispensable for Treg mediated suppression (13). On the other hand, FOXP3 suppresses IL-2 transcription, by binding to the IL-2 promoter (14, 15). As a result Treg do not produce IL-2, and even may act as a 'sink' for IL-2. Thus, competition for IL-2 between effector T cells (Teff) and Treg, which express a higher level of IL-2Rα chain (CD25) compared to Teff, may counteract proliferation of Teff (16, 17). Accordingly, Pandiyan et al. recently showed that in mice Treg consume IL-2 and thereby induce apoptosis in the Teff population (18, 19). This mechanism of apoptosis through cytokine deprivation was responsible for the suppressive function of Treg. Consistently, IL-2 and other IL-2Rγ-chain binding cytokines, such as IL-7, were able to overcome cell death (18), and, in earlier reports, have been shown to interfere with both murine and human Treg-mediated suppression (17, 20).

We aimed to determine whether apoptosis induction via cytokine consumption by Treg is an important mechanism for human Treg-mediated suppression of Teff. As it is not fully understood how human Treg mediate their suppressive action on Teff, we studied the suppressive capacity and induction of apoptosis by naturally occurring Treg from peripheral blood and compared it to, assumedly *in vivo* activated Treg from an inflammatory site, the synovial fluid, of JIA patients. Our findings demonstrate that apoptosis induction in Teff is not important for human Treg mediated suppression.

Materials and methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the Institutional Review Board of the UMC Utrecht. All patients provided written informed consent for the collection of samples and subsequent analysis.

Cells, medium and reagents

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers and JIA patients and synovial fluid mononuclear cells (SFMC) from the synovial fluid of JIA patients, after informed consent, using Ficoll Isopaque density gradient centrifugation (Amersham Biosciences, NJ, USA). RPMI 1640 containing 10mM HEPES (Seromed), 2mM L-glutamine 100 U/ml penicillin-streptomycin and 10% human AB serum was used as culture medium (all Invitrogen, Carlsbad, USA). Where indicated, IL-2 (1000 U/ml (= 60 ng/ml), Chiron, Uxbridge, UK) or IL-7 (10 ng/ml, PeproTech Inc, Rocky Hill, NJ, USA), were added.

Suppression assay

$CD4^+$ $CD25^-$ effector T cells (Teff), were magnetically isolated from PBMC using a CD4 T Lymphocyte Enrichment Set (BD Biosciences). Subsequently, $CD25^+$ T cells were depleted using CD25 Magnetic Particles (BD Biosciences). All magnetic cell isolations were performed according to the manufacturer's instructions. The $CD4^+CD25^-$ T cells were labeled with 3 μM CFSE for 10 min at 37°C and extensively washed. 25,000 Teff (Teff) were plated into anti-CD3-coated wells (OKT-3, 1.5 μg/ml), and to control for higher cell numbers in co-cultures (crowdedness) 50,000 Teff were plated (Teff+Teff). $CD4^+CD25^+CD127^-$ T cells were sorted as Treg from PBMC (21, 22) (with an average of 58% $FOXP3^+$ cells ± 13 % s.d.) or SFMC (23) (with an average of 24% $FOXP3^+$ cells ± 12% s.d.) by FACS Aria (BD Biosciences) and added in different ratios to Teff. T cell depleted, irradiated autologous PBMC (3500 Rad) were used as Antigen presenting cells (APC), 30,000 per well. Cells were cultured for 5 days and proliferation was measured by flow cytometry on a FACS Calibur (BD Biosciences). The levels of $FOXP3^+$ cells in the $CD4^+CD25^+CD127^{low}$ T cells directly isolated from PBMC or SFMC were lower than expected. This is due to an underestimation of the percentage of $FOXP3^+$ cells (See Figure S4). All data were analyzed using Cellquest software.

Flow cytometry staining

To determine levels of apoptosis, cells were stained with Annexin V PE and 7-AAD, using a staining kit according to the manufacturer's instructions (all BD Biosciences). $CFSE^+$ cells were gated to determine cell death within the Teff population. For FOXP3 analysis, PBMC were washed twice in FACS buffer (PBS containing 2% FCS and 0.1% sodium azide), adjusted to 0.5-1 x 10^6 cells/ml in FACS buffer and blocked with mouse serum (5 min at 4°C). Subsequently, the cells were incubated in 50 μl FACS buffer containing three appropriately diluted PE, FITC or PerCP labeled mAbs against human CD4 (clone RPA-T4), CD25 (clone 2A3), CD127 (clone hIL-7R-m21), all from BD Biosciences. For intranuclear staining of APC or Pacific Blue FOXP3 (clone PCH101), V450 FOXP3 (clone 259D, BD Bioscience) or Isotype Control, the cells were first surface stained, then fixed, permeabilized and stained using the FOXP3 staining kit (eBioscience) according to the manufacturer's instructions. Cells were analyzed on a FACS Calibur (BD Biosciences). All data were analyzed using Cellquest software.

Analysis of cytokine production by multiplexed particle-based flow cytometric assay

Cell culture supernatants were collected, stored at -80°C and processed within 1 month. Cytokine concentrations were measured with the Bio-Plex system in combination with the Bio-Plex Manager software, version 4.0 (Bio-Rad Laboratories, Hercules, CA, USA), which employs

the Luminex xMAP technology as previously described (24). The following cytokines were measured: IL-2, IL-5, IL-7, IL-10, IL-13, IL-17, tumor necrosis factor-α (TNF-α), and interferon-γ (IFNγ).

Statistical analysis

For statistical analysis of multiple groups One-way ANOVA or nonparametric ANOVA; Kruskal-Wallis test, were used. Bonferroni or Dunn's Multiple Comparison Test post test were used, to compare between 2 selected groups. To compare between two groups, non-parametric T-test, Mann Whitney was used. P values below 0.05 were considered significant.

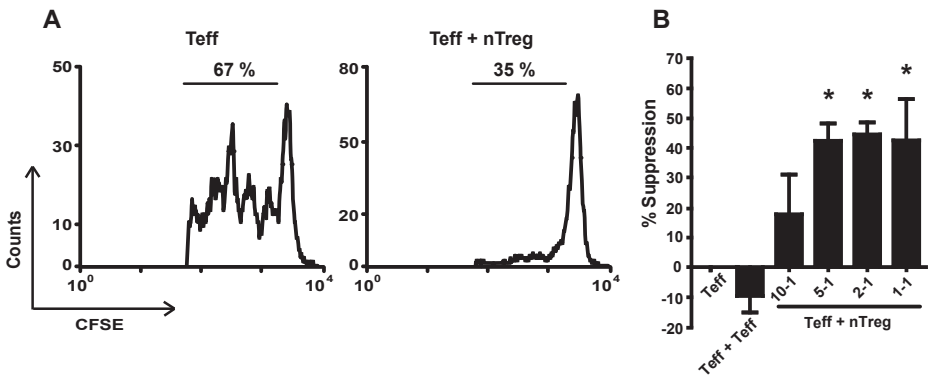
Results

nTreg are highly suppressive without inducing apoptosis in Teff

We first established that human Treg inhibit proliferation of activated Teff. CFSE labeled Teff were co-cultured for 5 days with a graded amount of $CD4^+CD25^+CD127^{low}$ naturally occurring Treg (nTreg), in 200 μl culture medium, and suppression of Teff proliferation and induction of Teff apoptosis were determined. As expected, nTreg inhibited proliferation of Teff, as measured by decreased CFSE dilution in Teff cells (**Figure 1A**). This suppression of proliferation increased with titrated amounts of Treg in the culture, in a dose-dependent manner (**Figure 1B**).

Next, cells from the same co-cultures were stained with 7-AAD and Annexin V and gated on CFSE+ cells (See **Figure S1 A, B**) to determine apoptosis in Teff. Only few apoptotic cells were found in cultures with Teff only, and the percentage of apoptotic cells did not increase upon the presence of more nTreg (**Figure 1C, D**), which was similar for the absolute number of apoptotic cells (Figure 1E). Thus under normal culture conditions, human nTreg do not induce apoptosis in Teff, while efficiently suppressing Teff proliferation.

We hypothesized that if cytokine consumption by Treg in the vicinity is responsible for apoptosis in Teff, culture of the same number of Teff and Treg in a smaller volume should enhance suppression mediated by apoptosis induction. Therefore, all further cultures were performed in 75 μl instead of 200 μl medium. Under these conditions the level of suppression was higher (up to 65% average at a 1-1 ratio) compared to normal culture conditions (up to 48% average at a 1-1 ratio) (**Figure 1F**). Furthermore, a larger number of Teff became apoptotic (up to 750 Annexin $V^+7\text{-}AAD^+$ cells average for Teff+Teff) (**Figure 1H**), but in the co-cultures with nTreg the percentage of apoptotic cells only slightly increased (**Figure 1G**), and the number of apoptotic Teff even decreased (**Figure 1H**). Although we show a low upregulation of Annexin V on highly activated cells (**Figure S3A**), the level of apoptosis per cell division was independent of the presence of Treg (**Figure S5**). To establish that day 5 was the appropriate timepoint to measure apoptosis in our assays, we also measured cell death on day 3 and 4. Consistently, on day 3 and 4 hardly any apoptosis was seen (**Figure S1 C, D**). Furthermore, we show that Teff in our assay are able to go into apoptosis, by titrating Sheath Fluid (BD Biosciences), containing ethanol into cultures with Teff (**Figure S2 A, B**), causing Teff apoptosis in a dose-dependent manner. Thus, apoptosis induction does not occur in Teff + nTreg co-cultures, whereas high levels of suppression are reached. Altogether, these data clearly demonstrate that apoptosis induction is not important for nTreg mediated suppression.



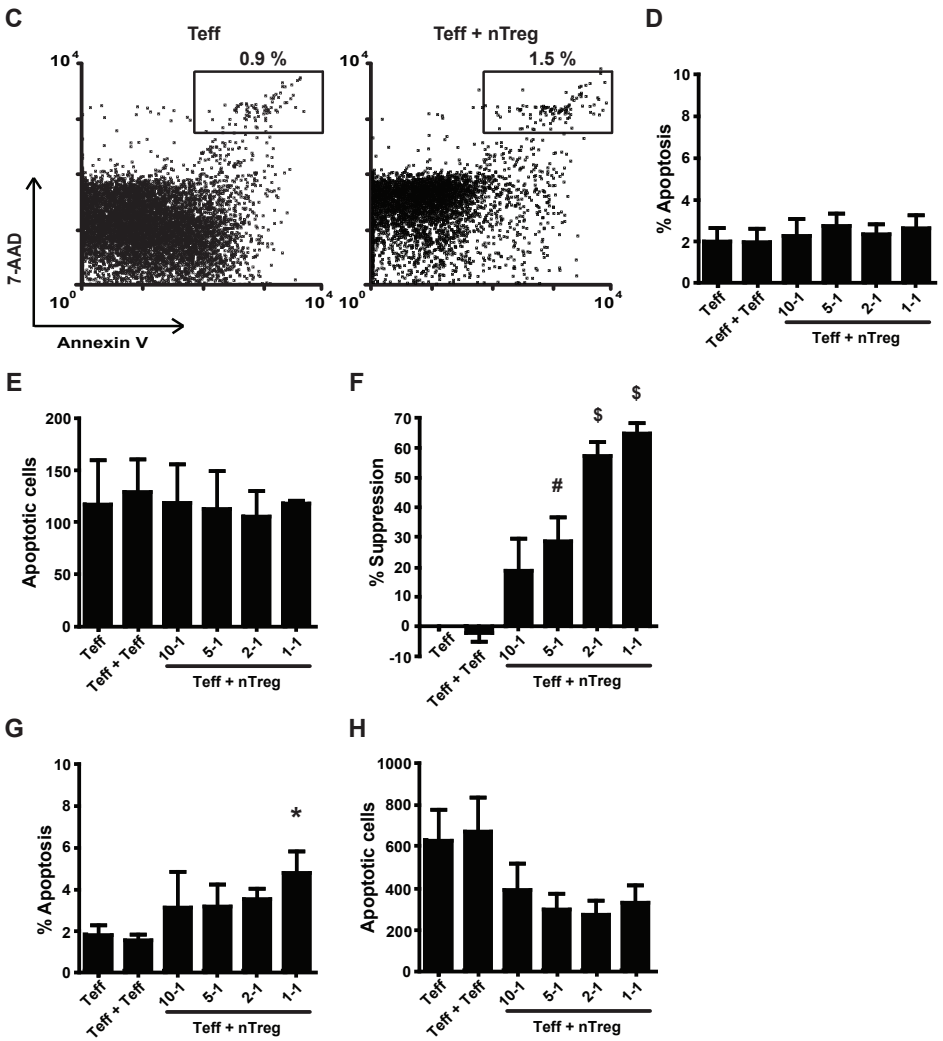


Figure 1. Naturally occurring Treg suppress Teff proliferation, but do not induce apoptosis. Cells were cultured in 200 μl medium for 5 days (n=3). (**A**) Proliferation of Teff measured by flow cytometry, cultured alone (left) or in co-culture with naturally occurring Treg (1-1) (right). 1 representative example is shown. (**B**) Level of suppression of Teff proliferation, calculated for several ratios of Teff + Treg, and Teff + Teff (ratio 1-1), compared to culture of Teff alone (suppression=0%). (**C**) Apoptotic Teff cells ($CFSE^+$) were measured after 7-AAD and Annexin V staining by flow cytometry analysis. Percentage of apoptosis in Teff cultured alone (left) and in co-culture with Treg (1-1) (right). 1 representative example is shown. (**D**) Average percentage, and (**E**) absolute number, corrected for cell input, of apoptotic Teff expressing 7-AAD and Annexin V, for several co-culture ratios of Teff + Treg, Teff + Teff (1-1) and Teff alone,. Cells were cultured in 75 μl medium for 5 days (n=9). (**F**) Level of suppression of Teff proliferation, calculated for several ratios of Teff + Treg, and Teff + Teff (ratio 1-1), compared to culture of Teff alone (suppression=0%). (**G**) Average percentage, and (**H**) absolute number, corrected for cell input, of apoptotic Teff cells expressing 7-AAD and Annexin V, for several co-culture ratios of Teff + Treg, Teff + Teff and Teff alone (n=9). Error bars represent means ± s.e.m., * P<0.05, # P<0.01, $ P <0.001.

IL-2 and IL-7 overcome suppression, without influencing apoptosis

In mice, cytokine consumption was suggested to be pivotal for Treg-mediated apoptosis in Teff and suppression. Therefore, we investigated whether absence of IL-2Rγ-chain binding cytokines plays a role in the induction of apoptosis in Teff and suppression by human nTreg. In co-cultures of Teff and nTreg we observed a clear decrease in IL-2, as well as other cytokines important for Teff function; IL-5, IL-13, IL-10, IFNγ, TNFα, but not IL-17 (**Figure 2A, C** (upper panel)). This lack of IL-17 suppression could be due to a resistance of Th17 cells to Treg mediated suppression (25, 26) The decrease of cytokines in the culture medium in the presence of Treg could be due to either a general suppression of Teff cytokine production, or to cytokine consumption.

To investigate this further, we studied whether exogenously added cytokines could affect apoptosis induction of Teff, or suppression of proliferation and cytokine production by Teff. When high concentrations of exogenous IL-2 or IL-7 were added, the proliferation of Teff cells increased (data not shown). Furthermore, suppression of Teff proliferation was abrogated in all co-culture ratios (**Figure 2B**), which is in line with studies describing abrogation of Treg mediated suppression by IL-2 and IL-7, by either Teff stimulation, or, in case of IL-2, abrogation of Treg anergy (17, 20, 27-29). The high levels of IL-2 or IL-7 abrogated nTreg-mediated suppression of cytokine production by Teff as well (**Figure 2C and Table S1**). Furthermore, it seems that both IL-2 and IL-7 increase cytokine production of Treg, which may have contributed to abrogation of suppression. In contrast, although the percentage of apoptotic cells seems to decrease (**Figure 2D and Figure S3B**), IL-2 and IL-7 did not decrease numbers of apoptotic Teff in co-cultures, instead the number of apoptotic cells was even significantly increased (**Figure 2E**) Thus, IL-2Rγ-chain binding cytokines prevent suppression of Teff proliferation and cytokine production, but this is not accompanied by a reduction in apoptosis. Although we cannot conclude from these data whether cytokine consumption is involved, this emphasizes that nTreg mediated suppression is independent of apoptosis induction in Teff.

Apoptosis induction in Teff is irrelevant for Treg function in inflammation

Last, we wished to establish the relevance of our findings on nTreg to human Treg function in ongoing inflammation. Therefore, we studied Treg from within a chronically inflamed environment, the synovial fluid of JIA patients (SF-Treg). In synovial fluid, Treg are abundantly present and highly activated, due to the chronic inflammation. Furthermore, Teff from the SF probably have a different activation state, which may be contributing to the ongoing inflammation in JIA. To make a reliable comparison between Treg from the peripheral blood and Treg from the site of inflammation, it is preferable to use the same Teff population in all assays. Therefore, we co-cultured SF-Treg with Teff obtained from peripheral blood of the same patient in 75 μl medium. Probably due a different cellular composition, which may be caused by contaminating activated T cells, suppression of Teff proliferation by SF-Treg was less compared to nTreg (**Figure 3A, B**), whereas suppression of Teff cytokine production was similar to nTreg (**Figure 3C, D**, see also **Tables S2** and **S3**). Still, similar as for nTreg, despite a slight increase of the percentage of apoptotic cells, a decreased number of apoptotic Teff was found in the presence of SF-Treg (**Figure 3E, F**). Altogether, even Treg from an inflammatory environment do not induce apoptosis in Teff cells to achieve suppression of Teff proliferation and cytokine production.

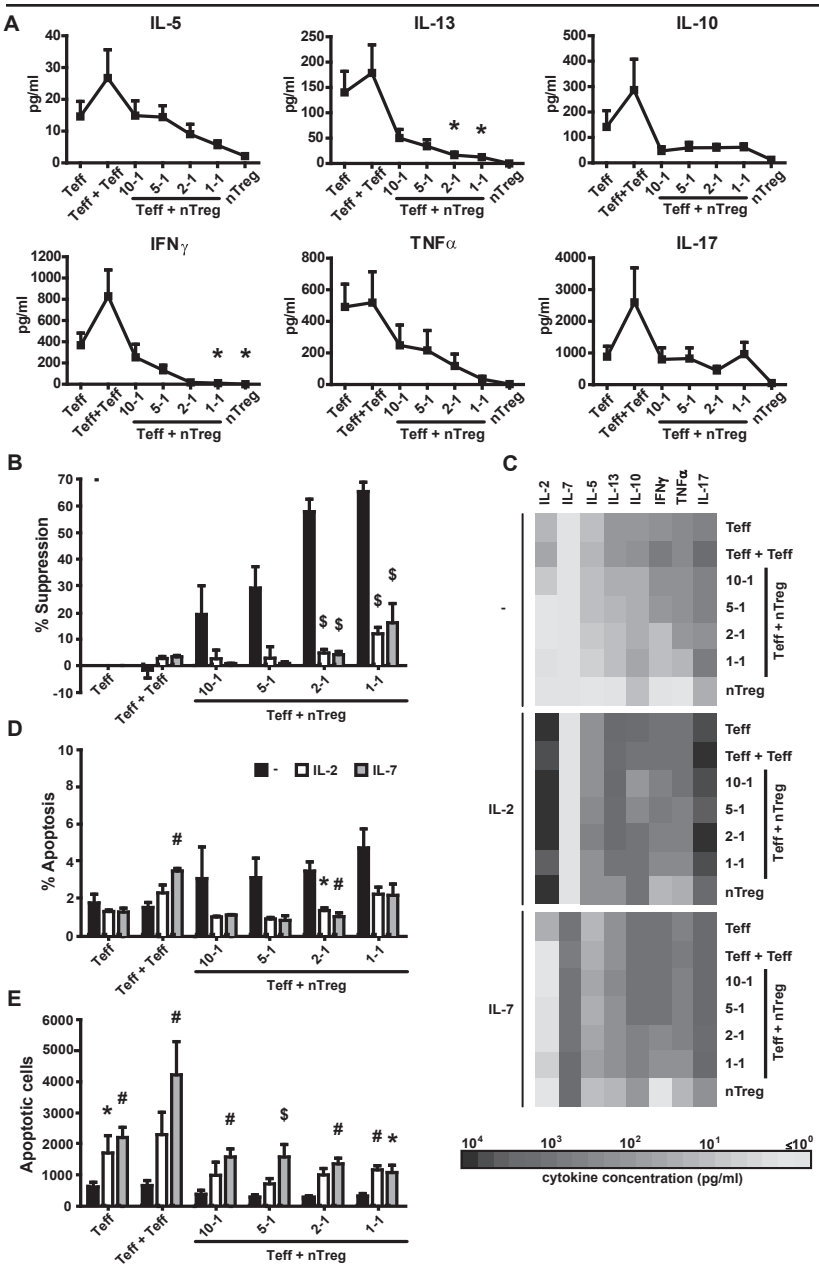


Figure 2. Exogenous IL-2 and IL-7 decrease suppression of Teff proliferation and cytokine production, but do not decrease apoptosis. Cells were cultured in 75 μl medium for 5 days (**A**) Levels of cytokines, present in culture medium, on day 5 of culture, in several co-culture ratios of Teff + Treg, Teff + Teff (1-1), and Teff alone (n=9). (**B**) Level of suppression of CFSE$^+$ Teff proliferation, calculated for several ratios of Teff + Treg, Teff + Teff (1-1) and Teff alone (suppression=0%), cultured without (black bars), or with IL-2 (white bars) or IL-7 (grey bars). Suppression was calculated by comparing co-cultures with Teff alone with equal cell culture conditions. (**C**) Mean levels of cytokines, present in culture medium on day 5 of culture, in medium (-), with addition of IL-2 (IL-2) or IL-7 (IL-7). A color profile of the means was made to show the differences between culture conditions (see also Table S1). (**D**) Average percentage, and (**E**) absolute number, corrected for cell input,of apoptotic CFSE$^+$ Teff cells, expressing 7-AAD and Annexin V, in several co-culture ratios of Teff + Treg and Teff alone, cultured without or with IL-2 or IL-7. (B-E, n=5). Error bars represent means ± s.e.m., * P<0.05, # P<0.01, $ P <0.001.

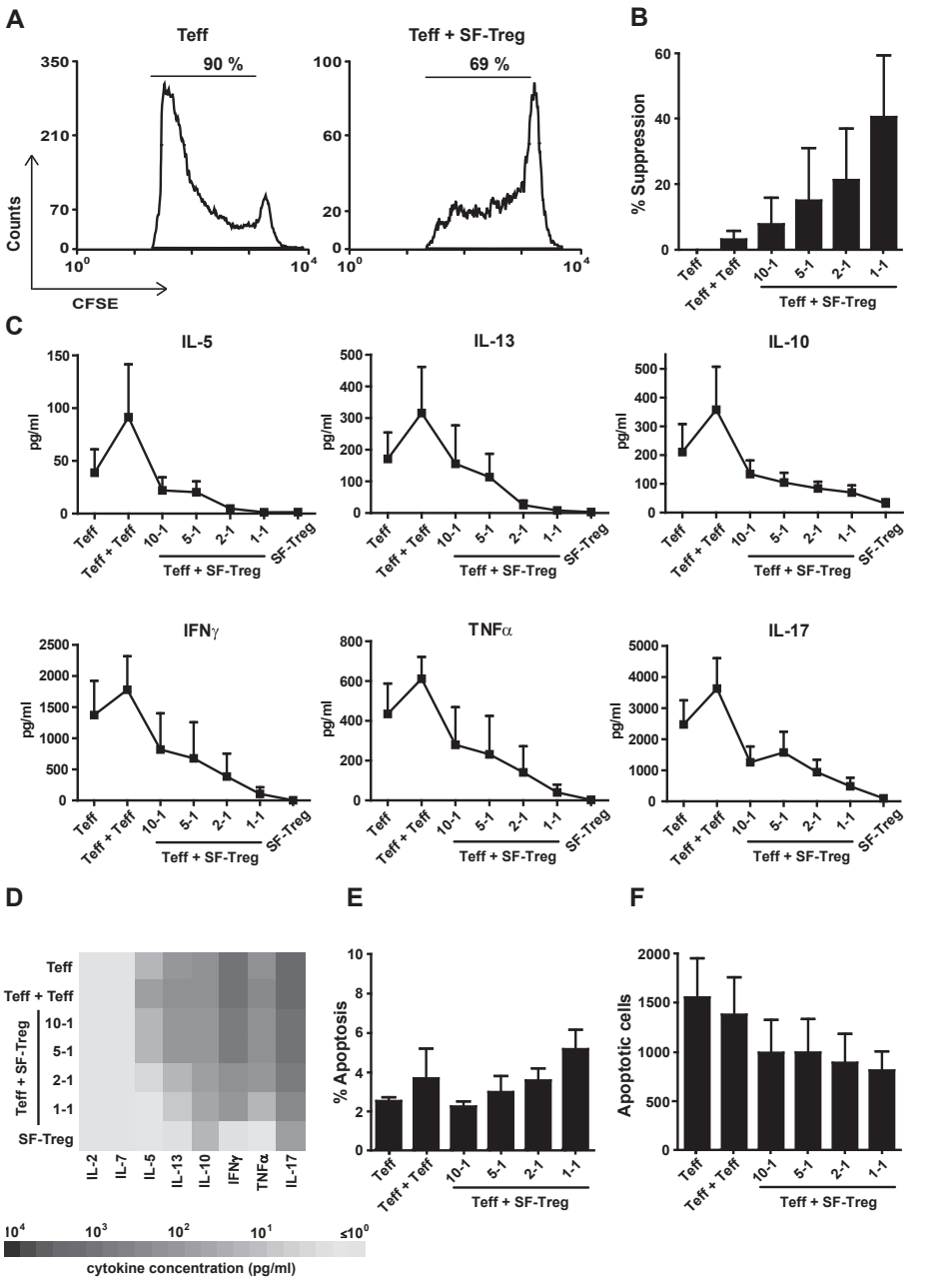


Figure 3. Synovial fluid-derived Treg suppress Teff proliferation and cytokine production, but do not induce apoptosis. Cells were cultured in 75 µl medium for 5 days. (**A**) Proliferation of CFSE$^+$ Teff measured by flow cytometry, cultured alone (left) or in co-culture with SF-Treg (1-1) (right). 1 representative example is shown. (**B**) Level of suppression of CFSE$^+$ Teff proliferation, calculated for several ratios of Teff + SF-Treg, and Teff + Teff (1-1), compared to culture of Teff alone (suppression = 0%). (**C**) Levels of cytokines, present in culture medium, on day 5 of culture, in several co-culture ratios of Teff + SF-Treg, Teff + Teff (1-1) and Teff alone. (**D**) Mean levels of cytokines, present in culture medium on day 5 of culture. A color profile of the means was made to show the differences between culture conditions (see also Table S2). (**E**) Average percentage, and, (**F**) absolute number, corrected for cell input, of apoptotic CFSE$^+$ Teff for several co-culture ratios of Teff + SF-Treg, Teff + Teff (1-1) and Teff alone (B-E, n=3). Error bars represent means ± s.e.m.

Discussion

Pivotal studies in mice models have pointed out that Treg are indispensable for the maintenance of peripheral immune tolerance. Also in humans a similar role of Tregs is likely, prompting discussions about their clinical applicability. Though comparable in many aspects, several differences between mouse and human Treg phenotype, function and mechanisms of suppression have been identified in the past few years. For instance, the expression of FOXP3 seems to be a more consistent marker for functional Treg in mice, than it is in humans (30-32). As for mechanisms of suppression, IL-35 production by Treg is important for suppression in mice (33), while IL-35 is not even expressed by human Treg (34). Since Treg are currently tested for therapeutic applications in humans, it is especially important to determine to what extend results obtained in mice can be translated to human Treg.

Recently, Pandiyan et al. exemplified a new mechanism of action of Treg in mice, namely their capacity to induce apoptosis in Teff, based on specific cytokine consumption as Treg can consume IL-2 produced by the Teff. Also, addition of IL-2 to co-cultures of Teff and Treg prevented the apoptosis of Teff. Though they did not directly show that addition of IL-2Rγ-chain binding cytokines, which diminished apoptosis, also prevented suppression *in vitro*, *in vivo* they did find that induction of Teff apoptosis is indeed important for Treg function. Furthermore, previous reports show that suppression *in vitro* by murine Treg is prevented by addition of IL-2Rγ-chain binding cytokines(17). Our current data show some similarities between the mouse and human system, but also reveal an essential difference between mouse and human Tregs; human Treg function is not mediated by apoptosis of Teff. Obviously, human experiments such as these are restricted to *in vitro* assays, and only limited numbers of cells are available. However, *in vitro* Treg assays, similar to those used for mice, can be performed with human cells as well and compared to data obtained in experimental models.

Similar to mice, we show that naturally occurring human Treg very efficiently suppress both proliferation and cytokine production by effector T cells, which can be reversed by addition of IL-2Rγ-chain binding cytokines. These results are consistent with earlier reports on human and murine Treg which show both inhibition of Teff IL-2 mRNA production, as well as Teff proliferation by Treg, and a decrease of suppression of Teff proliferation by addition of high levels of exogenous IL-2 (17, 35, 36). Also, Treg derived from a highly inflammatory environment, synovial fluid from the joints of JIA patients, suppress Teff proliferation and cytokine production. Obviously, mouse splenocytes differ in many aspects from human PBMC (37). Here we show that human Teff seem to be less prone to apoptosis than mouse Teff. When comparing cell death in cultures with only Teff, human Teff show hardly any apoptosis (2 %), whereas mouse Teff show a higher level of apoptotic cells (20 %) (18). And, importantly, we show that suppression by human Treg does not involve induction of apoptosis in Teff: the absolute numbers of apoptotic cells decrease in the presence of Treg.

IL-2 is an important cytokine for Treg function, both in mice and humans. However, we do not find a decrease of apoptosis in Teff upon addition of IL-2. This may again be due to the low level of apoptosis in Teff in general. However, it could also be explained by the fact that Teff do not necessarily require IL-2 to survive or become activated. This is confirmed by recent data obtained by *in vitro* tests on peripheral blood cells from a specific group of IPEX patients. In these patients Teff produce only low levels of IL-2 and, remarkably, the deficit in Treg function can be overcome by addition of IL-2 to cell cultures. Thus, the *in vivo* lack of Treg function could be explained by the decreased production of IL-2 by Teff in these IPEX patients (3, 38).Altogether, this suggests that in humans IL-2 is very important for Treg function, but is not required for Teff survival and function, as these Teff, despite low IL-2 production, are still highly activated and causing disease. We show here, in line with earlier publications, that addition of IL-2 and IL-7 abrogates suppression of both Teff proliferation and cytokine production. This could be due to a higher activation of the Teff, as the Teff cultured alone proliferate more and produce more cytokines in the presence of IL-2 and IL-7, or, in case of IL-2, to abrogation of Treg anergy. In addition, we do

not find a decrease of the added IL-2 in these cultures with Treg present. This suggests that IL-2 is not consumed by the Treg, although we can not exclude that the level of exogenous IL-2 is simply too high to detect consumption by Treg.

In conclusion, we here point out an important difference between human and murine Treg function: human Treg do not induce apoptosis in Teff to achieve suppression. With these data we emphasize that experimental data from mouse models should be carefully validated in human cells to identify discrepancies, and to ensure that further therapeutic applications are efficient and safe. This does not mean that Treg are less valuable targets for intervention. It could even be argued that if human Treg, instead of eliminating Teff by inducing apoptosis, render Teff either anergic, or even turn them into suppressor cells themselves (39, 40), may be able to exert a stronger bystander suppression in an ongoing inflammatory response.

Our functional data here support that Treg are excellent clinical targets to counteract autoimmune diseases. For optimal functional outcome in human clinical trials, future work should focus on the ability of Treg to suppress proliferation and cytokine production of Teff, rather than induction of Teff apoptosis.

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Supplementals

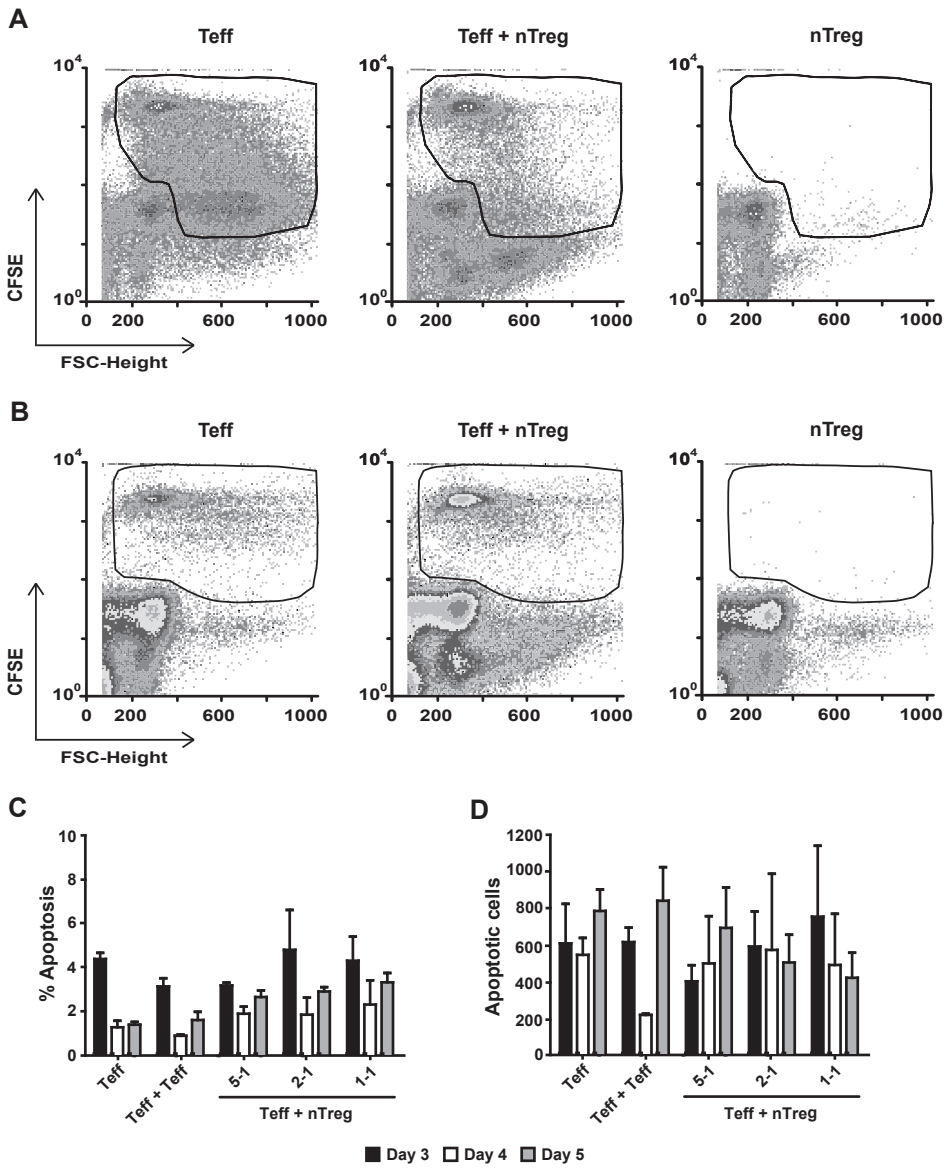


Figure S1. Proliferation and apoptosis of Teff after 3, 4, and 5 days of culture in the presence and absence of Treg. (**A**) Gated CFSE+ Teff in the presence of APC (left plot) or APC + Treg (middle plot) after 5 days of culture. For comparison, Treg only + APC are shown as well (right plot). 1 representative example of n = 9. (**B**) Gated CFSE+ Teff in the presence of APC (left plot) or APC + Treg (right plot) after 3 days of culture. For comparison, Treg only + APC are shown as well (right plot). 1 representative example of n=3. (**C**) Average percentage, and (**D**) absolute number (corrected for cell input) of apoptotic Teff, expressing 7-AAD and Annexin V, for several co-culture ratios of Teff + Treg, Teff + Teff and Teff alone after 3 (black bars), 4 (white bars) or 5 days (grey bars) of culture (n=3). Error bars represent means ± s.e.m.

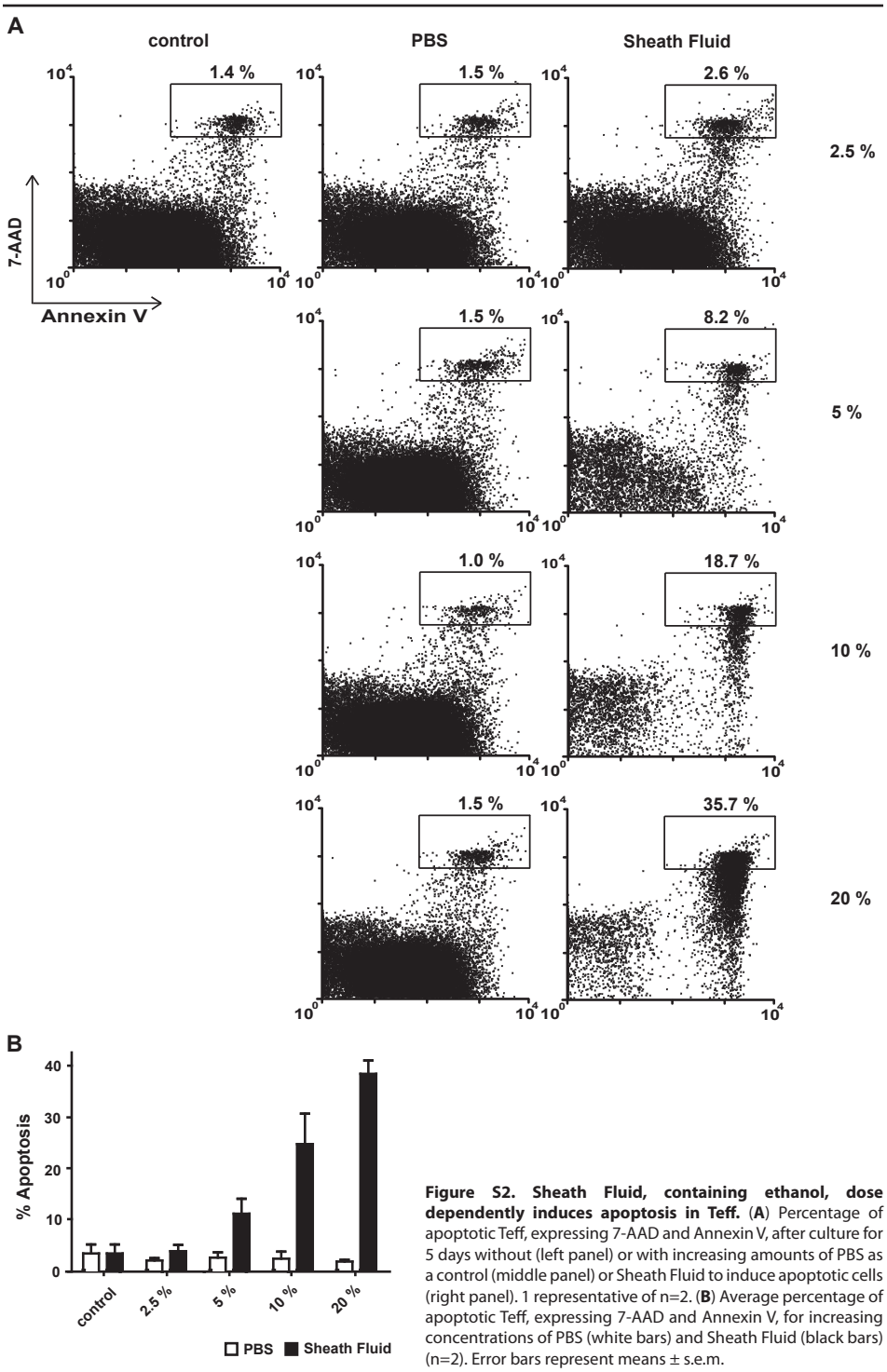


Figure S2. Sheath Fluid, containing ethanol, dose dependently induces apoptosis in Teff. (**A**) Percentage of apoptotic Teff, expressing 7-AAD and Annexin V, after culture for 5 days without (left panel) or with increasing amounts of PBS as a control (middle panel) or Sheath Fluid to induce apoptotic cells (right panel). 1 representative of n=2. (**B**) Average percentage of apoptotic Teff, expressing 7-AAD and Annexin V, for increasing concentrations of PBS (white bars) and Sheath Fluid (black bars) (n=2). Error bars represent means ± s.e.m.

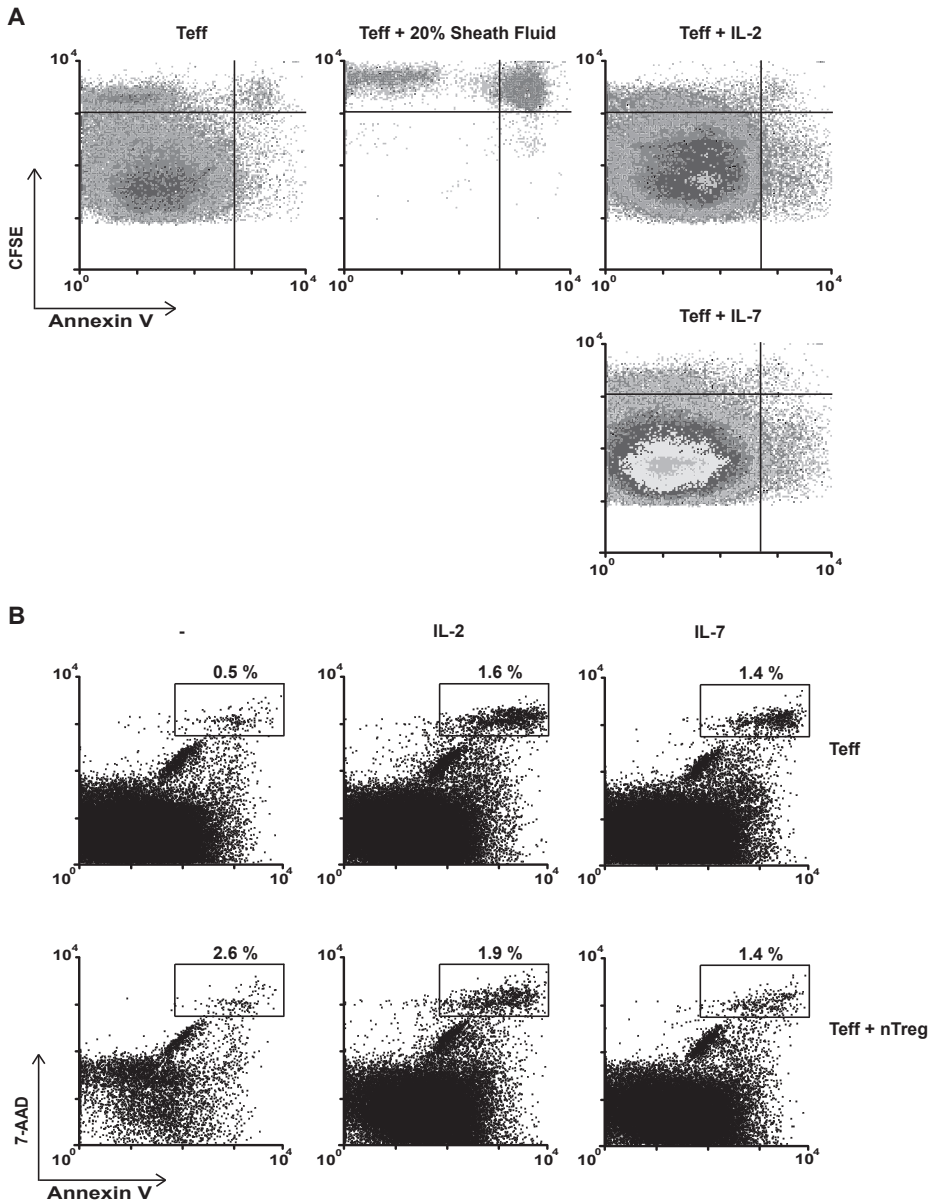


Figure S3. Annexin V expressing Teff and apoptotic Teff, expressing both Annexin V and 7-AAD, in the presence and absence of IL-2 and IL-7. (**A**) Annexin V expression of gated CFSE+ Teff cultured for 5 days without additional stimuli (left panel), in the presence of 20% Sheath Fluid to induce apoptotic cells (middle panel), or in the presence of IL-2 or IL-7 (right panel). 1 representative example for each condition is shown. (**B**) Percentage of apoptotic Teff, expressing 7-AAD and Annexin V, alone or in the presence of Treg (1-1) in the absence (left panel) or presence of IL-2 (middle panel) or IL-7 (right panel). 1 representative example of n=5.

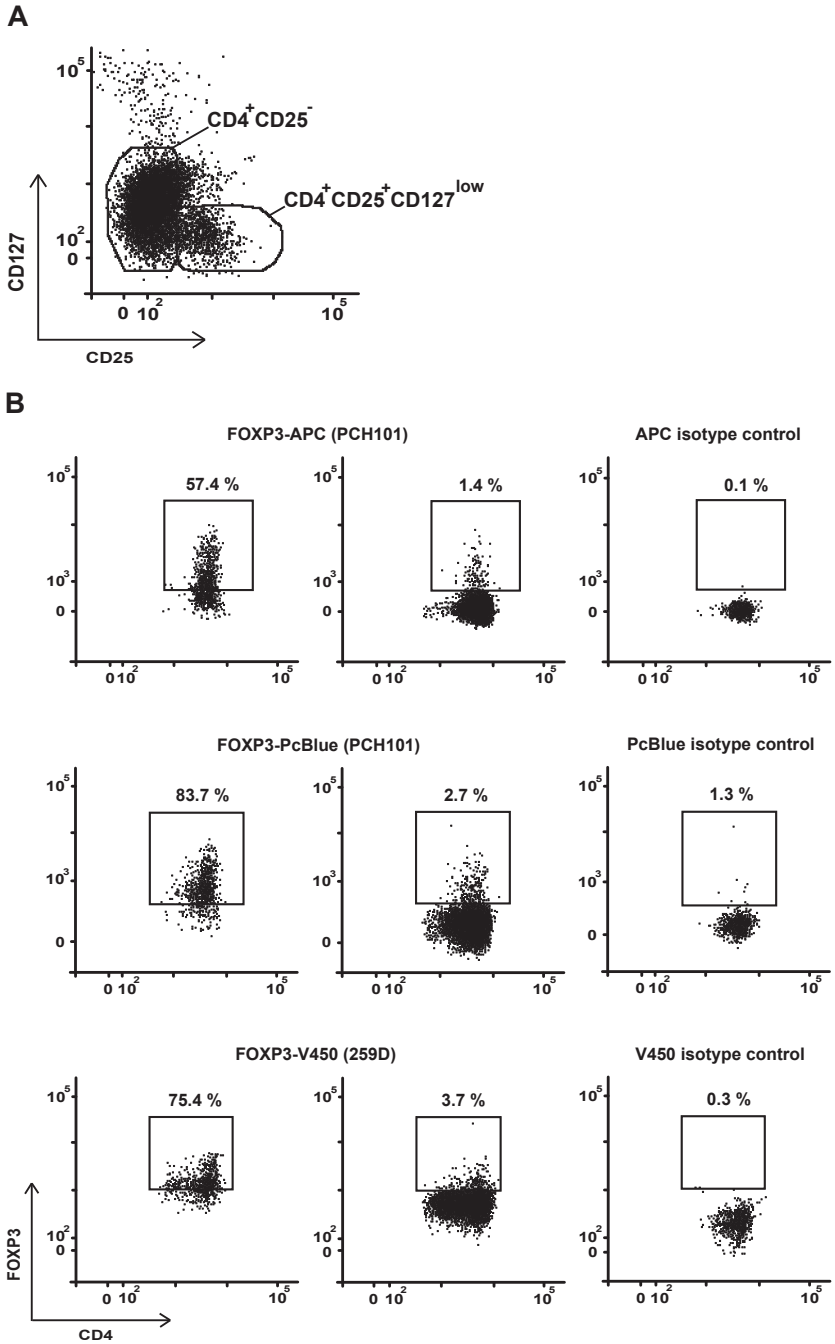


Figure S4. Percentage of FOXP3 expressing cells within the CD4+CD25+CD127low Treg population. (**A**) CD25 and CD127 expression of gated CD4+ T cells. The gate used for sorting the CD4+CD25+CD127low Treg population is indicated. For comparison of FOXP3 expression CD4+CD25- cells were gated. 1 representative example is shown. (**B**) FOXP3 expression measured by different FOXP3 antibodies within the CD4+CD25+CD127 low Treg population (left panel), within the CD4+CD25- cells (middle panel), and corresponding isotype controls gated on CD4+CD25+CD127 low Treg (right panel). 1 representative example of n=4.

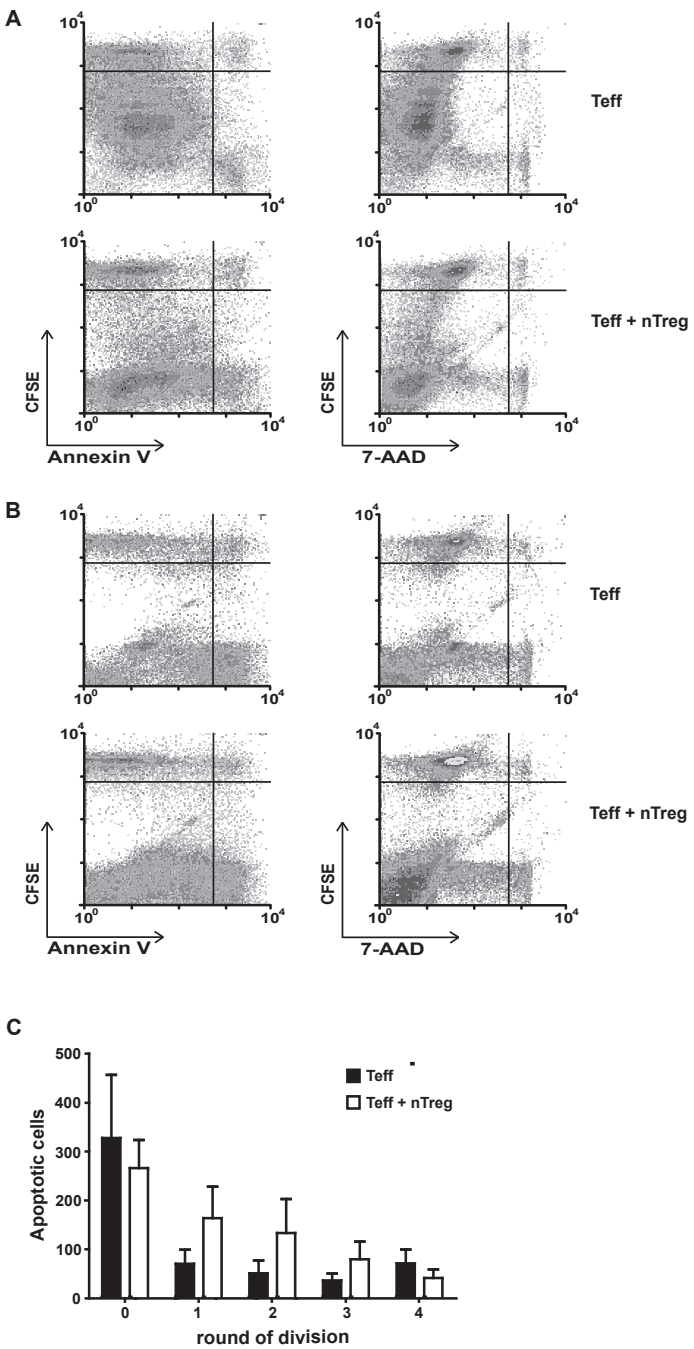


Figure S5. Annexin V and 7-AAD expressing cells and apoptotic Teff, expressing both Annexin V and 7-AAD, in the presence and absence of Treg. (**A**) Total cells expressing AnnexinV (left panel) and 7-AAD (right panel), in the presence and absence of Treg at day 5 of culture. 1 representative example of n=9. (**B**) Total cells expressing AnnexinV (left panel) and 7-AAD (right panel), in the presence and absence of Treg at day 3 of culture. 1 representative example of n=3. (**C**) Average absolute number of apoptotic Teff, expressing 7-AAD and Annexin per cell division (0=undivided cells), in the absence (black bars) and presence (white bars) of Treg at day 5 of culture. (n=4) Error bars represent means ± s.e.m.

Table S1. IL-2 and IL-7 inhibit nTreg mediated suppression of Teff cytokine production, but nTreg do not consume IL-2 or IL-7. Cells were culture in 75 µl medium for 5 days. Mean levels of cytokines, present in culture medium on day 5 of culture, in medium (-)(N=9), with addition of IL2 (IL-2)(N=5) or with addition of IL7 (IL-7)(N=5). or with addition of IL7 (IL-7)(N=5). All values (mean (SD)) are expressed in pg/ml. When values were compared to Teff only: * p<0.05, # p<0.01, $ p <0.001 (see table above per culture condition). When cytokine values in cultures +IL-2 or +IL-7 were compared to medium: * p<0.05, # p<0.01, $ p <0.001 ns=non-significant (see upper row of tables +IL-2 or +IL-7, significance is depicted per cytokine)

IL-2	IL-7	IL-5	IL-13	IL-10	IFNγ	TNFα	IL-17		
30.7 (45.1)	0.0 (0.0)	14.6 (14.3)	112.3 (99.6)	141.4 (192.2)	367.8 (340.1)	461.2 (403.6)	773.3 (856.9)	Teff	
78.3 (203.1)	0.0 (0.0)	26.7 (26.8)	142.8 (133.4)	286.4 (363.8)	828.3 (745.2)	486.8 (548.0)	2273.6 (2859.2)	Teff+ Teff	-
7.4 (5.3)	0.0 (0.0)	14.9 (12.3)	40.2 (35.8)	46.7 (44.8)	253.6 (324.4)	232.8 (319.6)	698.6 (847.7)	10-1	
2.9 (2.5)	0.0 (0.0)	14.4 (8.8)	27.2 (25.7)	59.3 (52.1)	131.2 (118.3)	202.6 (290.1)	721.4 (721.7)	5-1	
2.1# (1.7)	0.0 (0.0)	9.1 (8.8)	13.3* (13.2)	59.8 (36.2)	17.9 (20.7)	110.5 (198.7)	393.5 (314.9)	2-1	
3.5# (6.7)	0.0 (0.0)	5.6 (4.0)	10.1* (7.7)	61.3 (40.5)	10.9* (13.7)	32.1 (51.3)	844.2 (973.0)	1-1	
0.6 (1.0)	0.0 (0.0)	2.2 (0.3)	0.0 (0.0)	12.3 (5.3)	0.0* (0.0)	2.7 (3.4)	44.4 (28.4)	nTreg	
IL-2#	IL-7ns	IL-5$	IL-13$	IL-10*	IFNγ ns	TNFα#	IL-17#		
12878.2 (2170.6)	0.0 (0.0)	360.3 (177.5)	4034.3 (1244.5)	2356.2 (2832.7)	1036.7 (513.5)	1270.8 (560.8)	8328.4 (5514.3)	Teff	
8756.7 (2776.0)	0.0 (0.0)	267.6 (168.8)	2811.4 (1054.6)	1853.1 (594.7)	1478.4 (806.8)	1365.7 (985.3)	12036.4 (4362.6)	Teff+ Teff	+IL-2
13720.8 (4420.5)	0.0 (0.0)	298.7 (150.3)	4459.9 (262.8)	860.6 (822.5)	791.3 (122.1)	1022.6 (1262.0)	9417.6 (4929.7)	10-1	
11399.4 (3157.0)	0.0 (0.0)	455.1 (170.7)	3511.6 (1497.2)	885.9 (318.3)	893.5 (353.7)	697.4 (881.1)	6805.1 (5100.3)	5-1	
11642.7 (6578.4)	0.0 (0.0)	625.5 (364.7)	3082.3 (612.3)	1241.2 (973.0)	663.5 (223.4)	745.1 (419.3)	10086.6 (4898.7)	2-1	
6494.2 (7442.3)	0.0 (0.0)	394.5 (412.2)	1927.7 (1478.0)	2005.5 (1467.8)	513.6 (426.8)	509.0 (442.6)	9473.2 (6655.3)	1-1	
13213.0 (2457.5)	0.0 (0.0)	225.0 (184.6)	329.7# (159.2)	2214.1 (683.9)	26.0 (4.3)	49.6 (6.4)	5922.7 (1980.1)	nTreg	
IL-2 ns	IL-7$	IL-5 ns	IL-13 ns	IL-10#	IFNγ ns	TNFα ns	IL-17 ns		
52.0 (94.1)	2245.8 (417.7)	32.6 (11.0)	373.5 (103.5)	2114.2 (1872.5)	1126.4 (1042.2)	778.4 (293.3)	4869.1 (3014.3)	Teff	
2.5 (0.8)	983.3# (127.1)	43.2 (14.4)	334.7 (176.4)	1854.1 (1233.8)	1486.3 (887.6)	906.4 (345.3)	5021.8 (2421.1)	Teff+ Teff	+IL-7
2.2 (0.7)	2048.7 (557.2)	62.9 (36.9)	403.1 (100.4)	1708.6 (1795.6)	1131.3 (827.4)	662.3 (391.0)	4210.7 (2207.8)	10-1	
3.0 (0.2)	2103.1 (257.6)	45.5 (34.0)	331.2 (56.9)	2049.0 (1996.1)	1205.4 (1213.2)	794.1 (286.4)	5026.6 (1879.6)	5-1	
3.9 (5.3)	2479.4 (242.1)	62.8 (36.1)	282.7 (81.0)	861.4 (507.6)	570.7 (462.5)	621.3 (227.6)	3822.0 (867.8)	2-1	
5.1 (5.4)	3427.8# (655.3)	86.5 (85.1)	354.1 (128.4)	719.3 (430.3)	342.9 (305.5)	375.0 (117.4)*	4396.8 (2152.9)	1-1	
1.2 (0.8)	4590.8$ (77.1)	13.8 (7.4)	38.4 (17.0)	203.3 (53.4)	2.6 (0.6)	20.5* (2.6)	399.5 (152.2)	nTreg	

Table S2. SF-Treg suppress Teff cytokine production. Cells were cultured in 75 µ medium for 5 days (N=3). Mean levels of cytokines, present in culture medium on day 5 of culture. All values (mean (SD)) are expressed in pg/ml.

IL-2	IL-7	IL-5	IL-13	IL-10	IFNγ	TNFα	IL-17	
0.8 (0.9)	0.0 (0.0)	38.9 (38.1)	153.9 (130.3)	210.7 (168.3)	1372.5 (952.8)	380.7 (231.1)	2472.5 (1344.4)	Teff
0.6 (0.5)	0.0 (0.0)	91.5 (86.8)	284.6 (227.2)	357.9 (258.6)	1777.5 (933.6)	535.3 (166.50	3626.2 (1693.8)	Teff+ Teff
0.1 (0.1)	0.0 (0.0)	22.0 (21.4)	140.0 (189.4)	134.1 (81.9)	818.1 (1006.0)	244.5 (287.8)	1257.0 (877.1)	10-1
0.3 (0.6)	0.0 (0.0)	20.3 (17.7)	102.1 (115.5)	104.9 (58.8)	676.4 (1001.0)	202.8 (292.7)	1573.5 (1152.0)	5-1
0.1 (0.1)	0.0 (0.0)	4.8 (5.0)	22.8 (22.2)	83.9 (40.7)	383.7 (638.7)	123.5 (198.7)	940.6 (688.7)	2-1
0.0 (0.1)	0.0 (0.0)	1.3 (2.3)	7.3 (9.5)	70.0 (42.8)	105.8 (183.2)	35.0 (59.6)	487.4 (475.1)	1-1
0.0 (0.0)	0.0 (0.0)	1.5 (2.5)	3.0 (5.1)	32.0 (24.4)	3.4 (5.9)	2.4 (4.2)	96.3 (143.6)	nTreg

Table S3. The level of cytokine suppression for nTreg and SF-Treg. In this table we show the averages (SD) level of suppression of cytokines in the cultures. We compared per cytokine, per co-culture condition whether there was a significant difference in cytokine suppression between nTreg and SF-Treg, by Mann Whitney U test, * p< 0.05, # p<0.01.

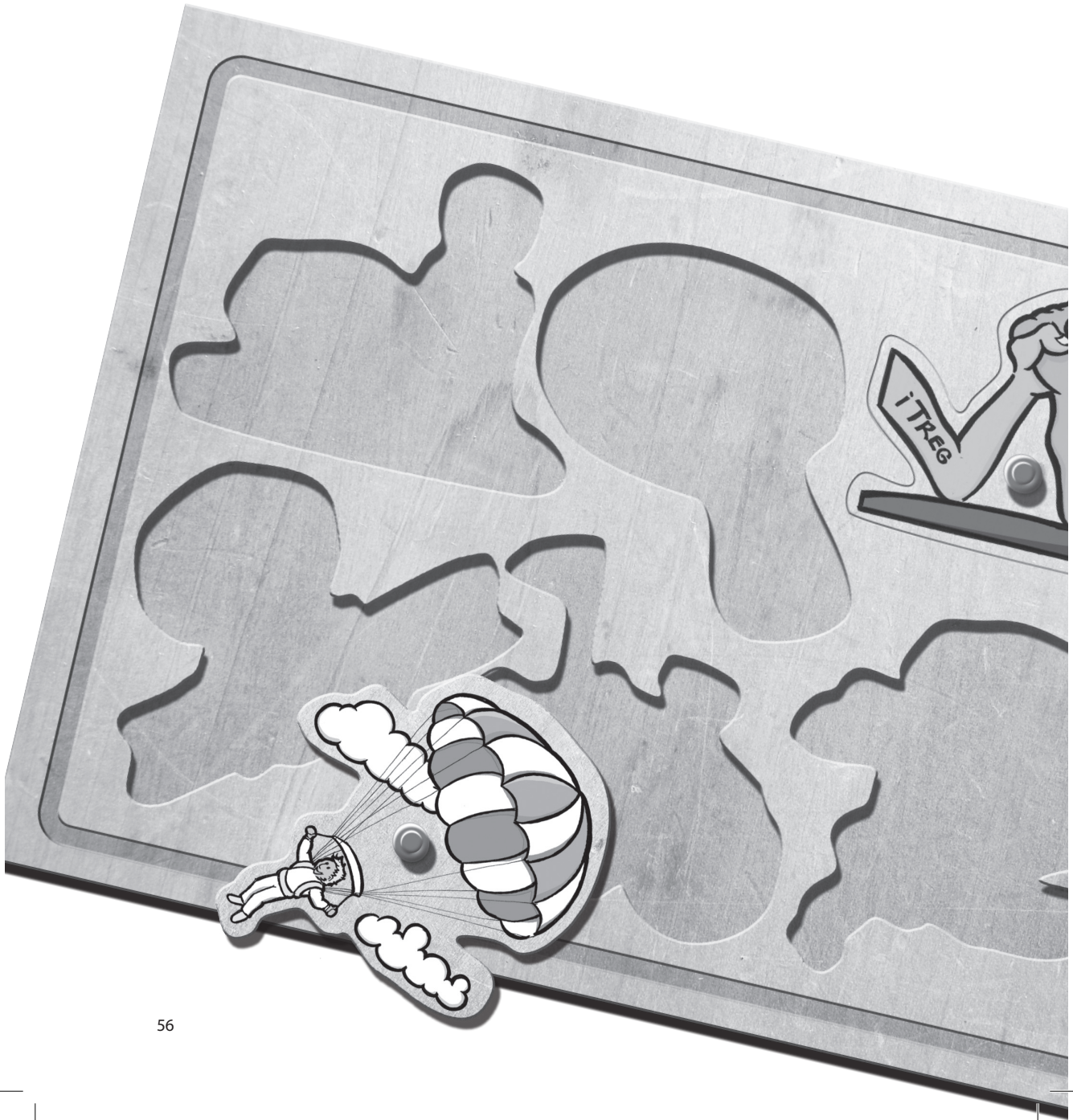
nTreg level of suppression

IL-5	IL-13	IL-10	IFNg	TNFa	IL-17	
0,0	0,0	0,0	0,0	0,0	0,0	Teff
-158,6# (209,8)	-32,3 (62,2)	-5940,3 (16939,9)	-275,1 (416,4)	-132065,5 (396125,5)	-74842,6 (223559,2)	Teff+Teff
-209,8 (346,7)	57,1 (37,2)	-5260,9 (13951,6)	64,1 (36,1)	-2939,6 (7919,8)	-64657,3* (170978,5)	10_1
-221,8 (258,1)	73,2 (15,7)	-1958,2 (4821,8)	78,9 (18,1)	57,2 (34,4)	-91260,8 (223069,9)	5_1
-95,6 (224,5)	78,4 (29,5)	-14056,6 (39414,3)	96,2 (4,8)	-2043,2 (6003,1)	-57914,0 (163598,7)	2_1
10,2 (94,4)	87,2 (11,5)	-14488,1 (43205,0)	96,3 (6,2)	83,0 (32,7)	-80248,3* (240507,0)	1_1

SF-Treg level of suppression

IL-5	IL-13	IL-10	IFNg	TNFa	IL-17	
0,0	0,0	0,0	0,0	0,0	0,0	Teff
-111,2# (98,6)	-124,5 (71,5)	-111,2 (98,6)	-44,8 (37,1)	-73,4 (90,5)	-59,0 (61,4)	Teff+Teff
23,2 (26,8)	43,5 (70,3)	23,2 (26,8)	57,4 (36,8)	52,1 (41,7)	52,2* (29,3)	10_1
33,9 (31,1)	57,2 (43,2)	33,9 (31,1)	68,3 (39,1)	63,3 (45,5)	43,1 (32,5)	5_1
38,5 (46,3)	90,1 (8,7)	38,5 (46,3)	83,5 (26,3)	78,4 (31,7)	65,4 (22,7)	2_1
50,2 (37,7)	97,0 (3,5)	50,2 (37,7)	95,6 (7,7)	94,0 (9,7)	81,0* (19,9)	1_1

3



Human induced CD4$^+$CD25$^+$FOXP3$^+$ T cells fail to suppress inflammation in mice with xenogeneic graft-versus-host disease

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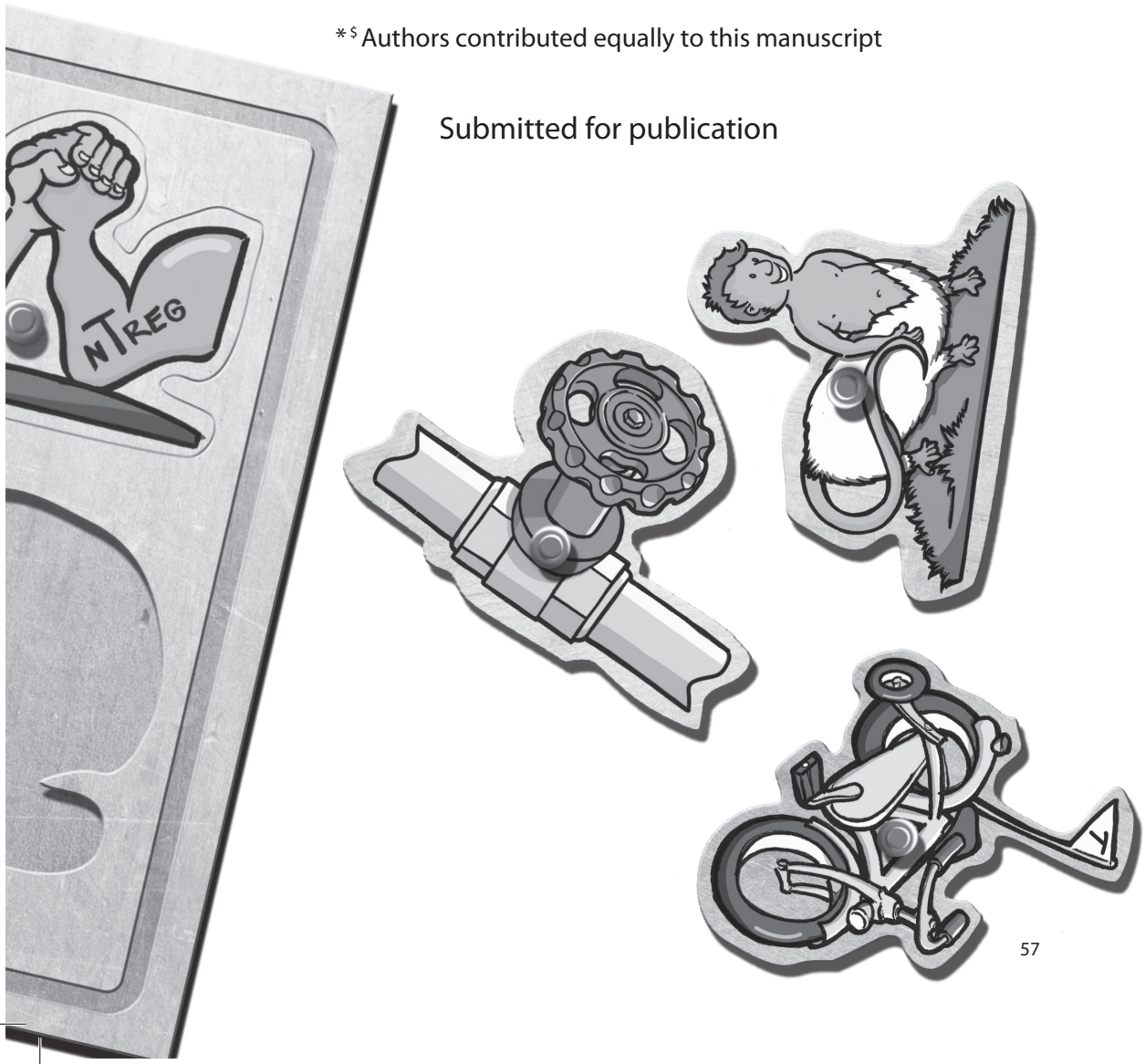


Abstract

$CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) are essential for immune homeostasis and prevent inflammatory diseases. Two subsets of Treg exist; thymus-derived naturally occurring Treg (nTreg), and peripherally induced Treg (iTreg). Induction and expansion of Treg are currently explored as potential therapy for human inflammatory diseases such as Graft-versus-Host Disease (GvHD). However, appropriate predictors of Treg functionality are lacking, since transient expression of FOXP3 does not result in suppressive capacity. In this study, we compared the phenotype and suppressive function of expanded human nTreg and iTreg. Both expressed similar levels of FOXP3, HLA-DR, CD62L, CD25, and CD45RO. iTreg expressed higher CTLA-4 and GITR, while only nTreg expressed Helios. PMA/ionomycin induced cytokine production was similar, and both types of Treg suppressed T cell activation in vitro. However, in a model for xenogeneic GvHD, induced by human PBMC in $RAG2^{-/-}\gamma\text{-}c^{-/-}$ mice, expanded nTreg suppressed disease, but iTreg failed. Moreover, iTreg lost FOXP3 expression shortly after administration, while nTreg maintained FOXP3. Thus, although expanded human nTreg and iTreg had similar properties in vitro, only nTreg maintained suppressive function and phenotype in vivo. These findings call for caution in the clinical use of iTreg and underscore that preclinical in vivo evaluation of cultured Treg is indispensable.

Introduction

$CD4^+CD25^+FOXP3^+$ Regulatory T cells (Treg) are important to maintain immune homeostasis. This has been extensively shown in several mouse models, such as Experimental Autoimmune Encephalomyelitis (EAE), diabetes, arthritis and Graft versus Host Disease (GvHD), in which the transfer of Treg can diminish disease (1-3). Furthermore, in several human inflammatory diseases, negative correlations are found between the presence of regulatory T cells and disease severity (4-6). For instance, in allogeneic stem cell transplantation the development of GvHD shows an inverse correlation with the presence of Treg. Furthermore, in diabetes mellitus type 1 patients treated with anti-CD3 antibodies resulting in enhanced Treg function, increased residual β-cell function and clinical improvement was observed (7, 8). Therefore, Treg are considered to be promising targets for the treatment or prevention of inflammatory diseases. A distinction in Treg poplulations can be made between natural Treg (nTreg), which are derived from the thymus, and peripherally induced Treg (iTreg). Since Treg constitute only a small proportion of peripheral blood T cells, several methods for in vitro Treg expansion have been described to enable their application in clinical trials. Later, methods for induction of Treg from $CD4^+CD25^-$ conventional T cells were described, since $CD4^+CD25^-$ T cells are present in large numbers and, therefore, could serve as a convenient source of Treg for therapy. These Treg expansion and induction protocols always include T cell receptor stimulation and co-stimulation through CD28, as well as addition of IL-2 , which in both human and mouse Treg are required for FOXP3 induction, as well as Treg survival, proliferation, and function (9-13). Nonetheless, there are also differences between these subpopulations (14, 15). For example, nTreg can be distinguished from iTreg by expression of transcription factor Helios, which is expressed only in nTreg (16). Furthermore, although expanded nTreg are suppressive in in vitro assays, human induced $FOXP3^+$ T cells do not consistently display suppressive function in vitro (17, 18), since activated effector T cells can also transiently express FOXP3. Moreover, it is unknown whether these cells exhibit suppressive function in vivo. In our view, preclinical testing of in vitro expanded or generated Treg in a reliable in vivo model is crucial for estimating the suitability of Treg for therapy. To this end, we previously developed a model for xenogeneic (x)–GvHD in $RAG2^{-/-}$commonγ-$chain^{-/-}$ mice. In this model, intravenous injection of human PBMC results in severe x-GvHD, and co-injection of freshly isolated human Treg suppresses x-GvHD development (19).

In this study we compared both the phenotype and suppressive function of iTreg and expanded nTreg. To this end, we analyzed expression of T cell activation and Treg markers, FOXP3 expression, cytokine production and in vitro suppressive capacitiy. Both Treg populations expressed similar levels of FOXP3 and other Treg markers, had similar cytokine secretion profiles, and equally suppressed T cell activation in vitro. However, only expanded nTreg, but not iTreg, suppressed x-GvHD due to a rapid loss of FOXP3 expression in iTreg in vivo. We conclude that this is an important observation as it shows that in vitro assays are insufficient to predict in vivo human Treg function. Our findings underscore the importance of preclinical in vivo evaluation of cultured Treg using validated in vivo models, such as our x-GvHD model, and it warrants serious caution for the use of expanded iTreg in human therapy for inflammatory disorders.

Materials and methods

Isolation of cells and expansion of Treg

Peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood of healthy volunteers or buffycoats (Sanquin Blood Bank, Amsterdam, The Netherlands), using Ficoll Isopaque density gradient centrifugation (Amersham Biosciences, NJ, USA). For iTreg induction, $CD4^+CD25^-$ T cells were magnetically isolated using CD4 T cell enrichment, followed by CD25 depletion (Miltenyi Biotec / BD IMAG). Platebound anti-CD3 (1.5 μg/ml, clone OKT3, eBioscience, CA, USA) and soluble anti-CD28 (2 μg/ml, clone CD28.2, eBioscience) were used for Treg induction. Where indicated, IL-2 (300 U/ml, Chiron, Uxbridge, UK) or TGFβ (5 ng/ml, eBioscience) were added. RPMI 1640 containing 10mM HEPES (Seromed), 2mM L-glutamine 100 U/ml penicillin-streptomycin and 10% Fetal Calf serum was used as culture medium for iTreg (all Invitrogen, Carlsbad, USA). Medium and soluble factors were refreshed every 3 or 4 days.
For nTreg expansion, $CD4^+CD25^{++}$ T cells were magnetically isolated by a human regulatory T cell isolation kit (Miltenyi Biotec, Auburn, CA, USA), and cultured with anti-CD3/anti-CD28 T cell expander beads (3 beads per cell, Invitrogen) in X-vivo medium (BioWhittaker, Walkersville, MA, USA), containing kanamycin (100 μg/ml), 5% human AB serum (Sanquin Blood Bank, Amsterdam, The Netherlands), TGFβ (5 ng/ml) and IL-2 (120 U/ml). Medium and soluble factors were refreshed every 3 or 4 days. On day 7 expander beads were removed from the culture and from day 10 cells were kept on low IL-2 up to day 13 or 14.

Mice and x-GvHD

$RAG2^{-/-}\gamma$-$chain^{-/-}$ mice on a Balb/c background were used as was previously described (19). X-GvHD was induced by intravenous injection human PBMC containing $10x10^6$ CD3+ cells. To test Treg suppressive capacity, 10×10^6 Treg were co-injected. Mice were scored for clinical GvHD symptoms and weighed twice per week. Weekly peripheral blood samples were drawn, starting in week 2.
For cell tracking with CFSE, iTreg and nTreg were labeled in PBS with 3 μM CFSE for 7 minutes at 37˚C and washed extensively with medium containing 10% FCS before injection. Mice were sacrificed three days after injection, and blood, spleen and bone marrow were collected for FACS analysis. All animal studies were conducted according to the current Dutch Law on animal experimentation.

Flow cytometry

For phenotype analysis of iTreg and nTreg, cells were washed twice in FACS buffer (PBS containing 2% FCS and 0.1% sodium azide), adjusted to $0.5\text{-}1 \times 10^6$ cells/ml in FACS buffer and blocked with mouse serum (5 min at 4°C). Subsequently, the cells were incubated in 50 μl FACS buffer containing 10% mouse serum and appropriately diluted PE, FITC, PerCP, APC, Pe-Cy7, Alexa647 labeled mAbs against human CD4 (clone RPA-T4, BD Biosciences), CD25 (clone 2A3, BD Biosciences), CD127 (clone HCD127, Biolegend), CCR4 (clone 1G1, BD Pharmingen), GITR (clone 110416, R&D), CD30 (clone Ber-H8, BD Biosciences), CD45RO (clone UCHL1, eBioscience), CD62L (clone DREG56, BD Pharmingen), CD95 (clone DX2, BD Biosciences), HLA-DR (clone L243, BD Biosciences). For intranuclear staining of FOXP3 with Pacific Blue, eFluor 450 or PERCP-Cy5.5-conjugated FOXP3 antibody (clone PCH101), V450 FOXP3 (clone 259D, BD Bioscience), the cells were first surface stained, then fixed, permeabilized and stained using a FOXP3 staining kit (eBioscience) according to the manufacturer's instructions. Other intracellular proteins were all co-stained in the FOXP3 staining protocol: CTLA-4 (clone BN13, BD Pharmingen), Helios (clone 22F6 , Biolegend).
For cell analysis in the x-GvHD model, whole blood stainings were performed (19). Cells were stained with CD19/CD3 Simultest (BD Biosciences), CD4/CD8 Simultest (BD Biosciences), anti-human CD45 (clone 2D1, BD Biosciences) and anti-mouse CD45 (clone 30-F11, BD Pharmingen).

Flow-Count Fluorospheres (Beckman Coulter, Ireland) were added to the samples to determine absolute cell numbers, as described before (19).
Data were analyzed with FACS DIVA software (BD Biosciences).

Analysis of cytokine production by multiplexed particle-based flow cytometric assay

Cells were stimulated for 48 hours in serum-free x-vivo medium with 10 ng/ml PMA and 500 ng/ml ionomycin. Cell culture supernatants were collected, stored at -80°C and processed within 1 month. Cytokine concentrations were measured with the Bio-Plex system in combination with the Bio-Plex Manager software, version 4.0 (Bio-Rad Laboratories, Hercules, CA, USA), which employs the Luminex xMAP technology as previously described (20). The following cytokines were measured: IL-2, IL-5, IL-7, IL-10, IL-13, IL-17, tumor necrosis factor-α (TNF-α), and interferon-γ (IFNγ).

Suppression assay

Suppressive capacity of Treg was tested in co-cultures with autologous PBMC, activated with anti-CD3 (1.5 μg/ml) for 6 days, the last 18h in the presence of [^{3}H]thymidine (1 μCi/well). The suppressive activity was determined by calculating the relative difference in proliferative response (mean [^{3}H]thymidine incorporation (cpm) of triplicate wells) between PBMC cultured alone and PBMC cultured in the presence of induced regulatory T cells. For CFSE based analysis, PBMC were labeled with 3 μM CFSE for 10 min at 37°C and extensively washed with 10% FCS containing medium, and proliferation of CD4$^+$ T cells was analyzed by flow cytometry. To exclude crowdedness-mediated suppression in the wells, extra PBMC were added instead of Treg.

Statistical analysis

For statistical analysis of multiple groups One-way ANOVA or nonparametric ANOVA; Kruskal-Wallis test, were used. Bonferroni or Dunn's Multiple Comparison Test post test were used, to compare between two selected groups. To compare between two groups, non-parametric T-test, Mann Whitney was used. P values below 0.05 were considered significant.

Results

iTreg express FOXP3 levels similar to expanded nTreg

In order to compare expanded natural Treg (nTreg) with induced and subsequently expanded Treg (iTreg), we first established the optimal culture conditions for induction of $CD4^+CD25^+FOXP3^+$ T cells from $CD4^+CD25^-$ T cells. We used anti-CD3 and anti-CD28 antibodies for T cell activation, and compared low level IL-2 (10 U/ml) with high level IL-2 (300U/ml). As expected, a high level of IL-2 induced much higher FOXP3 expression, already after 5 days of culture (**Figure 1A**) (21, 22). Since TGF-β is known to induce FOXP3 expression (21, 22), we investigated whether addition of exogenous TGFβ further increased FOXP3 expression. Cells were cultured in medium containing 10% fetal calf serum (FCS) for 7 days. Addition of TGFβ did not alter the level of FOXP3 expression. Since serum contains TGFβ, we also repeated the culture in serum-free medium, but again no change in FOXP3 expression was observed (**Figure 1B**), Thus, in our system, we continued to generate iTreg by stimulating $CD4^+CD25^-$ T cells with anti-CD3 and anti-CD28 antibodies in the presence of 10% FCS and 300 U/ml of IL-2 without addition of TGFβ.

Next, we compared FOXP3 expression of both iTreg and $CD4^+CD25^{++}$ nTreg before and after in vitro expansion. As expected, FOXP3 expression at the start of culture differed strongly; almost no $CD4^+CD25^-$ T cells expressed FOXP3 (< 1%, **Figure 1D**), while at least 50% of the $CD4^+CD25^{++}$ T cells expressed FOXP3. However, at the end of culture, both Treg cultures contained a similar percentage of $FOXP3^+$ cells (70%, **Figure 1C,D**). Furthermore, the FOXP3 content per cell within $FOXP3^+$ cells was similar in both populations (**Figure 1E**). Thus, after expansion iTreg and nTreg displayed a similar and stable FOXP3 expression.

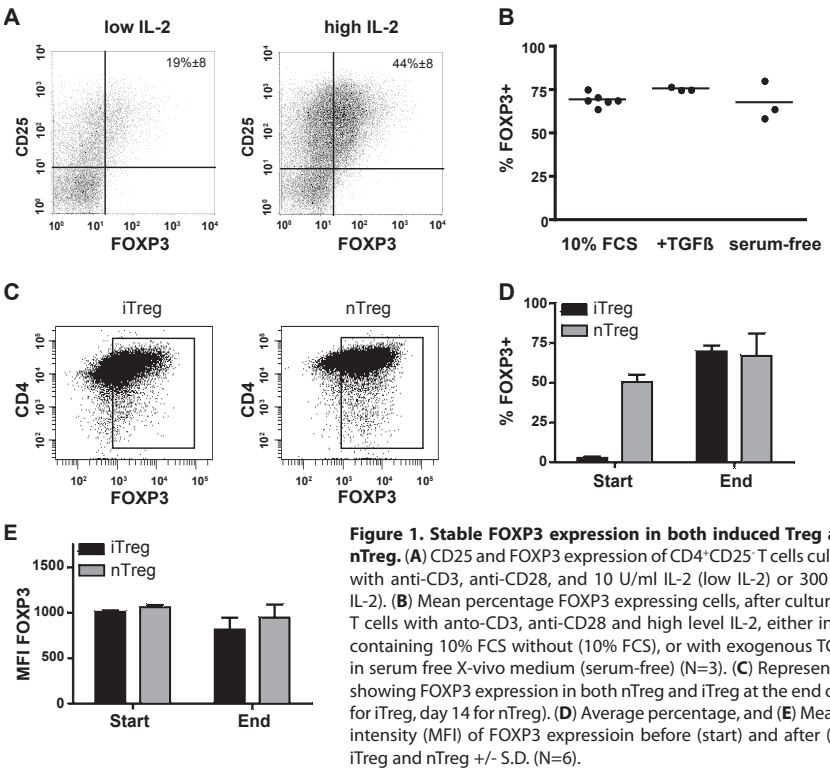


Figure 1. Stable FOXP3 expression in both induced Treg and expanded nTreg. (**A**) CD25 and FOXP3 expression of $CD4^+CD25^-$ T cells cultured for 5 days with anti-CD3, anti-CD28, and 10 U/ml IL-2 (low IL-2) or 300 U/ml IL2 (high IL-2). (**B**) Mean percentage FOXP3 expressing cells, after culture of $CD4^+CD25^-$ T cells with anto-CD3, anti-CD28 and high level IL-2, either in RPMI medium containing 10% FCS without (10% FCS), or with exogenous TGFβ (+TGFβ), or in serum free X-vivo medium (serum-free) (N=3). (**C**) Representative dot plots showing FOXP3 expression in both nTreg and iTreg at the end of culture (day 7 for iTreg, day 14 for nTreg). (**D**) Average percentage, and (**E**) Mean Fluorescence intensity (MFI) of FOXP3 expressioin before (start) and after (end) culture of iTreg and nTreg +/- S.D. (N=6).

Expanded nTreg and iTreg have a similar but not identical phenotype

In order to compare the two Treg subtypes more extensively, we compared the expression of several well-described T cell markers, associated with Treg function (CD25, CCR4, CTLA-4, GITR, CD127, HLA-DR), Treg origin (Helios (16)), and T cell activation and differentiation (CD62L, CD45RO) (**Figure 2A**). As expected, both iTreg and nTreg expressed low level of CD127 and high levels of CD25 (23, 24), and were mainly of memory phenotype as indicated by the expression of CD45RO in a substantial percentage (65-84%) of cells. Up to 20- 30% of both Treg populations co-expressed CD62L, HLA-DR and CCR4. As expected, Helios, was only expressed by nTreg (15-33%) but absent in iTreg. Remarkably, the expression of CTLA-4 and GITR, two molecules frequently associated with Treg function were significantly higher on iTreg than on nTreg ($p<0.01$). Thus, although both Treg populations had a very similar expression of various markers associated with a Treg phenotype, and only the higher levels of expression of CTLA-4 and GITR in Treg suggested a difference between these Treg populations.

Expanded nTreg and iTreg produce similar levels of cytokines

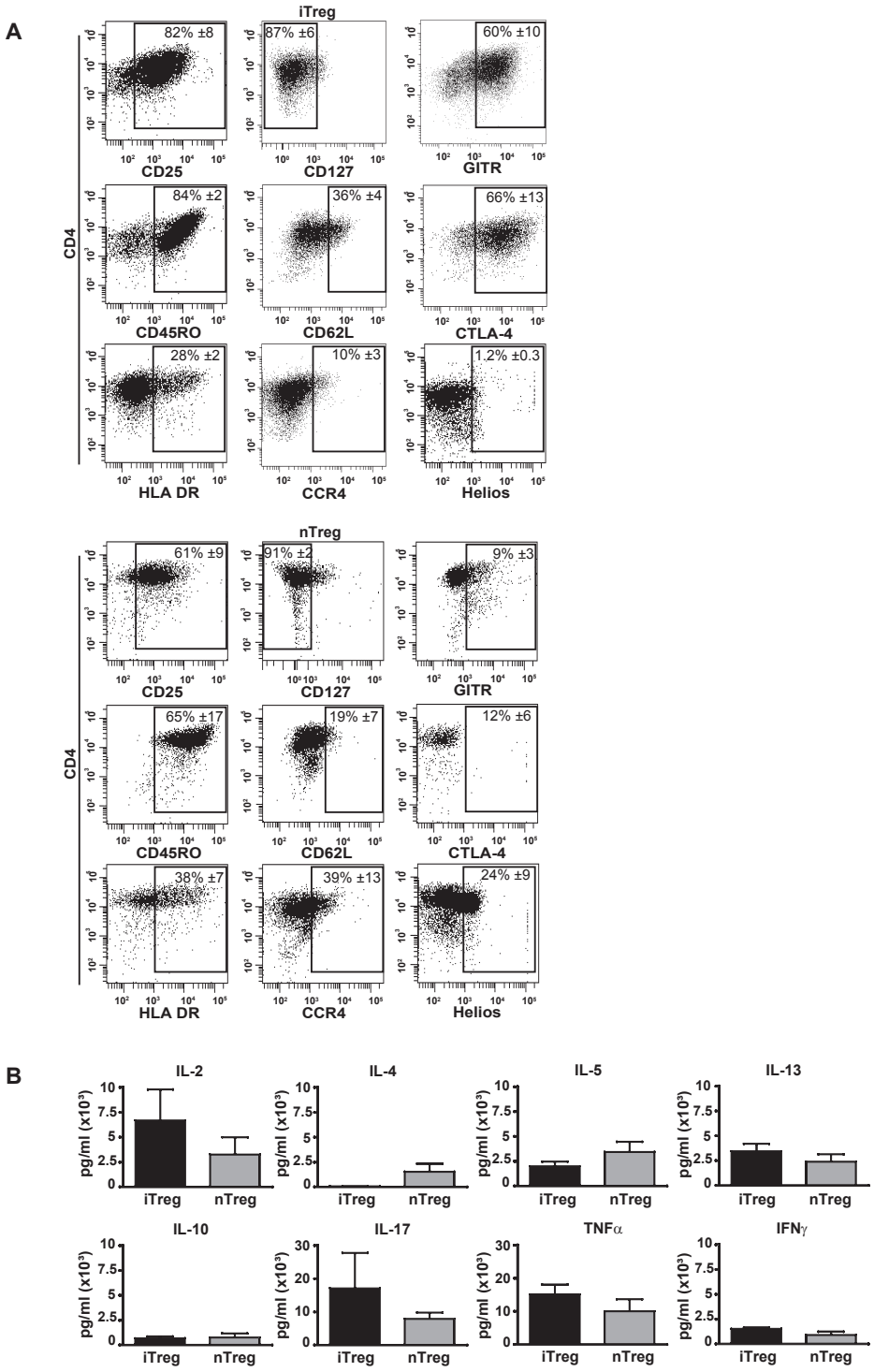
Since cytokines secreted by T cells are frequently associated with their functions, we next determined the cytokine secretion profiles of expanded nTreg and iTreg using a multiplex based cytokine analysis of culture supernatants after stimulation of Treg with PMA and ionomycin (**Figure 2B**). No significant differences in IL-2, IL-4, IL-5, IL-13, IL-10, IL-17, TNFα, and IFNγ secretion were detected between iTreg and nTreg. Although the capacity to produce cytokines (after stimulation with PMA and ionomycin) of nTreg and iTreg was similar at the end of culture, the cytokine profiles indicated that both Treg types were capable of producing high levels of inflammatory cytokines. All T cell cytokines, except IL-10, were highly produced at the same time, and comparable to cytokine levels produced by $CD4^+CD25^-$ effector T cells (Teff), suggesting that the cells are not driven towards one particular T helper phenotype (**Figure 2C** and **Table S1**). Thus, both expanded nTreg and iTreg produced high levels of various T cell cytokines, in similar amounts.

Both iTreg and nTreg suppress T cell responses in vitro

For obvious reasons, the most important parameter for Treg function is to compare the suppressive capacity of both iTreg and expanded nTreg. To this end, we first conducted classical in vitro suppression assays, both with ^3H thymidine incorporation and CFSE labeling as a measure for effector T cell proliferation. In ^3H thymidine incorporation assays, iTreg and nTreg equally suppressed T cell proliferation up to 70% (**Figure 3A**). This was confirmed by CFSE assays, which showed clear suppression of proliferation of CFSE labeled $CD4^+$ T cells by both iTreg and nTreg (**Figure 3B**). Furthermore, iTreg cultured for 3 or 7 days longer still suppressed in vitro (data not shown), indicating that suppressive capacity of iTreg was stable during in vitro culture.

nTreg, but not iTreg, suppress x-GvHD in vivo

To gain a better insight into the therapeutical value of in vitro generated Treg, we extended the analysis of iTreg and nTreg suppressive function to our humanized mouse model for x-GvHD. In this model, we injected human PBMC to induce lethal x-GvHD and co-injected iTreg or nTreg to analyze their capacity to suppress the development of x-GvHD. Control mice receiving no cells survived throughout the experiment as expected, while mice receiving only PBMC developed severe x-GvHD and survived no longer than three weeks (**Figure 3C**). Co-injection of nTreg significantly prevented development of x-GvHD. Remarkably however, iTreg treatment did not delay development of x-GvHD at all. The failure of iTreg to control x-GvHD was reflected in other x-GvHD scores, including fur-shape and mobility (**Figure 3D**). Furthermore, ex vivo analyses of peripheral blood collected from mice demonstrated that nTreg, but not iTreg, could suppress the elevation of human T cell numbers (**Figure 3E**). Thus, while both expanded nTreg and iTreg equally suppressed T cell activation in vitro, only expanded nTreg were able to prevent x-GvHD. This indicated that in vitro analysis of Treg suppressor function was not sufficient to predict the in vivo capacity of these cells.


A
iTreg
82% ±8
87% ±6
60% ±10
CD25
CD127
GITR
84% ±2
36% ±4
66% ±13
CD45RO
CD62L
CTLA-4
28% ±2
10% ±3
1.2% ±0.3
HLA DR
CCR4
Helios
CD4
nTreg
61% ±9
91% ±2
9% ±3
CD25
CD127
GITR
65% ±17
19% ±7
12% ±6
CD45RO
CD62L
CTLA-4
38% ±7
39% ±13
24% ±9
HLA DR
CCR4
Helios
CD4
B
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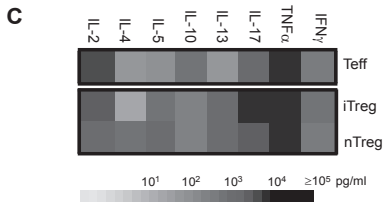

Figure 2. Phenotype and cytokine production profile of cultured nTreg and iTreg. At the end of culture nTreg and iTreg were stained for flow cytometry, or were stimulated with PMA and ionomycin, followed by analysis of cytokine secretion in supernatants (**A**) Representative dotplots for several T cell markers at the end of culture of nTreg (left panel) and iTreg (right panel). Mean expression of T cell markers at the end of culture is presented in the gates +/- S.D. (iTreg N=5, nTreg N=6). (**B**) Cytokine excretion by iTreg and nTreg. Shown are averages +/- S.E.M. (N=6). (**C**) Color profile of average cytokine excretion comparing nTreg, iTreg and CD4+CD25- effector T cells (Teff) (iTreg N=5, nTreg N=6, Teff N=6).

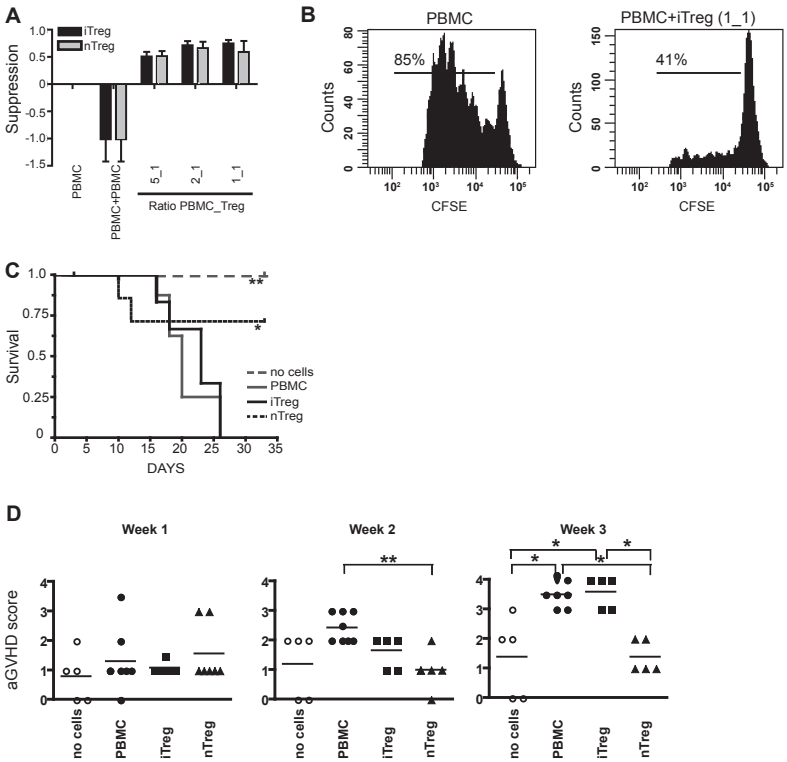

Figure 3. nTreg and iTreg both suppress in vitro, but only nTreg suppress x-GvHD. nTreg and iTreg were cultured and tested in an in vitro suppression assay (**A**) Average level of suppression of T cell proliferation by nTreg and iTreg, determined by 3H Thymidine incorporation (N=5). (**B**) PBMC were labeled with CFSE and stimulated with anti-CD3.Representative histograms of proliferation of gated $CD4^+$ T cells in cultures with PBMC alone (left histogram), or in co-cultures of PBMC with iTreg (right histogram). iTreg and nTreg were cultured and co-injected with PBMC into $RAG2^{-/-}\gamma\text{-}c^{-/-}$ mice to test in vivo suppressive capacity in x-GvHD. (**C**) Survival Curve of mice receiving no cells (no cells), only PBMC (PBMC), PBMC and iTreg (iTreg), PBMC and nTreg (nTreg). (**D**) Average absolute numbers of cells per week. (**E**) Average absolute numbers of cells per microliter blood in week 2. Shown are averages ± S.D. (N=8 for PBMC, iTreg, nTreg, N=5 for no cells).

iTreg lose FOXP3 expression in vivo

In order to establish the cause of the lack of suppressive capacity of iTreg in vivo, we performed another in vivo x-GvHD experiment using CFSE labeled Treg, allowing us to monitor their numbers and levels of FOXP3 expression. Three days after injection of PBMC with or without labeled iTreg or nTreg, mice were sacrificed, and blood and spleen cells were harvested and analyzed by flow cytometry. CFSE$^+$Treg were clearly visible in the groups with iTreg and nTreg, and could be distinguished from the non-labeled PBMC (**Figure 4A**). In both blood and spleen, percentages of FOXP3$^+$ cells were higher in the nTreg treated group compared to iTreg (**Figure 4B**). Furthermore, percentages of FOXP3 expressing Treg, compared to the percentage FOXP3$^+$ cells within the Treg populations at time of infusion (day 0), were much lower in iTreg compared to nTreg in blood (iTreg 4%, nTreg 35%), but also in spleen (iTreg 28%, nTreg 41%) (**Figure 4C**). Furthermore, cell numbers of FOXP3$^+$ Treg were 4-fold higher in blood and spleen of mice that received nTreg, compared to recipients infused with iTreg (**Figure 4D**). Thus, although iTreg maintained stable high levels of FOXP3 when cultured with high IL-2 in vitro, we found a dramatic decrease of FOXP3 expression in iTreg shortly after injection, which probably resulted in iTreg being unable to suppress x-GvHD.

Taken together, phenotypically iTreg and nTreg seemed similar and both had excellent suppressive function in vitro. However, in vivo iTreg rapidly lost FOXP3 expression and suppressive function, while nTreg maintained FOXP3 expression and suppressed x-GvHD.

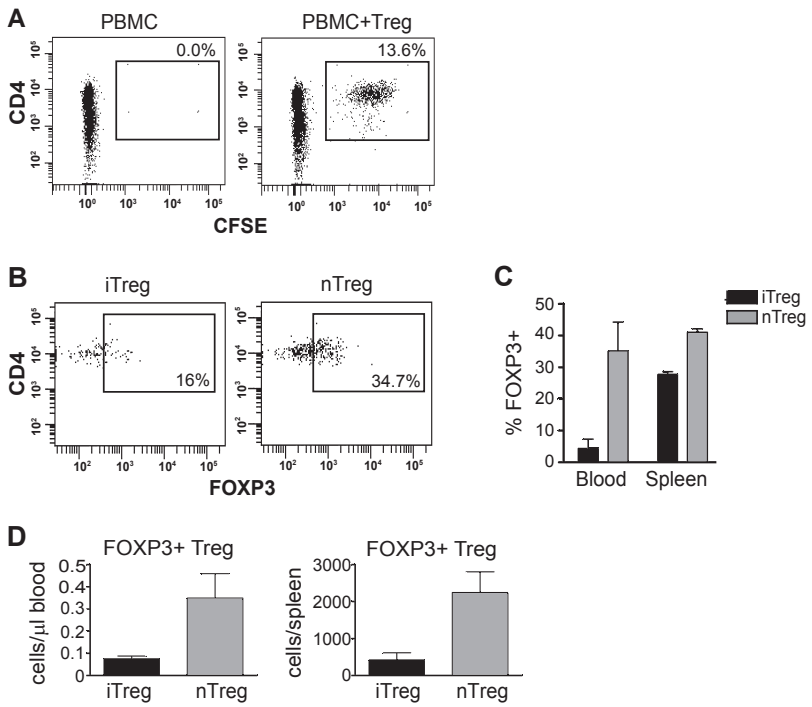

Figure 4. FOXP3$^+$ iTreg are unstable in vivo. iTreg and nTreg were labeled with CFSE and co-injected with PBMC into RAG2$^{-/-}\gamma$-c$^{-/-}$ mice. Stability of FOXP3 expression was analyzed in the CFSE labeled Treg populations. (**A**) Representative dotplots showing analysis of human cells from spleen from mice injected with PBMC alone (left dotplot), or PBMC and CFSE$^+$ Treg (right dotplot). The gate contains CFSE$^+$ Treg. (**B**) Representative dotplots showing FOXP3 expression for gated CFSE$^+$ iTreg (left dotplot) or CFSE$^+$ nTreg (right dotplot) from spleen. (**C**) Percentage of FOXP3$^+$ cells in gated CFSE$^+$ Treg relative to FOXP3 expression on day of injection. (**D**) Absolute numbers of FOXP3$^+$ Treg per micro liter blood (left graph) and per spleen (right graph). Figures show averages ± S.D. (N=3 for PBMC, N=2 for iTreg, N=2 for nTreg)

Discussion

Numerous studies in experimental models have demonstrated that Treg play a pivotal role in the preservation of the immune balance. Therefore, Treg are promising candidates for immune therapy of human inflammatory diseases. This has resulted in the development of various approaches such as antigen specific therapy and cellular therapy to augment Treg function or numbers (8, 25, 26). Despite significant progress in the field of Treg research in experimental models, 14 years after the initial description of Treg by Sakaguchi (27), this effort has not resulted in a tangible therapeutic application in humans. There are several reasons for this lack of translation. First of all, the relevance of Treg for human diseases inevitably depends on either mouse models, or in vitro human studies, which may not be directly translatable to the human in vivo situation. Therefore, the interpretation of these studies obviously is difficult. Data are mostly lacking on the in vivo behavior (expansion and mode of action) of Treg in humans, especially during conditions of inflammation. For example, it is still unclear whether the increased numbers of $FOXP3^+$ T cells found at inflammatory sites in most human autoimmune diseases (5, 28) reflects a deficiency of Treg function or is due to the infiltration of T effector cells that transiently express FOXP3. Because of a lack of a unique marker for true Treg, in vitro suppressive function is considered the golden standard for true Treg. Here we show that even this golden standard is not sufficient to unequivocally define Treg identity, since in vitro suppression by expanded iTreg did not translate to in vivo suppression. Despite extensive analyses, we did not find any specific in vitro properties in either of the Treg subtypes that could have predicted this difference in in vivo suppressive function. For instance, Treg associated markers such as CD25, FOXP3, CD127, HLA-DR, CD62L, were all expressed in a similar fashion. Moreover, while iTreg expressed more CTLA-4 and GITR, which could theoretically suggest a better suppressive function, this rather indicated that iTreg were highly activated cells. Both Treg subtypes had the capacity to produce a high variety of cytokines after PMA and ionomycin stimulation, which was similar between both populations, showing apparent resemblance of iTreg and nTreg. While Helios was only expressed by nTreg, it was only observed in 33% of the nTreg. Furthermore, Helios is an intracellular transcription factor, thus not suitable for selection of Treg for therapeutic goals. Taken together, there remains no unique marker to differentiate expanded iTreg from nTreg and more importantly, to select functional Treg.

Thus, in vitro Treg properties, even in vitro suppressive capacity, cannot directly be translated to in vivo functionality. The reason for this could lie in the stability of in vivo FOXP3 expression. However, similar to suppressive function, in vivo FOXP3 expression could not be predicted from in vitro studies, since FOXP3 expression in iTreg remained both stable and high when cultured with high IL-2. However, once administered in vivo, FOXP3 expression decreased dramatically, which may have resulted in the loss of Treg function. These results correspond with a recently published report on murine iTreg, in which a rapid loss of FOXP3 expression was also observed after injection in vivo (29). During in vitro assays, suppression of T cell activation is already established immediately after T cell activation; when Treg are added to cultures with readily activated effector T cells (for murine cells already from 12 hours upon antigenic stimulation (30)), they are unable to suppress effector T cell activation. iTreg expressed high FOXP3 levels at the end of culture and thus were likely functionally suppressive, before they had lost FOXP3 expression. However, in the x-GvHD model activation of T cells takes longer than in vitro. In vivo, three days after iTreg were administered FOXP3 expression was almost completely lost, while the disease symptoms occurred much later. In vitro we observed that continuous high levels of IL-2 are required to induce FOXP3 expression. Thus, iTreg lose FOXP3 expression rapidly once they are no longer contained in culture with high levels of IL-2, and therefore are probably unable to suppress long-lasting inflammation in vivo.

Taken together, here we show that in vitro suppression assays do not predict in vivo Treg suppressive capacity. Until a unique marker is found that can better predict the in vivo function of true Treg, the data presented in this study cast serious doubts on the use of polyclonally

induced Treg for in vivo immune therapy in humans. Therefore, preclinical in vivo evaluation of cultured Treg using validated in vivo models, such as our x-GvHD model, is required before proceeding to clinical testing.

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Supplementals

Table S1. Cytokine production by iTreg and nTreg, compared to Teff. $CD4^+CD25^-$ effector T cells (Teff), iTreg and nTreg were stimulated up to 48 hours with pma and ionomycin. Culture supernatants were analyzed by multiplexed particle-based flow cytometric assay. Means (s.e.m.) of N=6 (nTreg and Teff), or N=5 (iTreg) are given.

IL-2	IL-4	IL-5	IL-10	IL-13	IL-17	TNFα	IFNγ	
9876 (3759)	182 (86)	253 (94)	1671 (663)	155 (83)	4192 (1856)	10211 (3252)	845 (336)	Teff
6673 (3125)	71 (24)	1984 (476)	667 (208)	3438 (754)	17087 (10703)	15075 (3037)	1521 (119)	iTreg
3252 (1737)	1522 (816)	3426 (1040)	764 (405)	2384 (756)	7901 (1880)	10019 (3638)	892 (350)	nTreg


CD30

CD30 discriminates heat shock protein 60 induced FOXP3^{+}CD4^{+} T cells with a regulatory phenotype

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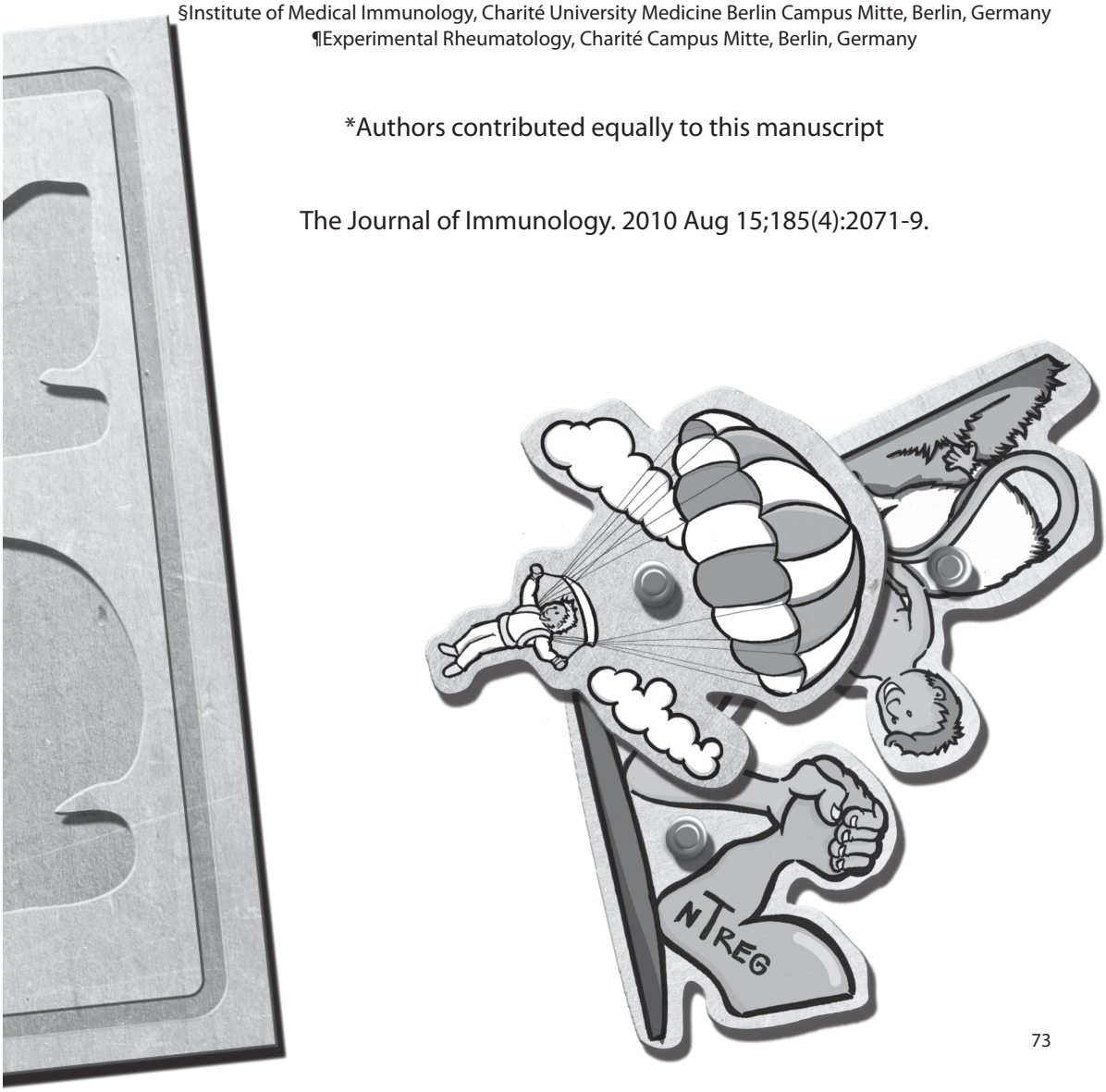

Abstract

In many animal models the manifestations of inflammatory diseases can be prevented by the adoptive transfer of CD4$^+$FOXP3$^+$ regulatory T cells (Treg). CD4$^+$FOXP3$^+$ Treg can be obtained by isolation and expansion of polyclonal naturally occurring Treg, or by antigen specific activation of CD4$^+$CD25$^-$FOXP3$^-$ T cells. Two major obstacles are hampering the translation of this latter protocol into therapeutic application. First, there is a lack of knowledge on relevant autoantigens. Second, the resulting population is contaminated with activated CD4$^+$ T cells that transiently express FOXP3, but gain no regulatory function. Therefore these cells may not be safe for clinical application. Here we demonstrate that highly suppressive FOXP3$^+$ Treg can be induced in vitro by the activation of CD4$^+$CD25$^-$ T cells with the self-antigen human heat shock protein 60 (HSP60). The activation induced suppressive FOXP3$^+$ Treg can be distinguished by surface-expression of CD30 from non-suppressive FOXP3$^+$ effector cells. We confirm that the induced CD30$^+$FOXP3$^+$ Treg recognize HSP60 epitopes, and that the induction of Treg by HSP60 is enhanced by signaling via TLR4 on APC. These findings have implications for the generation and isolation of pure populations of antigen specific Treg, with the potential to prevent and treat human inflammatory diseases.

Introduction

Owing to intense research during the past decade, $CD4^+FOXP3^+$ regulatory T cells (Treg) have emerged as a central T cell population for preserving peripheral tolerance. Reduced frequencies or impaired function of this Treg cell population leads to a wide range of autoimmune disorders in animals (1) and putatively also in humans (2-7). This indicates that an imbalance between autoreactive T cells and Treg may contribute to the pathogenesis of such diseases. Thus, learning how to manipulate this balance, by either expanding the pool of $CD4^+FOXP3^+$ Treg and/or enhancing their suppressive activity has obvious therapeutic relevance.

In many animal models the onset or progression of inflammatory (auto) immune diseases can be prevented by an adoptive transfer of Treg. This approach has been successfully applied in the treatment of experimental autoimmune encephalitis (EAE) (8), experimental type I diabetes, arthritis (9) and colitis (10). Of specific interest are studies suggesting that the success of Treg-based immunotherapy depends on the antigen specificity of the Treg (11, 12). For example, studies utilizing non-lymphopenic mouse models of autoimmune diabetes showed that organ-specific Treg are superior in disease protection compared to polyclonal Treg (11, 13). The use of antigen-specific Treg may also avoid dampening of immune responses to tumors and infectious agents, which are possible side effects of transferring large numbers of polyclonal Treg. Since transferred antigen-specific Treg can suppress T cells through bystander suppression, therapeutic Treg do not require specificity for the antigen initiating the disease. However, the Treg need to be specific for tissue-antigens capable of activating Treg. Here lays one of the most critical issues with respect to translating the successful results of Treg based immunotherapy in transgenic mice to human autoimmune diseases; in most human autoimmune diseases relevant auto-antigens have not been identified yet, despite intensive research. In addition, the establishment of Treg cell lines to predefined antigens is technically challenging. Antigen specific $FOXP3^+$ Treg can be generated *in vitro* by activating $CD4^+CD25^-FOXP3^-$ T cells in the presence of TGFβ. However, FOXP3 can also be transiently expressed in effector T cells without gain of regulatory function (14-16). The resulting population therefore contains a mixture of $FOXP3^+$ Treg and $FOXP3^+$ effector cells and may not be safe for clinical application. It was recently shown that the two $FOXP3^+$ cell types can be distinguished by the methylation pattern at the FOXP3 locus (17). However, a surface marker that enables selective sorting of activation induced $FOXP3^+$ Treg is still lacking.

Earlier studies suggested that heat shock proteins (HSPs) are good targets for antigen-specific Treg based immunotherapy in human inflammatory disease (18). HSPs are evolutionary strongly conserved proteins present in all eukaryotic and prokaryotic cellular organisms. They are expressed both constitutively and under stressful conditions, such as ultraviolet radiation, infections and malignancies (19-21). Besides being potent activators of the innate immune system (22, 23), HSPs have important immune regulatory effects. The human 60-kDa heat shock protein (HSP60) molecule can down regulate T cell migration (24) and inhibit the secretion of proinflammatory cytokines by activated T cells (25). This anti-inflammatory effect may be mediated by a direct TLR2 mediated effect of HSP60 on intrinsic Treg, resulting in enhancement of regulatory T cell function (26). Furthermore, extensive studies in animal models, as well as Juvenile Idiopathic Arthritis (JIA) patients (27-29), have provided evidence that specific T cell responses against HSPs, especially HSP60, are associated with anti-inflammatory regulation (30). In almost all models of experimental arthritis, including adjuvant arthritis (AA), pre-immunization with HSP65 protects animals from arthritis (31-33). This protection is mediated by the activation of self-HSP-specific T cells with regulatory characteristics and capable of down regulating inflammation (31, 34). Also in arthritis-patients specific T cell responses against HSP60 are found. These T cell responses against endogenously produced HSP60, which is abundantly expressed in the synovial lining cells of the patients (35), are associated with a good prognosis (28, 36). Thus, HSPs fulfil two non-redundant criteria for antigen-specific Treg based immunotherapy, namely selective expression at sites of inflammation and immunogenicity.

In the present report, we explored the potential of human HSP60 as a target for Treg based immunotherapy. We found that suppressive HSP60-specific FOXP3$^+$ Treg can be generated in vitro and distinguished from FOXP3$^-$ cells and from FOXP3$^+$ effector cells without regulatory function by the surface expression of CD30. We therefore conclude that CD30 represents a marker that separates contaminating HSP60 induced FOXP3$^+$ effector T cells from immune suppressive FOXP3$^+$ Treg. The HSP60-mediated differentiation to a Treg phenotype was enhanced by TLR4 signalling on APC. These findings may allow the generation and isolation of pure populations of HSP60-specific Treg and could have implications for the development of Treg-based immunotherapy for human inflammatory diseases.

Materials and methods

Participants

Buffy coats were taken from healthy volunteers (n=15) and 20 ml blood samples from patients with Juvenile Idiopathic Arthritis (JIA, n=6) or Rheumatoid Arthritis (RA, n=1). The JIA patients were followed up at the department of Pediatric Immunology and Rheumatology and the RA patient at the department of Rheumatology of the University Medical Center Utrecht, the Netherlands. The study was approved by the local institutional review board, and oral consent was obtained from all healthy volunteers and patients or their parents.

Cells, medium and reagents

PBMC were isolated using Ficoll Isopaque density gradient centrifugation (Amersham Biosciences, New Jersey, USA). Very low endotoxin RPMI 1640 (Seromed, Berlin, Germany) containing 10mM HEPES (Seromed), 2mM L-glutamine (Seromed), 20 μg/ml streptomycin, 60 mg/ml penicillin and 10% human AB-serum was used as a culture medium. Low endotoxin HSP60 (<0.3 pg/μg protein) was obtained as a kind gift from dr Ruurd van der Zee, from the Faculty of Veterinary Medicine, Utrecht, the Netherlands, and from StressGen (Victoria, BC Canada). Measurement of the endotoxin levels in the used HSP60 samples was contracted out to Cambrex (Rapid Endo-test, Cambrex, Verviers, Belgium). In some experiments TLR4 blocking antibody (clone HTA152, Serotec, Oxford, UK) or TLR2 blocking antibody (clone TL2.1, Biolegend, San Diego, USA) was used. For these experiments, APC or $CD4^+CD25^-$ T cells were incubated (45 minutes, 37°C) with 20 μg/ml TLR4 blocking antibody before addition to the cultures (37). The survival of the cells was measured at day 7 by staining the cells with 7-AAD (Becton Dickinson Biosciences (BD), San Jose, CA, USA).

Flow cytometry

PBMC were washed twice in FACS buffer (PBS containing 2% FCS) and adjusted to 0.5-1 x 10^6 cells/ml in staining buffer (FACS buffer containing 0.1% sodium azide) and blocked with mouse serum (30 min at 4°C). Subsequently, the cells were incubated in 50μl FACS buffer containing three or four appropriately diluted PE, FITC, CY or APC labeled mAbs against human CD4 (clone RPA-T4), CD25 (clone 2A3), CD30 (clone Ber-H83), CD127 (clone hIL-7R-m21), CCR4 (clone 1G1), GITR (glucocorticoid-induced TNFR related protein, clone 110416) and TLR-4 (HTA125). For intracellular staining of CTLA-4 (clone BN13) the cells were first surface stained, then fixed in Cytofix/Cytoperm solution (20 min, 4ºC) and washed twice in Perm/Wash solution (Cytofix/perm kit, BD, San Jose, CA), followed by incubation with anti-CTLA-4, anti-IL-10, or anti-IFNγ mAb. For FOXP3 staining (clone PCH101) the cells were first surface stained and subsequently treated with a FOXP3 staining kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. GITR mAb was obtained from R&D Systems (Wiesbaden, Germany). TLR4 mAb was obtained from Serotec (Oxford, UK) and FOXP3 mAb from eBioscience (San Diego, CA, USA). All other mAbs were obtained from Becton Dickinson (San Jose, CA, USA). Stained mononuclear cells were diluted in sheath fluid and run on a FACSCalibur (BD). CellQuest software (BD) was used for analysis.

Induction of regulatory T cells

$CD4^+CD25^-$ T cells from healthy donors were obtained by a first step of CD4 T cell enrichment and a second step of CD25 negative selection using CD4 T cell isolation kit, CD25 microbeads and VarioMACS selection columns (Miltenyi Biotec, Bisley, Surrey, UK). Contamination of the $CD4^+CD25^-$ T cell fraction was <3%. Irradiated (3500 Rad) $CD4^+$ T cell depleted PBMC were used as antigen-presenting cells (APC). $CD4^+CD25^-$ T cells and APC (1:3) were cultured in the presence of human HSP60 (10 μg/ml) for 7 days. After 7 days the cells were evaluated on the expression of the above-described markers. Furthermore, $CD4^+CD25^-$ and induced $CD4^+CD25^+$ T cells

were sorted by FACS and analyzed on the expression of mRNA FOXP3. The purity of the sorted populations, determined by FACS reanalysis of an aliquot of sorted cells, was 97% on average.

Suppression and proliferation assays

The 3% $CD4^+CD25^+$ T cells with the brightest expression of CD25 were tested in co-culture experiments on suppressive function and compared to $CD25^+CD30^+$ and $CD25^+CD30^-$ $CD4^+$ T cells. Fresh $CD4^+CD25^-$ effector T cells (3-5x10^3) from the same donor and induced $CD25^{bright}$, $CD25^{bright}CD30^-$, $CD25^{bright}CD30^+$. $CD4^+$ T cells were directly sorted by FACS (FACS Vantage, BD Biosciences, San Jose, CA, USA) into plate-bound anti-CD3-coated wells (OKT-3, 1.5 μg/ml), in different ratios. T cell depleted, irradiated PBMC (3500 Rad) from the same donor were used as APC (1:3). The cells were incubated at 37°C for 6 days, the last 18h in the presence of [^{3}H] thymidine (1 μCi/well). The suppressive activity was determined by calculating the relative difference in proliferative response (mean [^{3}H]thymidine incorporation (cpm) of triplicate wells) between $CD4^+CD25^-$ T cells cultured alone and $CD4^+CD25^-$ T cells cultured in the presence of induced regulatory T cells. For CFSE assays, CD4+CD25- effector T cells (Teff) isolated by magnetic selection, labelled with 2 μM CFSE, plated 25,000 per well, and irradiated T cell depleted PBMC were used as APC, plated 25,000 per well. Alternatively, 10,000 CFSE labelled PBMC were used as effector cells and plated per well. Induced Treg were isolated by FACS sorting and added in different ratios to the cultures. Cells were stimulated with anti-CD3 (clone OKT3). On day 4 of culture $CD4^+$ T effector proliferation was analyzed by FACS Canto (BD Biosciences). Percentage of suppression was calculated by comparing the percentage of proliferated $CD4^+CD25^-$ T cells in wells with $CD4^+CD25^-$ T cells cultured alone and $CD4^+CD25^-$ T cells cultured in the presence of HSP60 induced regulatory T cells.

Stability of FOXP3 expression and cytokine production

To determine FOXP3 stability and cytokine secretion by CD30 expressing cells and $CD30^-$ cells, $CD25^+CD30^+$ and $CD25^+CD30^-$ $CD4^+$T cells were FACS sorted and cultured separately in presence of 1.5 μg/ml plate bound anti-CD3. For FOXP3 analysis cells were stained on day 7. Supernatants were taken on days 3, 5 and 7 for cytokine analysis by multiplex immuno assay (see below).

Antigen specific suppression assay

To assess specificity of Treg suppression, $CD4^+CD25^-$ effector T cells were isolated by magnetic selection, labelled with 2 μM CFSE, plated 25,000 per well, and irradiated T cell depleted PBMC were used as APC, plated at 25,000 cells per well. Induced Treg were isolated by FACS sorting and added in different ratios to the cultures. Cultures were either stimulated with 10μg/ml human HSP60, or with 1.5 μg/ ml Tetanus Toxoid. On day 7 of co-culture, T effector proliferation was analyzed by FACS Canto (BD Biosciences). Percentage of suppression was calculated by comparing the percentage of proliferated $CD4^+CD25^-$ T cells in wells with $CD4^+CD25^-$ T cells cultured alone and $CD4^+CD25^-$ T cells cultured in the presence of HSP60 induced regulatory T cells.

Methylation of FOXP3 Treg Specific Demethylated Region (TSDR)

To determine the methylation of the FOXP3 TSDR, only healthy male donors were included. DNA was isolated from sorted $CD25^+CD30^+$ and $CD25^+CD30^-$ $CD4^+$ T cells, induced by HSP60, using QiaAmp DNA Mini Kit (Qiagen). Demethylation of the FOXP3 TSDR was determined according to the previously published methods (38).

mRNA analysis by quantitative PCR

Total RNA from FACS sorted cells was isolated using Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometer and adjusted to a concentration of 500 ng/ml. First-strand cDNA was synthesized from total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV, Promega, Madison WI, USA) with 1 μg/μl Oligo (dT) and 10 mM dNTP's

(both Amersham Pharmacia Biotech AB, Uppsala, Sweden). The reaction mixture was incubated at 40°C for 90 minutes followed by incubation at 70°C for 15 minutes. To ensure the fidelity of mRNA extraction and reverse transcription to first-strand cDNA all samples were subjected to real time PCR amplification with primers specific for the constitutively expressed gene beta-2 microglobulin (β2m). For FOXP3 and β2m transcripts real-time quantitative PCR was performed with a LightCycler (Roche Diagnostics, Mannheim, Germany) based on specific primers and general fluorescence detection with SYBR Green. The following primer combinations were used; IL10 Forward 5′ TGAGAACAGCTGCACCCACTT 3′ , Reverse 5′ GCTGAAGGCATCTCGGAGAT 3′ , IFNγ Forward 5′ GCAGAGCCAAATTGTCTCCT 3′, Reverse 5′ ATGCTCTTCGACCTCGAAAC 3′, FOXP3 Forward 5′ TCAAGCACTGCCAGGCG 3′, FOXP3 Reverse 5′ CAGGAGCCCTTGTCGGAT 3′ and β2m Forward 5′ CCAGCAGAGAATGGAAAG TC 3′, β2m Reverse 5′ GATGCTGCTTACATGTCT CG 3′. All PCR reactions were performed using LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). A pool of cDNA from tetanus toxoid stimulated human PBMC was used as a standard and normalization to β2m was performed for each sample. Semi-quantitative levels of FOXP3 are expressed as percentage of the FOXP3 expression of the cDNA pool.

Analysis of cytokine production by multiplexed particle-based flow cytometric assay

Cell culture supernatants were collected, stored at -80°C and processed within 1 month. Cytokine concentrations were measured with the Bio-Plex system in combination with the Bio-Plex Manager software, version 4.0 (Bio-Rad Laboratories, Hercules, CA, USA), which employs the Luminex xMAP technology as previously described (39). The following cytokines were measured: IL-2, IL-4, IL-5, IL-10, IL-13, IL-17, tumor necrosis factor-α (TNF-α), and interferon-γ (IFNγ).

T cell capture and artificial APC

This technique is extensively described earlier (40). Compared to the previously described protocol a few improvements were made. In short, PBMC of DR4 positive arthritis patients were cultured with or without human HSP60. After 7 days the cells were prestained with anti-CD4-CY and subsequently incubated with Artificial Antigen-Presenting Cells (aAPCs), loaded with DR4, hsp60 peptides and antibodies to CD28 with or without LFA1 for 2 hours at 37°C.

The aAPCs were prepared as follows. Phosphatidylcholine and cholesterol (Sigma) were combined in a glass tube at a molar ratio of 7:2. The solvent was evaporated under an Argon stream for 30 min and dispersed at a final concentration of 10 mg/ml in 140 mM NaCl and 10 mM Tris·HCl, pH 8 (buffer A) containing 0.5% sodium deoxycholate. Monosialoganglioside-GM1 (Sigma G-7641) was added at a final concentration of 0.28 mM. The solution was sonicated until clear and was stored at -20°C. Liposomes were formed through dialysis at 4°C against PBS in a 10-kDa Slide-A-Lyzer (Pierce) for 48h. Biotinylated recombinant MHC was incubated with the peptide (6h, room temperature). The resulting MHC-peptide complexes were incorporated in rafts, engineered on the aAPC surface. The rafts were constructed by mixing biotinylated HLA-DR4 molecules, biotinylated antibodies to CD28 and anti-LFA-1, and biotinylated Cholera toxin subunit B-FITC conjugated (CTB-FITC; Sigma) in the appropriate (equal) molar ratio. Next, neutravidin (NA; Pierce) was added in a molar ratio of four biotinylated moieties per molecule of NA. CTB-FITC was used to visualize T cells bound by the aAPCs. After incubation (1.5h at room temperature), the Raft-NA mixture was added to the liposomes for 2h, again at room temperature and washed 3 times in PBS. As negative controls, empty liposomes (blanco), liposomes with anti-CD19 (α-CD19), or anti-CD28 (α-CD28) were used. Finally, the aAPCs were incubated with the stained cells, washed twice and resuspended in FACS buffer. CD4-CY and CTB-FITC high double positive cells were sorted by FACS (FACS Vantage, Becton-Dickinson, San Jose, CA, USA) and analyzed on the expression of mRNA FOXP3, IL-10 and IFNγ.

Statistical analysis

One-way ANOVA analysis with Bonferroni's multiple comparison post test was used to compare (multiple) means of mRNA FOXP3 levels in sorted CD25 and CD30 expressing cells. The Wilcoxon rank test, $\alpha = 0.05$, two-tailed, was used to compare mRNA FOXP3 expression before and after TLR4 block. Paired T Test was used to compare Mean Fluorescence Intensity of FOXP3 expression between $CD30^{+}FOXP3^{+}$ and $CD30^{-}FOXP3^{+}$ T cells, and to compare cytokine secretion between $CD30^{+}$ and $CD30^{-}$ cells.

Results

Activation of CD4$^+$CD25$^-$ T cells with human HSP60 induces a clear population of CD4$^+$FOXP3$^+$ T cells.

Stimulation of human CD4$^+$CD25$^-$ T cells can result in considerable FOXP3 expression and development of suppressor activity (41-43). We examined the potential of human HSP60 to generate FOXP3$^+$ Treg by stimulating CD4$^+$CD25$^-$ T cells obtained from healthy adults and JIA patients. In both groups similar results were seen. Stimulation of CD4$^+$CD25$^-$ T cells with HSP60 resulted in the expression of CD25, in intermediate and bright levels, suggestive of two populations (**Figure 1A**). Flow cytometric staining for FOXP3 showed that all CD4$^+$CD25^{bright} T cells and most CD4$^+$CD25$^{intermediate\ (int)}$ T cells (82.2% ± 2,9%, mean ± SEM) induced after activation with HSP60 were FOXP3$^+$, whereas only a minority of the CD4$^+$CD25$^-$ T cells exhibited FOXP3 expression (**Figure 1A**). These findings were confirmed by the measurement of FOXP3 mRNA levels in sorted CD4$^+$ T cell populations (**Figure 1B**). Thus, HSP60 can induce expression of CD25 and FOXP3 in CD4$^+$ T cells. Next, we tested the suppressive capacity of the HSP60 induced CD4$^+$CD25^{bright} T cells as described before (2). Clear suppression was found in only three of the six donors, even though in all six donors 100% of the CD4$^+$CD25^{bright} T cells expressed FOXP3 (**Figure 1C**).

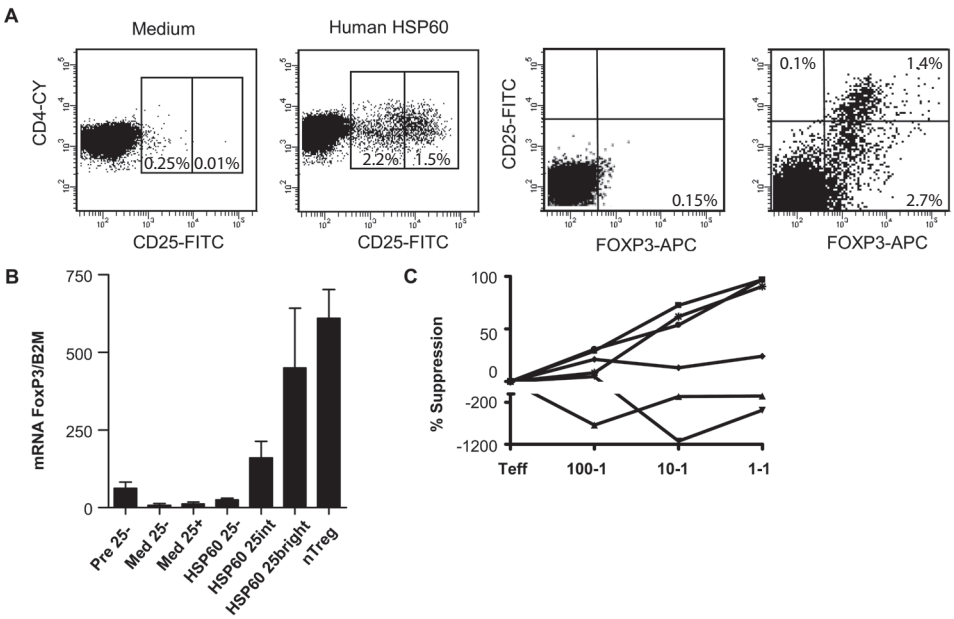

Figure 1. Induction of CD4$^+$FOXP3$^+$ Tregs with human HSP60. (**A**) Flow cytometry analysis of CD4+CD25- T cells obtained from a healthy control and stimulated during 7 days with medium or human HSP60 in the presence of APC. Shown is a representative example of 12 experiments (6 healthy controls and 6 JIA patients). (**B**) Semi-quantitative RT-PCR analysis of FoxP3 expression in sorted CD4$^+$ T cells. Pre 25-: CD4$^+$CD25$^-$ T cells before stimulation, Med 25- and med 25+: CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cells after 7 days of culture in medium only. HSP60 CD25-, 25int and 25bright: CD4$^+$CD25$^-$, CD4$^+$CD25^{intermediate} and CD4$^+$CD25^{bright} T cells after 7 days of culture with human HSP60. nTreg: ex vivo isolated natural occurring CD4$^+$CD25^{bright} Treg. Shown is the mean ± SD of six experiments with blood of healthy controls. One-way ANOVA with Bonferroni correction: no significance. (**C**) Suppression assays (n=6) with freshly isolated CD4$^+$CD25$^-$ effector T cells (Teff) and HSP60 induced CD4$^+$CD25^{bright} T cells in ratios (Teff:Treg) as indicated on the x-axis. Given is the percentage suppression of the proliferative response of CD4$^+$CD25$^-$ T cells cultured in the presence of CD4$^+$CD25^{bright} T cells compared to CD4$^+$CD25$^-$ T cells cultured alone. The maximum ^{3}H count per assay varied from 3330 to 25.600 counts per minute.

Surface expression of CD30 discriminates activation induced FOXP3$^+$ Treg

Despite the high prevalence of FOXP3$^+$ cells, HSP60 induced CD4$^+$CD25^{bright} T cells lacked suppressive capacity in half of the donors. Since FOXP3 can be transiently expressed in activated cells without acquiring regulatory function, this finding may indicate that, in addition to contamination with FOXP3$^-$ cells, the induced CD25^{bright} T cell population contains FOXP3$^+$ effector T cells without regulatory function. Whether the HSP60 induced CD25^{bright} T cells are suppressive depends on the proportion of functional Treg within this population. Thus, to rely on CD25^{bright} expression alone is not sufficient in identifying activation induced Treg for use in suppressor function tests or for the isolation of therapeutic Treg, even though all of these cells express FOXP3. We therefore sought to find a surface marker that correlates with suppressive function rather than induced FOXP3 expression.

CD30 is a member of the TNF receptor (TNFR) superfamily and originally described as a marker of Reed-Sternberg cells in Hodgkin's disease (44, 45). While in peripheral blood the expression of CD30 is extremely low to zero; it is expressed upon activation on T- and B-lymphocytes. Though, engagement of CD30 by its ligand has shown to provide costimulatory signals to activated T cells and to enhance cytokine production and secondary proliferative responses (46), the exact function of CD30 in mature T cells is still unknown. Of interest are reports suggesting that CD30 is required for or contributes to immune regulation (36, 47-49). In a previous study we showed that CD30$^+$ T cells present in the synovial fluid of JIA patients correlate with a favorable disease course (36). Furthermore, in mice CD30 signaling on CD4$^+$CD25$^+$ Treg has shown to be important in the prevention of allograft rejection and acute graft versus host disease (50, 51). These studies suggest a role for CD30 in activation induced regulatory cells and prompted us to study the expression of CD30 in our system. First we analyzed the relationship between the expression of CD30 and FOXP3 by FACS and RT-PCR in CD4$^+$CD25$^-$, CD4$^+$CD25^{int} and CD4$^+$CD25^{bright} T cells obtained after 7 days cultures of HSP60 stimulated CD4$^+$CD25$^-$ T cells. Single cell analysis by FACS showed that CD30 expression is restricted to FOXP3 expressing cells and that 56% (range 53-62%) of the FOXP3 expressing T cells coexpress CD30 (**Figure 2A**). Furthermore, the expression of FOXP3 per cell was higher in CD30 expressing cells, compared to the CD30$^-$ FOXP3 expressing cells (Figure.2*C*). These data were confirmed by RT-PCR. Although no significance was reached (one-way ANOVA with Bonferroni correction), RT-PCR showed a clear trend towards higher mRNA FOXP3 levels in T cell subsets coexpressing CD30 (**Figure 2B**).

The restricted expression of CD30 to FOXP3$^+$ cells enabled us to compare the suppressive capacity of activation-induced CD25^{bright}, CD25$^+$CD30$^-$ and CD25$^+$CD30$^+$ T cells. Interestingly, only CD4$^+$ T cells coexpressing CD30 manifested regulatory function *in vitro,* whereas CD4$^+$CD30$^-$ T cells did not, and for CD25^{bright} only half of the donors (3 out of 6) showed suppression, whereas 2 donors showed high proliferation of Treg, causing an average negative level of suppression (**Figure 2D**; for CD25^{bright}, see also **Figure 1C**). Additionally, we showed a similar pattern in suppression assays using CFSE dilution as readout, discriminating of CD4$^+$ effector T cells proliferation from Treg proliferation. The difference in *in vitro* suppressive capacity between CD30$^+$ and CD30$^-$ CD4$^+$ T cells was less pronounced in this system (**Figure 2E**). This fits with other reports that especially for induced Tregs in vitro suppressive assays are not as consistent as for freshly isolated Tregs (52).

Furthermore, we tested stability of FOXP3 expression within the CD30$^+$ cells. Indeed after 7 days of separate culture of sorted CD30$^+$ cells, the cells that maintained CD30 on their surface were all FOXP3$^+$ (**Figure S1**). A demethylated state of the Treg-specific demethylated region (TSDR) within the FOXP3 locus, relates to Treg function and stability of FOXP3 expression. However, TSDR demethylation was similarly low in both HSP60 induced CD25$^+$CD30$^+$ T cells and CD25$^+$CD30$^-$ T cells (data not shown).

Next, we tested cytokine production by both CD30$^+$ and CD30$^-$ T cells after stimulation with anti-CD3, similar to stimulation in suppression assays. Although not significant, CD30 expressing cells seem to excrete less IL-2 (p=0.1), probably due to high FOXP3 expression. Both CD30$^+$ and CD30$^-$ cells excreted high levels of IL-17, which was a little higher in the CD30$^+$ cells. For

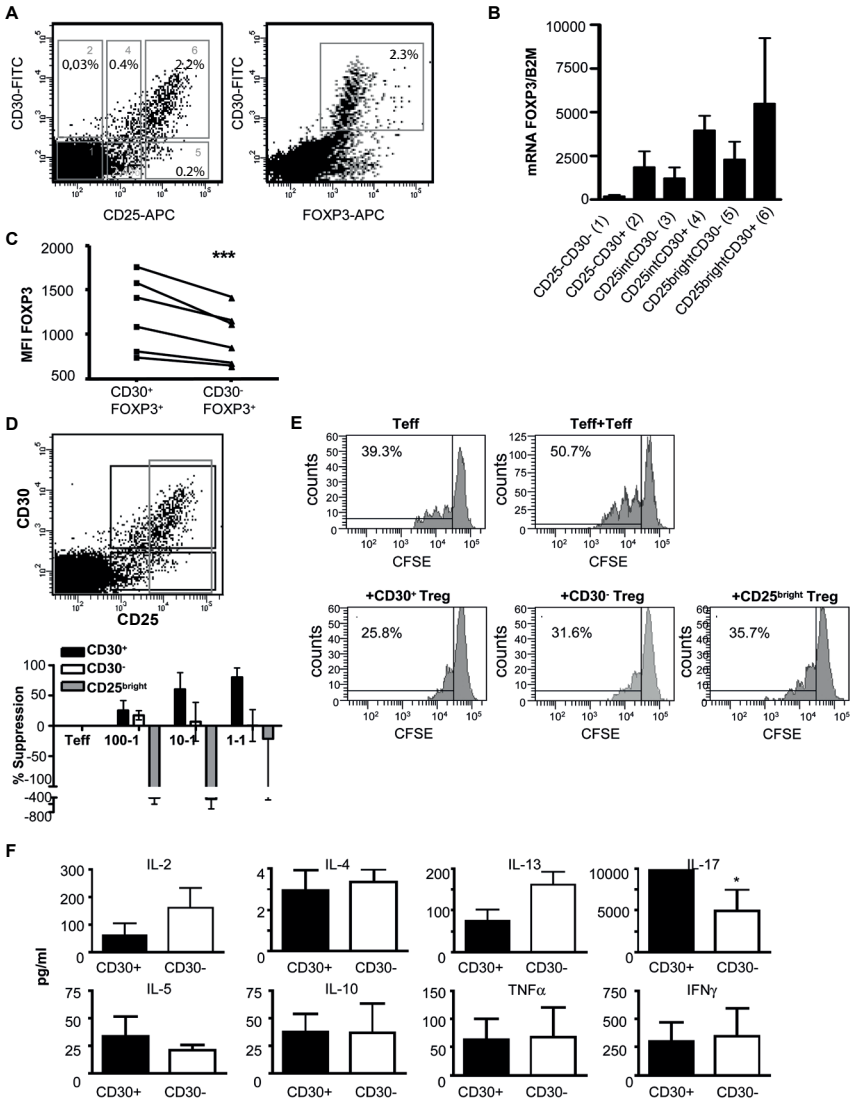

Figure 2. FOXP3 mRNA expression and suppressive function of HSP60 induced CD+CD25+CD30+ T cells. (**A**) Flow cytometric staining of CD25, CD30 and FOXP3 on CD4+CD25- T cells activated with HSP60 in the presence of APC. (**B**) Semi-quantitative RT-PCR analysis of FOXP3 mRNA in sorted CD4+ T cells, derived after six day cultures of CD4+CD25- T cells in the presence of human HSP60. Shown is the mean ± SD of six experiments with blood of healthy controls. The sortgates for the six populations analyzed are given in (A). (**C**) FOXP3 Mean Fluorescence Intensity (MFI) of gated $CD4^+CD25^+CD30^+FOXP3^+$ T cells compared to $CD4^+CD25^+CD30^-FOXP3^+$ T cells derived after 7 day cultures of CD4+CD25- T cells in the presence of human HSP60 for 6 healthy donors. (**D,E**) Comparison of suppressive potential of $CD4^+CD25^+CD30^+$, $CD4^+CD25^+CD30^-$ T cells and $CD4^+CD25^{bright}$ derived after 7 day cultures of $CD4^+CD25^-$ T cells in the presence of human HSP60. The dotplot shows the used sortgates. (**D**) Four separate experiments with blood of 3 healthy donors are shown for $CD25^+CD30^+$ and $CD25^+CD30^-$ and 6 experiments with blood of 6 donors for $CD25^{bright}$. Level of suppression of effector T cell (Teff) proliferation, calculated for several ratios of Teff + Treg, compared to culture of Teff alone (suppression=0%). (**E**) Effector T cells were labelled with CFSE. Percentage of Teff proliferation, and Teff + Teff, compared to culture of Teff with Tregs. Histograms from 1 out of 10 donors are shown. (**F**) $CD4^+CD25^+CD30^+$ T cells and $CD4^+CD25^+CD30^-$ T cells were sorted on day 6 and cultured separately for 5 days in the presence of anti-CD3. Supernatants were collected out of triplicates of cultured wells and measured by multiplexed particle-based flow cytometric assay. Mean cytokine levels in supernatants are depicted of 3 healthy controls. Shown are means +/- s.e.m., *p<0.05, **p<0.01, ***p<0.001.

IL-4, IL-5, IL-10, IL-13, IFNγ and TNFα excretion, no significant difference between $CD30^+$ and $CD30^-$ cells was observed (**Figure 2F**). Cytokine excretion profiles of both populations are very different from freshly isolated $CD4^+CD25^+CD127^{low}$ T cells (Treg), which produce only low levels of cytokine, but are comparable to $CD4^+CD25^-$ T cells (Teff), except for a higher IL-17 and lower TNFα production (see **Table S1**, and our previously published data (53)).
Other markers tested were CD127, CD27, GITR and CTLA4, all described to play a role in Treg function (54-56). CD127 held our interest particularly, because it is a marker used for the identification of natural occurring $CD4^+FOXP3^+$ Treg (54). However, none of these markers, including $CD127^{low}$, showed a similar pure positive correlation with FOXP3 expression in activation induced Treg (**Figure 3**). Furthermore, $CD127^{low}$ expression did not correlate with CD30 expression, and $CD25^+CD127^{low}$ cells had a lower expression of FOXP3 (**Figure S2**). We therefore concluded that cell surface expression of CD30, but not $CD127^{low}$, CD27, CTLA-4, or GITR, allows a distinction of activation induced $FOXP3^+$ T cells.

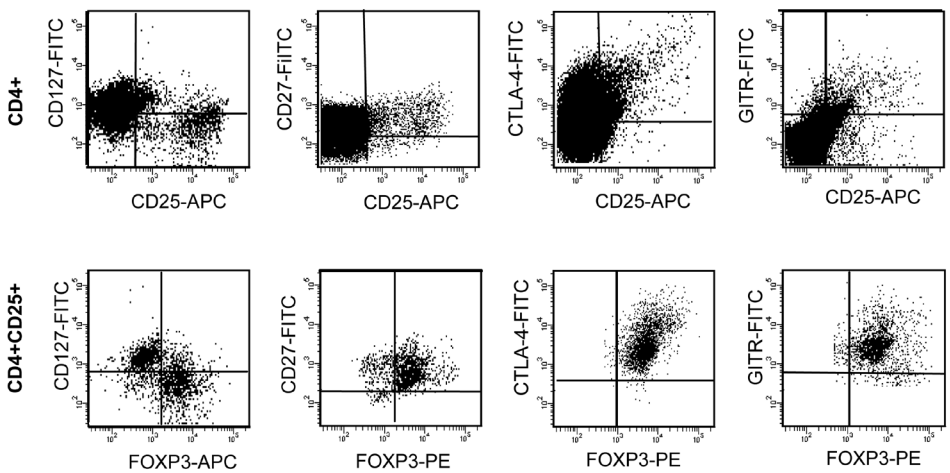

Figure 3. Phenotype of HSP60 induced T cells. Flowcytometry of $CD4^+CD25^-$ T cells cultured for 7 days with human HSP60. Cells were gated on the expression of CD4 (top panels) or $CD4^+CD25^+$ (lower panels) and analyzed on the expression of CD25, CD27, CD127, GITR, CTLA4 and FOXP3. A representative example of six experiments with blood of healthy controls is shown.

The induction of $CD4^+$ $FOXP3^+$ Treg through activation is enhanced by TLR4 triggering on APC

Besides being an antigen for T cells HSP60 can also function as an innate ligand for TLR2 (24) and TLR4 (57, 58). Both TLR2 and TLR4 are abundantly expressed on cells of the innate immune system, such as DCs and macrophages. In our experiments, HSP60 induced FOXP3 expression required the addition of APC (**Figure 4A**). To test whether TLR2 or TLR4 ligation on APC plays a role in the induction of FOXP3, we pretreated APC with neutralizing anti-TLR2 or anti-TLR4 mAb before addition to the cultures. Interestingly, blocking TLR4 on APC resulted in a significant reduction of CD25 and FOXP3 mRNA expression, while blocking TLR2 on APC had no such effect (**Figure 4B**). Although several batches of recombinant human HSP60 were shown to contain minimal residual LPS and LPS-lipoproteins (59), it is unlikely that TLR4 ligation with contaminating LPS played a role in our assays. The highly purified recombinant human HSP60 used in this study contained undetectable amounts of bacterial endotoxin (i.e. <0,003 EU/μl or <0.3 pg/μg protein). We therefore conclude that HSP60 can enhance activation induced $FOXP3^+$ Treg induction via TLR4 ligation on APC.

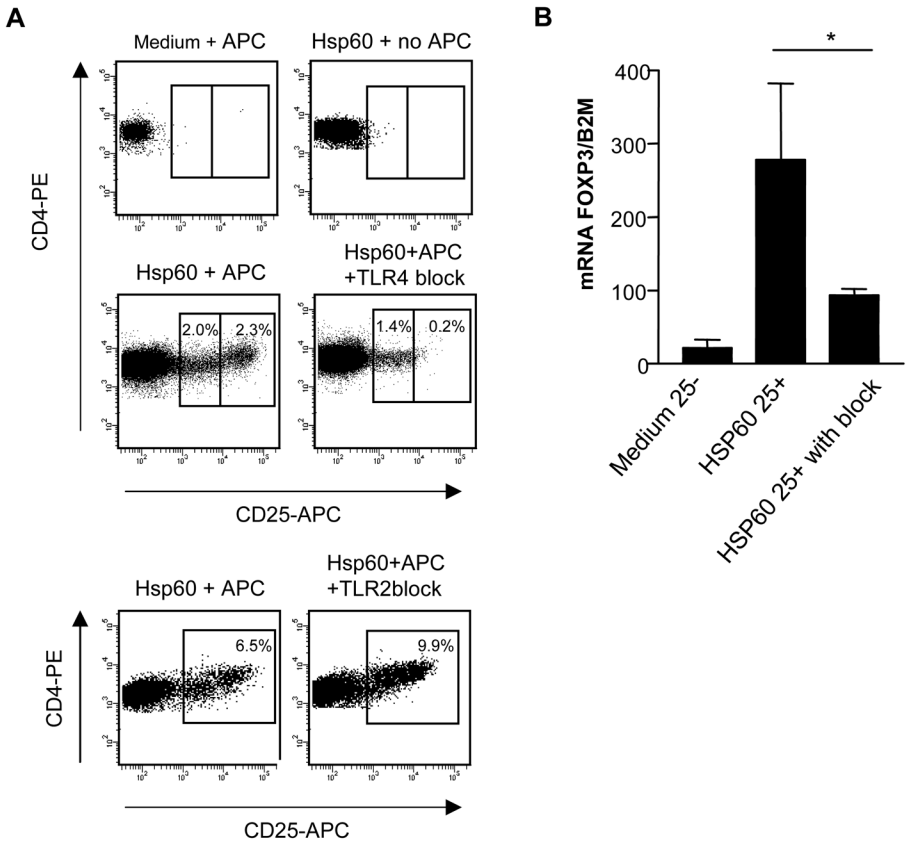

Figure 4. The effect of TLR4-block on the induction of CD4$^+$CD25$^+$ Tregs. (**A**) Flowcytometry of CD4$^+$CD25$^-$ T cells cultured for 7 days in the presence or absence of HSP60, APC and TLR4 block or TLR2 block. (**B**) mRNA FOXP3 expression in CD4$^+$CD25$^-$ T cells cultured with medium only and in CD4$^+$CD25$^+$ T cells obtained from CD4$^+$CD25$^-$ T cell cultures stimulated with HSP60 and with or without the addition of TLR4 block. Shown is the mean ± SD of six experiments with blood of healthy controls. * P<0.03, Wilcoxon rank test.

The activation induced CD4$^+$CD30$^+$ Treg are antigen specific

Since HSP60 functions both as TCR- and TLR4-ligand, we wanted to exclude a pure innate effect and evaluated whether we could induce CD4$^+$CD30$^+$FOXP3$^+$ T cells by activating CD4$^+$CD25$^-$ T cells with peptides from HSP60. Healthy controls (n=2) were selected on their responsiveness to previously identified pan-DR binding peptide-epitopes from human HSP60 (60, 61). In both donors activation of CD4$^+$CD25$^-$ T cells with a peptide of HSP60 resulted in a similar CD30 and FOXP3 expression pattern as activation with the whole protein (**Figure 5A**). This shows that CD4$^+$CD30$^+$FOXP3$^+$ Treg development is the result of activation through the TCR and that TLR signaling enhances but is redundant for Treg development.

In addition, the antigen-specificity of the HSP60 induced CD4$^+$CD30$^+$FOXP3$^+$ Treg was evaluated using the so-called T cell capture technique. This technique uses artificial APC (aAPC) consisting of liposomes with a high relative density of MHC-peptide complexes enabling the capture of low avidity class-II restricted Ag-specific T cells (40). CD4$^+$CD25$^-$ T cells derived from the peripheral blood of three healthy volunteers (**Figure 5B,C**), two DR4+ JIA patients and one DR4+ RA patient were stimulated with HSP60, in the presence of APC. We next used CTB labeled aAPC containing DR4 molecules loaded with a peptide of HSP60 to isolate T cells able to recognize HSP60 epitopes

from the cultures. Cells were stained with fluorescence labeled aAPC, CD4 and CD30. The aAPC-positive cells were analyzed on the expression of CD30 (**Figure 5B** and **Figure S3**),and after sorting on the expression of IL-10, IFNγ, and FOXP3 mRNA by RT-PCR (**Figure 5D**). A comparison in mRNA content was made with *ex vivo* isolated CD4$^+$CD25$^-$ T cells and CD4$^+$CD25^{high}CD127^{low} natural Treg. In each experiment HSP60 peptide specific T cells expressing CD30 and FOXP3 mRNA could be identified, producing high levels of IL-10 and no IFNγ, which is compatible with intracellular staining of total HSP60 induced Treg, showing that CD30 expression correlates with high IL-10 and low IFNγ (**Figure S4**). This indicates that at least part of the HSP60 induced CD4$^+$CD30$^+$FOXP3$^+$ T cells recognize HSP60 epitopes and have a regulatory cytokine-production profile. In order to test specific suppressive capacity, we undertook suppression assays in which stimulation of the effector T cells with specific antigen (HSP60), or aspecific antigen (Tetanus Toxoid) was used. As these antigens activate only few effector cells, Teff proliferation is similarly low for both antigens, compared to anti-CD3 stimulation. Therefore optimal suppression is already reached at a co-culture ratio of 10-1. HSP60 responses were suppressed similar compared to Tetanus Toxoid responses (**Figure 5E**). This suggests that HSP60 induced CD4$^+$CD25$^+$CD30$^+$ T cells are able to perform bystander suppression, as well as specific suppression.

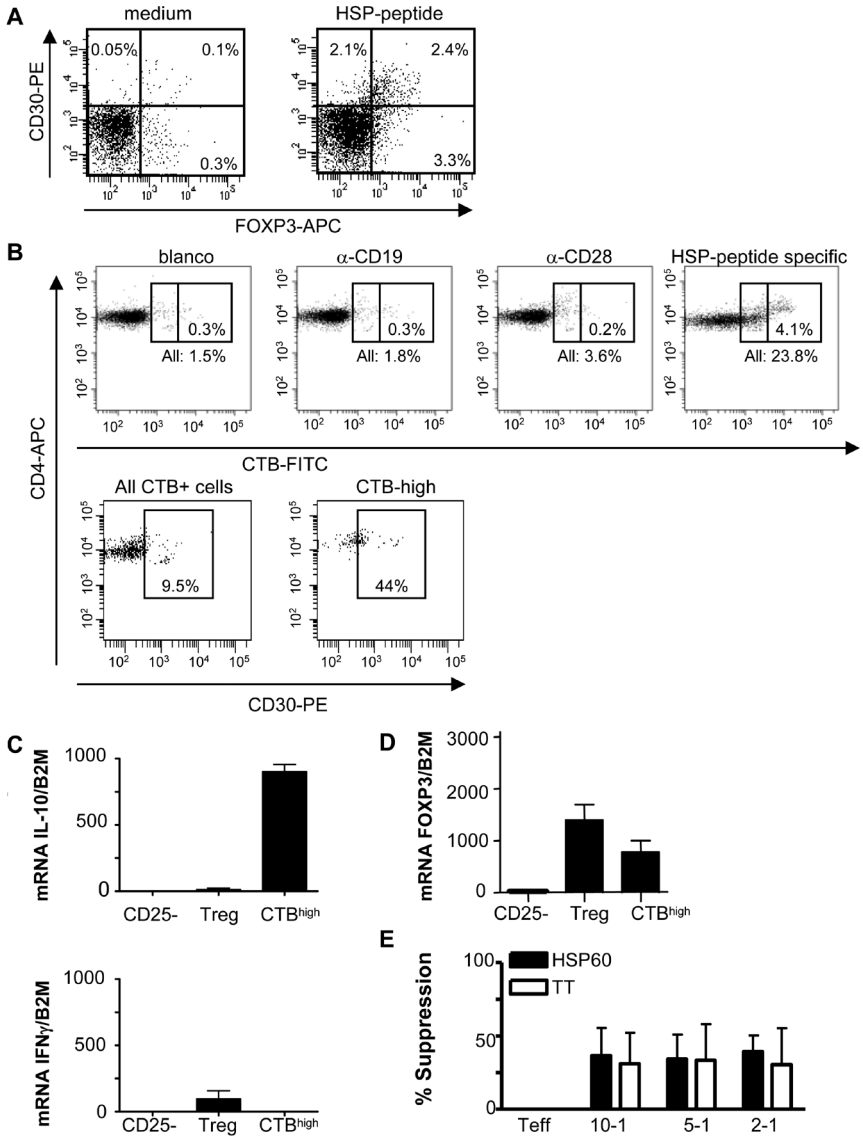

Figure 5. The identification and isolation of HSP60 epitope specific Treg by T cell capture. (**A**) $CD4^+CD25^-$ T cells were stimulated with medium only (left dotplot) or a peptide of HSP60 (right dotplot) in the presence of irradiated APC. (**B**) $CD4^+CD25^-$ T cells were stimulated with HSP60 for 7 days. Peptide specific T cells were identified using CTB-labeled aAPC. One representative example of 3 experiments with blood of 3 healthy donors is shown. The upper left 3 dotplots are all negative controls for a-specific binding to the aAPC, and represent T cells incubated with either empty liposomes (blanco), aAPC loaded with anti-CD19 (α-CD19), or aAPC loaded with anti-CD28 (α-CD28). The upper right dotplot shows specific binding of T cells incubated with aAPC loaded with HSP60 peptide (HSP-peptide specific). The lower two dotplots show CD30 expression on CTB positive cells. (**C,D**) Analysis of FoxP3 mRNA expression by RT-PCR in ex vivo isolated $CD4^+CD25^-$ T cells (CD25-), $CD4^+CD25^+$ nTregs (Treg) and FACS sorted HSP60-epitope specific T cells (CTB^{high}) derived from (**C**) 2 healthy donors, or (**D**) 3 patients after culture of $CD4^+CD25^-$ T cells with HSP60 in the presence of HSP60. Shown is the mean ± SD of the experiments. (**E**) $CD4^+CD25^-$ T cells were stimulated with HSP60 for 7 days. $CD4^+CD25^+CD30^+$ T cells were sorted and cocultured with Teff stimulated with HSP60 or TT. Cells from 3 different donors were tested. Level of suppression of Teff proliferation, calculated for several ratios of Teff + Treg, compared to culture of Teff alone (suppression=0%). Shown is the mean ± SD of the experiments.

Discussion

While antigen- and/or site-specific suppression of inflammation seems the ultimate goal of immunotherapy for specific inflammatory diseases, knowledge on relevant antigens is still lacking in most cases. In addition, thus far it has not been possible to identify and isolate pure activation induced Treg populations due to the lack of an appropriate surface marker that can distinguish FOXP3$^+$ Treg.

In the present study we show that the activation of CD4$^+$CD25$^-$ T cells with human HSP60 results in *de novo* generation of a subset of FOXP3 expressing T cells. In addition, we show that within this population the surface-expression of CD30 marks a very homogenous population of activation induced FOXP3$^+$ T cells with a stable expression of high levels of FOXP3, leading to suppressive function *in vitro*.

The exact function of CD30 on mature T cells is unknown. Previous in vitro studies have shown that CD30-CD30L interaction has effect on both co-stimulation and cytokine production (62, 63) and recent *ex vivo* studies implicated a role for CD30$^+$ T cells in the regulation of autoimmunity and tumor rejection (36, 47-49). In mice, CD30 signaling plays a role in the suppression of allograft rejection and acute GVHD (50, 51). In line with these studies, our data now strongly point at an immunoregulatory role for CD30 on activation induced Treg.

The antigen-specificity of the HSP60 induced FOXP3$^+$ Treg was confirmed by identifying HSP60 peptide specific T cells among the induced CD4$^+$CD30$^+$ T cell population using the T cell capture technique (40, 64). To find antigen specificity was of special interest since HSP60 can have a TCR-mediated effect, but also functions as a ligand for TLRs. TLR4 triggering on APC indeed had an enhancing effect on the induction of CD4$^+$CD30$^+$FOXP3$^+$ Treg. This observation adds to previous reports showing that ligation of TLRs on APC modulates immune regulation by Treg. For example, TLR4 triggered DCs have shown to induce Treg proliferation by a cooperative action of IL-1 and IL-6 (65). And TLR9 induced IL-6 production by DCs releases effector T cells from Treg-mediated suppression (66). Thus, depending on the local milieu, TLR signaling on APC confers signals that either augment or attenuate T cell mediated immunoregulation.

In addition to this indirect, APC-mediated effect, TLRs have shown to control regulatory function via a direct effect on Treg. Zanin-Zhorov et al. previously showed that direct TLR2 triggering of Treg enhances their suppressive capacity (26). Like *ex vivo* isolated Treg, HSP60 induced Treg express high levels of TLR2 and no TLR4 (data not shown). Neither HSP60, nor LPS, exerts any increased proliferation or survival of human CD4$^+$CD25$^+$ Treg (I. de KLeer and Y. Vercoulen, unpublished data). It therefore seems unlikely that an additional innate effect of HSP60 on the proliferation and/or survival of *de novo* generated HSP60 specific CD4$^+$CD25$^+$ Treg plays a role in our assays.

Furthermore, both total CD30$^+$ and the induced HSP60 peptide-specific Treg produce high levels of IL10 and low IFNγ, and are not only able to suppress HSP60 specific responses, but also responses to Tetanus Toxoid. This may be because we did not select HSP60 peptide specific Treg for these assays, or because the highly activated state of the Treg in the HSP60 cultures enables them to suppress Teff responses to different antigens. This suggests that these HSP60 induced Treg are suitable to suppress independent of the antigen that is causing inflammation. However, given the uncertainty in interpreting human *in vitro* Treg phenotype and functional assays (52), we realize that our findings can not be directly translated into predicting suppressive function *in vivo*.

Altogether, these data suggest a model whereby *de novo* generated Treg that are the result of prolonged or repeated innate and/or specific T cell activation by HSPs at sites of tissue damage, are involved in controlling the spread of an inflammatory response. This may explain the high numbers of Treg isolated from sites of inflammation in various human inflammatory diseases (2). The here described ability of HSP60 to drive *de novo* induction of Treg *in vitro* and the identification of CD30 as a surface marker for activation induced Treg may prove useful in new therapeutic approaches for human inflammatory diseases.

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Supplementals

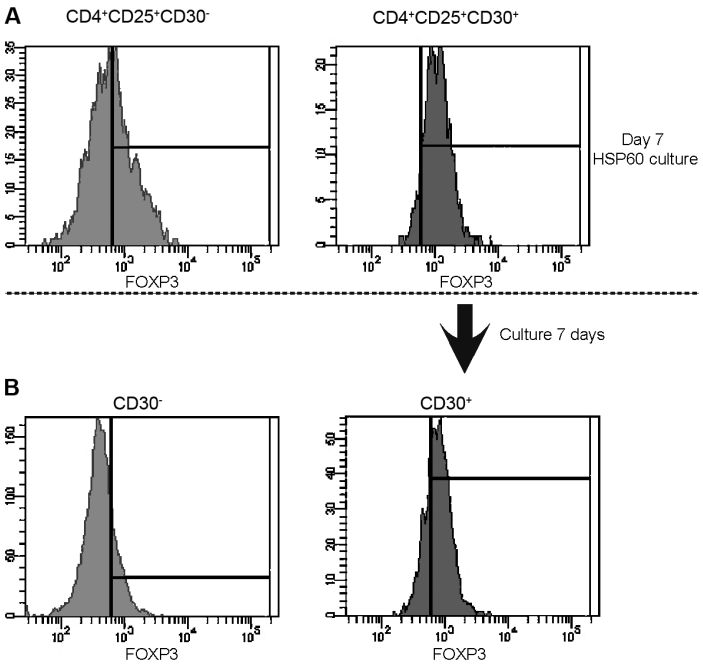

Figure S1: FOXP3 expression is stable within CD30 Expressing cells. (**A**) CD25$^+$CD30$^+$CD4$^+$ T cells were FACS sorted and cultured in presence of 1.5 µg/ml platebound anti-CD3. (**B**) After 7 days of culture CD30 and FOXP3 expression was analyzed..

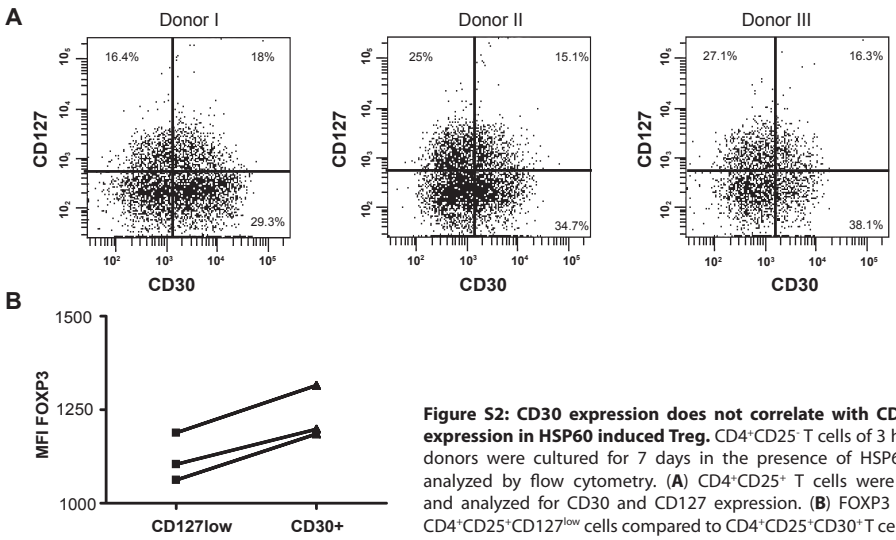

Figure S2: CD30 expression does not correlate with CD127^{low} expression in HSP60 induced Treg. CD4$^+$CD25$^-$ T cells of 3 healthy donors were cultured for 7 days in the presence of HSP60 and analyzed by flow cytometry. (**A**) CD4$^+$CD25$^+$ T cells were gated and analyzed for CD30 and CD127 expression. (**B**) FOXP3 MFI in CD4$^+$CD25$^+$CD127^{low} cells compared to CD4$^+$CD25$^+$CD30$^+$T cells.

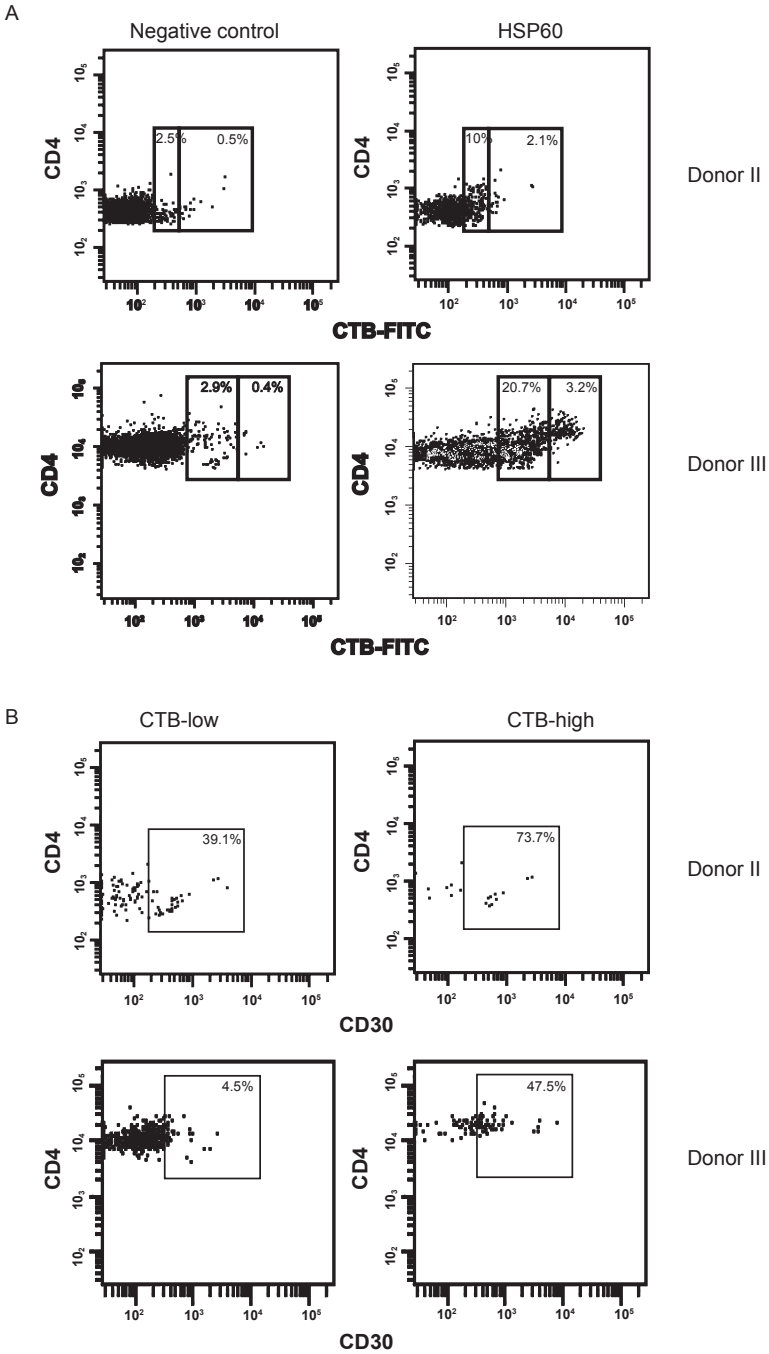

Figure S3: HSP60 induced Treg are specific for HSP60 epitopes and express CD30. $CD4^+CD25^-$ T cells of 2 donors were activated for 7 days in the presence of HSP60. T cell capture was performed and analyzed by flow cytometry. (**A**) Percentages of specific $CD4^+$ T cells, binding with low affinity (left gate) or with high affinity (right gate). Left dotplots are negative controls, right dotplots are incubated with liposomes presenting HSP60 peptide. (**B**) Percentages of CD30 expressing T cells within HSP60-epitope specific T cells. Left dotplots display the T cells binding with low affinity, right dotplots show the T cells binding with high affinity, gated as in (**A**).

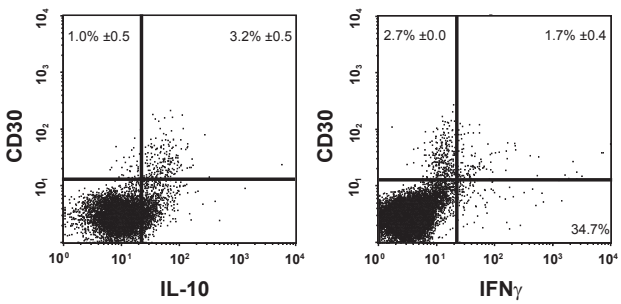

Figure S4: HSP60 induced CD30⁺Treg express high IL-10 and low IFNγ. $CD4^+CD25^-$ T cells of 2 healthy donors were activated for 7 days in the presence of HSP60 and analyzed by flow cytometry. (**A**) Representative dotplots are shown, of gated $CD4^+$ T cells, analyzed for CD30, intracellular IL-10 (left dotplot), and IFNγ (right dotplot).

Table S1. Mean levels of cytokines, present in culture medium on day 5 of culture with anti-CD3 of fresh $CD4^+CD25^-$ T cells (Teff), fresh $CD4^+CD25^+CD127^-$ T cells (Treg), HSP60 induced $CD4^+CD25^+CD30^+$ Tcells ($CD30^+$), HSP60 induced $CD4^+CD25^+CD30^-$ T cells ($CD30^-$). All values (mean (S.E.M.)) are expressed in pg/ml.

IL-2	IL-4	IL-5	IL-13	IL-10	IFNγ	TNFα	IL-17	
30.7 (15.0)	**4.4** (0.3)	**14.6** (4.8)	**112.3** (33.2)	**141.4** (64.1)	**367.8** (113.4)	**461.2** (134.5)	**773.3** (285.6)	**Teff**
0.6 (0.6)	**0.5** (0.3)	**2.2** (0.2)	**0.0** (0.0)	**12.3** (3.1)	**0.0** (0.0)	**2.7** (2.0)	**44.4** (16.4)	**Treg**
64.0 (42.9)	**2.9** (1.0)	**33.9** (17.8)	**74.9** (26.7)	**37.5** (16.8)	**297.8** (173.2)	**63.3** (37.9)	**10000** (0.0)	**$CD30^+$**
161.2 (71.9)	**3.4** (0.6)	**21.0** (4.5)	**162** (31.2)	**36.6** (26.8)	**344.8** (250.4)	**67.4** (53.9)	**4964** (2520)	**$CD30^-$**

5
CD30
NTREG
iTREG

Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization

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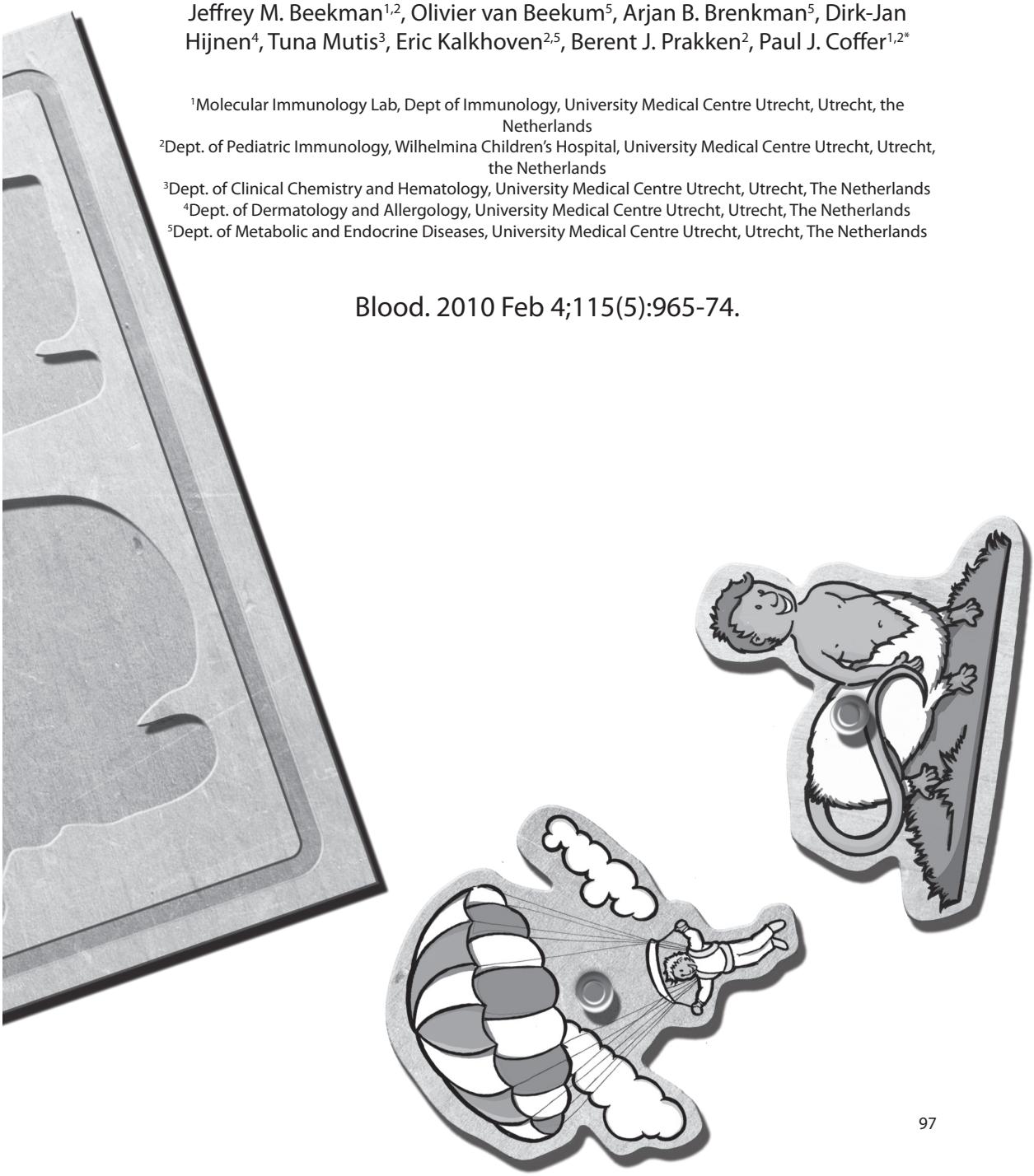

Abstract

Regulatory T cells (Tregs) are a specific subset of lymphocytes that are critical for the maintenance of self-tolerance. Expression levels of the transcription factor FOXP3 have been causally associated with Treg differentiation and function. Recent studies show that FOXP3 can also be transiently expressed in effector T cells, however, stable FOXP3 expression is required for development of a functional Treg suppressor phenotype. Here, we demonstrate that FOXP3 is acetylated and this can be reciprocally regulated by the histone acetyltransferase p300 and the histone deacetylase (HDAC) SIRT1. Hyperacetylation of FOXP3 prevented poly-ubiquitination and therefore proteasomal degradation, dramatically increasing stable FOXP3 protein levels. Moreover, using mouse splenocytes, human peripheral blood mononuclear cells, T cell clones and skin-derived T cells, we demonstrate that treatment with HDAC inhibitors resulted in significantly increased numbers of functional Treg cells. Taken together, our data demonstrate that modulation of the acetylation state of FOXP3 provides a novel molecular mechanism for assuring rapid temporal control of FOXP3 levels in T cells, thereby regulating Treg numbers and functionality. Manipulating FOXP3 acetylation levels could therefore provide a new therapeutic strategy to control inappropriate (auto)immune responses.

Introduction

Regulatory T cells (Tregs) are a specific subset of lymphocytes that play a crucial role in the maintenance of self-tolerance(1;2). These $CD4^+CD25^+$ cells can be distinguished from conventional T cells by the expression of a distinct subset of molecules, both on their cell surface, as well as intracellularly(3;4). The transcription factor FOXP3 is crucial for Treg differentiation and function, and various FOXP3 mutations, both in scurfy mice and IPEX (immune dysregulation polyendocrinopathy, enteropathy, X chromosome-linked syndrome) patients result in the development of complex autoimmune disease, due to Treg deficiency(5;6). Ectopic expression of FOXP3 in $CD4^+CD25^-$ T cells has also been shown to induce a suppressive phenotype, suggesting that stable FOXP3 expression is sufficient for development of functional Tregs (2;3;7).

FOXP3 expression is not unique to lymphocytes, recent reports demonstrate that respiratory, thymic, prostate and mammary epithelium cells also express FOXP3, although expression levels were low compared to Tregs(8). Furthermore, it has been reported that *in vitro* TCR stimulation of $CD4^+CD25^-$ effector T (Teff) cells can result in transient FOXP3 expression, which does not however generate T cells with a suppressive phenotype(9;10). In contrast, TCR stimulated $CD4^+CD25^-$ cells expressing high and stable FOXP3 levels develop suppressive capacity, illustrating that persistent FOXP3 expression is likely an essential step in the conversion of Teff cells into Tregs(11;12).

Since FOXP3 is an essential transcription factor for maintenance of immune homeostasis, its activity must be tightly and specifically regulated. However, surprisingly little is currently understood concerning post-translational regulation of this transcription factor. A recent study has demonstrated that FOXP3 can interact with Histone deacetylase (HDAC) 7 and 9, and with the histone acetyl transferase (HAT) TIP60(13). While these data suggest that FOXP3 is able to form a multiprotein complex containing both HAT/HDAC molecules, the functional relevance of these observations remains to be further clarified. Furthermore, mice treated with HDAC inhibitor Trichostatin A (TSA) have increased numbers of functionally improved Tregs correlating with reduced disease severity in an induced colitis model as well as a increased donor-specific allograft tolerance in a cardiac and islet transplant model(14). However, the molecular mechanism underlying improved Treg function by TSA treatment remains unclear, and it is not evident whether these are direct or indirect effects.

Here, we demonstrate that FOXP3 acetylation can be reciprocally regulated by the HAT p300 and the HDAC SIRT1. We show that FOXP3 protein has a short half-life and that acetylation prevents proteasomal degradation, dramatically increasing FOXP3 levels. Furthermore, modulating SIRT activity in mouse and human primary T cells regulates FOXP3 protein levels as well as the number and suppressive capacity of Tregs. Taken together, directly modulating the acetylation state of FOXP3 provides a novel molecular mechanism for assuring rapid temporal control of FOXP3 levels in T cells. Increasing FOXP3 acetylation levels may thus be a critical switch in the generation of induced Tregs from activated peripheral T cells.

Materials and Methods

Antibodies, DNA constructs and reagents.

The following antibodies were used: mouse anti-FOXP3 clone PCH101 for FACS analysis (eBioscience, San diego, CA), rabbit anti-p300 (Santa Cruz N15, Palo Alto, CA), rabbit anti-acetyl-lysine (Cell signaling, Danvers, MA), mouse anti-Flag M2 from Sigma (Zwijndrecht, The Netherlands), Mouse anti-hemaglutinin (HA) clone 12CA5 from Santa Cruz, mouse anti-tubulin (Sigma) and anti-Myc monoclona mice Ab were made using a hybridoma cell line. FOXP3 was cloned from MIGR1-FOXP3 (kindly provided by Dr. S. Sakaguchi(7)) into pMT2 that already contained a Flag tag resulting in pMT2-Flag-FOXP3. Using side-directed mutagenesis the pMT2-Flag-FOXP3ΔE250 mutant was constructed. pcDNA3 (Invitrogen Carlsbad,CA), pcDNA3-HA-p300, pcDNA3-HA-TIP60 (kindly provided by Dr. D. Trouche(15)), 6xHis-p300 (kindly provided by Dr. W.L. Kraus(16)), pRSV-NFATc/A(17) and Myc-SIRT1(18) (both kindly provided by Dr. B.M.T. Burgering) have been described earlier. pCDNA3-p300-HA was generated by cloning a Not I-Hind III fragment from CMVβ-p300-HA (a gift from Dr. R. Eckner) into the respective cloning sites of pCDNA3. PEI (#23966) was purchased from Polysciences inc (Eppelheim, Germany). Trichostatin A (TSA), nicotinamide (NAD) cyclohexamide (CHX), epoxomycin, lactacystin and MG132 were from Sigma.

Transfection of cells and luciferase assays.

HEK293 cells were maintained in DMEM (Invitrogen) supplemented with 8% heat-inactivated FCS, penicillin and streptomycin (Invitrogen) at 37°C and 5% CO_2. Cells were grown to 50% confluence in 6 wells-plates (Nunc, Roskilde, Denmark) and transfected with a mixture of 1.5 µg DNA and 7.5 µl PEI overnight, next day cells were washed twice with PBS and cultured for 24 hours in medium. Cell lysates were prepared for Western blot analysis. For the luciferase assay cells were transfection Calcium-phosphate was used with 1 µg IL-2 promoter luciferase reporter from Panomics (Fremont, CA) 0.5 µg of pMT2-FOXP3, 0.5 µg pcDNA3-HA-p300, pcDNA3-NFATc/A or 0.5 µg pcDNA3 empty vector and 7 µg pMT2 empty vector and 0.05 µg pRLTK *renilla*, (Promega, Leiden, the Netherlands) to normalize for transfection efficiency. Cells were transfected in a 6-well plate, 3 days after transfection the cells were washed twice with PBS and lysed in 50 µl passive lysis buffer for 15 minutes, insoluble cell debris were spun down and the supernatant fraction was assayed for luciferase activity using Dual-Luciferase Reporter Assay System (Promega, Leiden, The Netherlands)(19).

In vitro acetylation assay.

GST fusion proteins were induced and purified an described previously(20). The wild-type and catalytic AT2 mutant p300 proteins were synthesized in Sf9 cells by using a baculovirus expression system and purified as previously described. 1 µg GST-FOXP3, 0.5 µl (10nCi) [14C]-AcetylCoA (Perkin Elmer, Groningen, The Netherlands) and 2 µg His(6x)-p300 or His(6x)-p300mutAT2 were incubated in AlPH buffer for 40 minutes at 30°C. The reaction was stopped by 10 µl 5x sample buffer. Samples sample were analyzed by SDS PAGE, and Kodak XB films (Rochester, NY)

Confocal studies.

HEK 293 cells were cultured on poly-L-lysine-coated (Sigma) microscope glasses, fixed in PBS containing 3% paraformaldehyde (Merck, Nottingham, United Kingdom) for 15 minutes at 15°C and subsequently 100% methanol (Merck) for 30 minutes at -20°C. Cells were preincubated with 10% normal Goat serum (Jackson West Grove, PA) before mouse anti-FOXP3 (5 µg/ml ; eBioscience) and of rabbit anti-p300 (5 µg/ml) was added for 1 hour, followed by PBS washes and incubation with 2 µg/ml Goat-anti-mouse-Cy3 (Jackson) or Goat-anti-rabbit-Cy5 (Jackson) conjugates (all antibody stainings were in PBS with 10% Goat serum). Slides were then washed extensively, and cells were mounted in mowiol containing 3% DABCO followed by a glass cover as described(21). Cells were examined with a Zeiss LSM 710 microscope (Oberkochen, Germany).

Western blots.

Cells were lysed in Laemmli buffer [0.12 mol/L Tris-HCL (pH 6.8), 4% SDS, 20% glycerol, 0.05 µg/µL bromphenol blue, 35 mmol/L ß-mercaptoethanol] and boiled for 5 minutes and the protein concentration was determined. Equal amounts of sample were analyzed by SDS-PAGE, electrophoretically transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA), and probed with the respective antibodies. Immunocomplexes were detected using enhanced chemiluminescence (Amersham, Buckinghamshire, United Kingdom).

Isolated primary T cells.

T cells from human PBMC's. PBMCs were isolated by Ficoll density-gradient centrifugation (Pharmacia, Uppsala, Sweden) and CD4 positive cells were isolated by MACS. Cells were cultured in for 7 days with RPMI 1640 supplemented with 2 m*M* L-glutamine, 100 units/ml of penicillin/streptomycin (Gibco BRL, Gaithersburg, MD), and 10% (volume/volume) AB_ heat-inactivated (60 minutes at 56°C) human serum (Sanquin Blood Bank, Amsterdam, The Netherlands), and stimulated with plate bound anti-CD3 (1.5µg/µl), 300 IU/ml human IL-2 and 2 µg/ml anti-human CD28 in combination with 10 mM NAM or 5 µM resveratrol. Cells were cultured in triplicate in round-bottomed 96-well plates (Nunc, Roskilde, Denmark) at 37°C in an atmosphere of 5% CO2 with 100% relative humidity.

T cells from human skin. Normal human skin was obtained from patients undergoing cosmetic surgery procedures. Three-dimensional matrices (Statamatrix) were obtained from Cell Sciences (Singapore). Explant cultures were established as described(22). Skin was cut into very small fragments and placed on the surface of a matrix. Each matrix was placed into 1 well of a 24-well plate in 2 ml/well of Iscove modified medium (Mediatech, Herndon, VA) with 20% heat-inactivated fetal bovine serum (FBS; Sigma, St Louis, MO), penicillin and streptomycin, and 3.5 ml/l b-mercaptoethanol. Cultures were fed 3 times a week by careful aspiration of 1 ml of culture medium and replacement with fresh medium. Cells were harvested at 12 days. IL-15 (10 ng/ml) and IL-2 (5 IU/ml) (R&D Systems, Benicia, CA) were added and refreshed with each feeding, in combination with 10 mM NAM or 5 µM resveratrol.

T cells from mouse splenocytes. Spleens were removed from healthy euthanized C57BL/6 mice, CD4 positive T cells were isolated from splenocytes using MACS separation. Cells were cultured for 5 days in flat bottom plates coated with mu-aCD3 (1ug/ml in pbs) in IMDM supplemented with 2 m*M* L-glutamine, 100 units/ml of penicillin/streptomycin (Gibco BRL), and 10% (volume/volume) heat-inactivated (60 minutes at 56°C) FC serum, 0.2 ng/ml murine IL-2 and 0.5 µg/ml anti-murine CD28 and 2 ng/ml TGFβ in combination with 10mM NAM or 5 µM resveratrol. Cells were cultured in triplicate in round-bottomed 96-well plates (Nunc) at 37°C in an atmosphere of 5% CO2 with 100% relative humidity.

T cell clone. The CD4+ T cell clone N3CA8 was generated as described elsewhere(23), and cultured for 6 days with RPMI 1640 supplemented with 2 m*M* L-glutamine, 100 units/ml of penicillin/streptomycin (Gibco BRL, Gaithersburg, MD), and 10% (volume/volume) heat-inactivated pooled human serum (Sanquin Blood Bank, Amsterdam, The Netherlands) in 48-wells tissue culture plates (Becton Dickinson). The T cell clone was stimulated with 40 IU/ml human IL-2 and anti-CD3/anti-CD28 Dynabeads (invitrogen) at a bead: T cell ration of 1:10 in combination with 5 mM NAM or 2.5 µM resveratrol. Staining of surface markers and FOXP3 for flowcytometry was done according to the manufacturer's protocol (eBioscience).

Suppression assay

Mice CD4 T cells were cultured as described above and stained by anti-mouse CD4 PercP and anti-mouse CD25 APC (clone RM4-5 and PC61, respectively; both from BD Pharmingen). To determine suppression of proliferation by measuring CFSE dilution within the CD4 T effector cell population, we labeled freshly isolated splenocytes with 2 µM CFSE, for 7 minutes at 37˚C. 10.000 labeled splenocytes per well in a 96-well plate were stimulated with 1 ug/ml soluble anti-

CD3, and 2000 $CD4^+CD25^+$ sorted cells were added. On day 4 cells were harvested, stained with anti-CD4, and CFSE dilution was measured on FACS CANTO (BD Biosciences).

Quantitative PCR

mRNA was isolated using the trizol according to the manufacturer's protocol (Invitrogen), cDNA synthesis was performed using IScript cDNA synthesis kit (Bio-Rad, Hercules, CA). cDNA samples were amplified using SYBR green supermix (BIO-Rad), in a MyiQ single-color real time PCR detection system (Bio-Rad) according to the manufacturer's protocol. The following primers were used: FOXP3 fw: TCAAGCACTGCCAGGCG, FOXP3 rv: CAGGAGCCCTTGTCGGAT, β2M fw: CCAGCAGAGAATGGAAAGTC, β2M rv: GATGCTGCTTACATGTCTCG. To quantify the data, the comparative Ct method was used. Relative quantity was defined as $2^{-\Delta\Delta Ct}$. β2-microglobulin was used as reference gene.

Statistical analysis.

Statistical analysis was performed using the Mann-Whitney test (Prism GraphPad Software, San Diego, CA). $p<0.05$ was considered statistically significant.

Results

FOXP3 interacts with and is acetylated by p300

To assess whether FOXP3 can be acetylated, cells were transfected with a Flag-tagged FOXP3 construct and incubated with the HDAC inhibitors (HDACi) Trichostatin A (TSA) and nicotinamide (NAM). Together these HDACi can inhibit a majority of HDACs. FOXP3 acetylation was analyzed by Flag immunoprecipitation and immunoblotting using an anti-acetyl lysine antibody. We observed a basal level of FOXP3 acetylation in the absence of HDACi, which was increased by addition of TSA/NAM in a dose-dependent manner (**Figure 1A**). To determine whether the ubiquitously expressed histone acetyl transferase (HAT) p300 could acetylate FOXP3, cells were transfected with FOXP3 and increasing amounts of p300, and FOXP3 acetylation was again analyzed. Indeed, addition of p300 dose-dependently increased FOXP3 acetylation (**Figure 1B**). Since a recent study demonstrated interaction between FOXP3 and the HAT TIP60(13), we wished to determine whether TIP60 could also acetylate FOXP3 in living cells. Lysates were prepared from HEK 293 cells co-transfected with Flag-FOXP3 and HA-TIP60 or HA-p300. Immunoblots reveal that in contrast to p300, TIP60 co-transfection did not increase FOXP3 acetylation (**Figure 1C**). We next determined whether p300 and TIP60 could associate with FOXP3 by co-immunoprecipitation after co-transfection of cells with Flag-FOXP3 and HA-p300 or HA-TIP60. Both p300 and TIP60 were found to associate with immunoprecipitated FOXP3 (**Figure 1D+E**). Finally, we tested p300 for its ability to directly acetylate FOXP3 using an *in vitro* acetylation assay. GST-FOXP3 fusion protein coupled to sepharose beads was incubated with [^{14}C]-labeled acetyl-CoA and p300, or an acetylase dead p300mutAT2, samples were separated on SDS-page and analyzed by autoradiography (**Figure 1F**). Incubation of FOXP3 with p300 but not the catalytically inactive variant, resulted in FOXP3 acetylation. These data demonstrate that although both TIP60 and p300 can associate with FOXP3, FOXP3 acetylation is selectively mediated by p300.

Acetylation regulates FOXP3 protein levels

Since acetylation of the related Foxo Forkhead transcription factors has been reported to mediate their subcellular localization(18;24), we analyzed FOXP3 subcellular localization under conditions of hyperacetylation. Using confocal microscopy we found FOXP3 to be exclusively localized in the nucleus (**Figure 2A**, upper panel). In contrast to previous reports analyzing Foxo transcription factor localization, co-transfecting cells with p300 did not influence FOXP3 localization (**Figure 2A**, lower panel). However, ectopically-expressed as well as endogenous p300 co-localized with FOXP3 in the nucleus, underscoring a role for p300 in nuclear FOXP3 acetylation.

To examine a possible role for p300 in FOXP3-mediated transcriptional activity we performed transcription reporter assays in which cells were transfected with NFAT and an IL-2 promoter luciferase reporter together with FOXP3 in the absence or presence of p300(25) (**Figure 2B**). FOXP3 expression resulted in clear repression of IL-2 promoter activity, while the IPEX mutant FOXP3 ΔE250, which does not dimerize, was not transcriptionally functional as expected. Co-transfection of p300 significantly increased FOXP3 transcriptional repression ($p=0.008$), indicating an increased functionality of acetylated FOXP3.

Since p300 mediated acetylation resulted in increased FOXP3 activity, FOXP3 protein expression levels were analyzed. Cells were transfected with FOXP3, incubated with HDAC inhibitors and FOXP3 levels were determined by immunoblot analysis. We observed a dramatic, dose-dependent increase in FOXP3 protein levels in HDACi treated cells (**Figure 2C**). Similarly, p300 co-transfection increased FOXP3 protein levels in a dose-dependent manner (**Figure 2D**). Expression levels of the related Foxo3 transcription factor, which has previously been described to be acetylated, was unaffected by HDACi treatment or p300 co-transfection(18) (**Figure 2E**). These results demonstrate that p300-dependent FOXP3 acetylation can regulate its protein expression level.

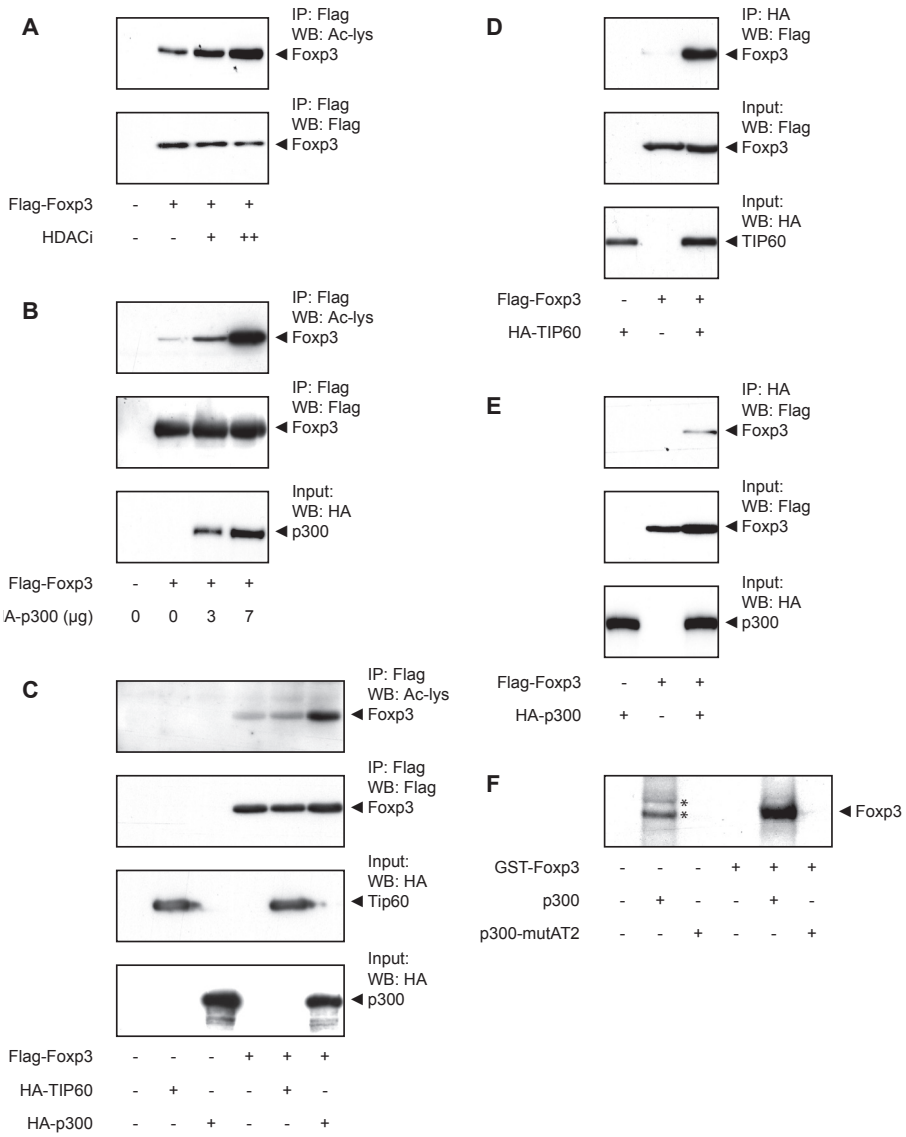

Figure 1. p300 promotes FOXP3 acetylation. (**A**) Flag immunoprecipitation from lysates from Flag-FOXP3 transfected cells. HEK 293 cells were treated with HDAC inhibitors (HDACi) +: 50 nm Trichostatin A (TSA) and 1 mM nicotinamide (NAM); or ++: 250 nm TSA and 5 mM NAM for 16 hours. Equal amounts of immunoprecipitated FOXP3 were separated by SDS-PAGE and western blots were probed for acetylated lysines (Ac-Lys) or Flag. (**B**) Cells were transfected with Flag-FOXP3 and/or HA-p300. Flag-FOXP3 was immunoprecipitated with anti-Flag beads and analyzed using acetyl lysines (Ac-Lys), Flag or HA antibodies. (**C**) Cells were co-transfected with Flag-FOXP3 and HA-p300 or HA-TIP60 and immunoprecipitated using anti-Flag beads. Immunoblots were analyzed with acetyl-lysine (Ac-Lys) antibody, anti-Flag or anti-HA. Cells were co-transfected with Flag-FOXP3, HA-Tip60 (**D**) or HA-p300 (**E**). Cell lysates were co-immunoprecipitated using anti-HA beads and analyzed using anti-Flag or anti-HA. (**F**) GST-FOXP3 fusion protein coupled to sepharose beads was incubated with [14C] labeled acetylCoA and p300 or the acetylase dead p300mutAT2, samples were separated on SDS-page and analyzed using films sensitive for radioactivity. Results depicted are representative of at least 3 independent experiments. ∗, aspecific band. IP, immunoprecipitation. WB, Western blot.

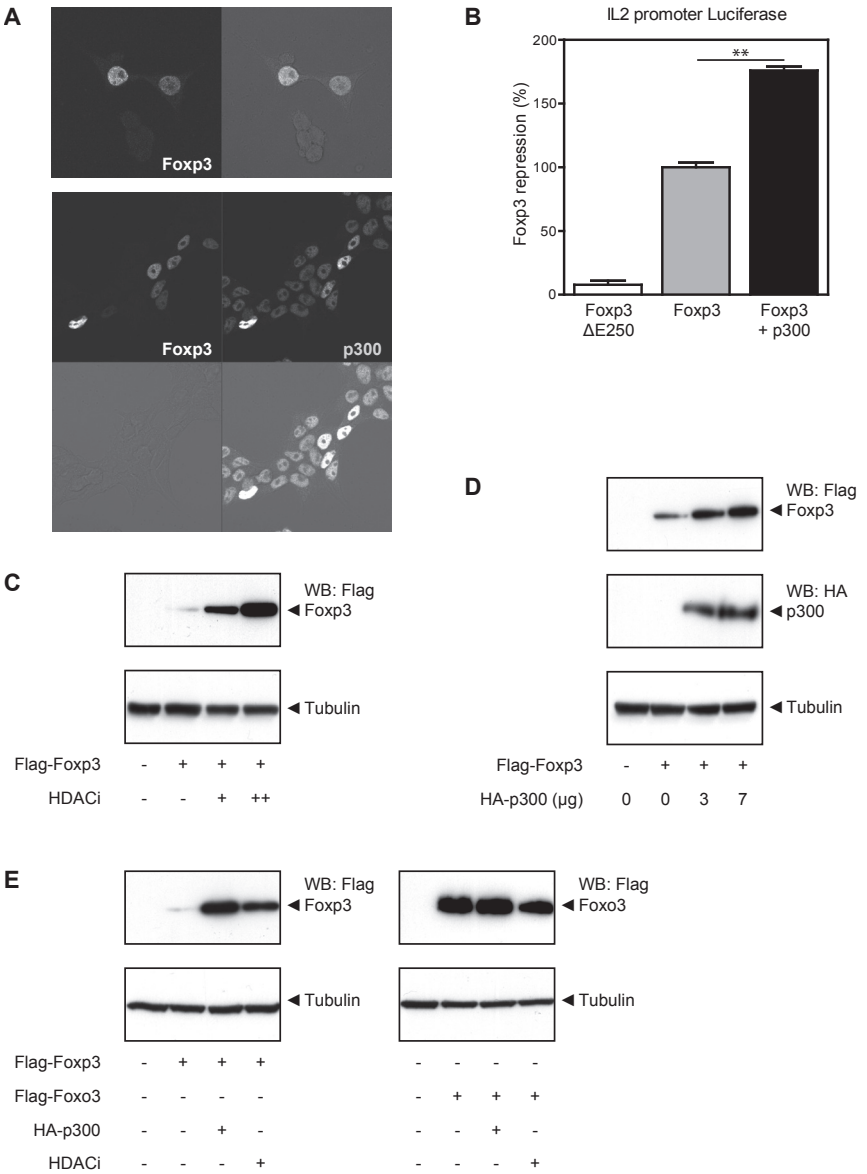

Figure 2 Acetylation modulates FOXP3 protein levels (**A**) Representative examples of cells that were transfected with only FOXP3 (green; upper panel). Subcellular distribution of cells that were co-transfected with FOXP3 (green) and p300 (red) are shown in the lower panel. p300 was localized using an anti-p300 antibody that recognizes both endogenous and ectopically expressed p300. Co-localization of FOXP3 and p300 is indicated in yellow. (**B**) FOXP3 function was assessed by evaluating IL-2 promoter reporter activity. IL-2 promoter luciferase activity was analyzed in HEK 293 cells by co-transfecting NFAT with FOXP3 del E250 (white bar), FOXP3 (grey bar), or FOXP3 with p300 (black bar). Repression of IL-2 luciferase activity by wild type FOXP3 was set as 100%, values were all normalized for co-transfected Renilla. (**C**) HEK 293 cells were transfected with Flag-FOXP3 and treated with HDAC inhibitors (HDACi) +: 50 nm TSA and 1 mM NAM or ++: 250 nm TSA and 5 mM NAM. Immunoblots were probed for Flag expression or tubulin as loading control. (**D**) Cells were transfected with Flag-FOXP3 and increasing amounts of HA-p300. Western blots were incubated with antibodies against Flag, HA or tubulin as indicated. (**E**) HEK 293 cells were transfected with Flag-FOXP3 or Flag-Foxo3 with or without HA-p300. Cells were treated with 100 nM TSA and 2.5 mM NAM for 16 hrs (HDACi). Data shown are representative of at least 3 independent experiments. A double asterisk indicates $p<0.01$.

Since it has been described that FOXP3 function depends on homo-dimerization(26), we also investigated whether dimerization is also necessary for p300 mediated acetylation, Flag-FOXP3 or Flag-FOXP3ΔE250 were immunoprecipitated and acetylation levels were analyzed. FOXP3ΔE250 acetylation was comparable to that of wild-type FOXP3 in the presence or absence of p300 (**Figure S1A**). To determine whether acetylation of Flag-FOXP3ΔE250 also resulted in increased protein expression levels, cells transfected with Flag-FOXP3 or Flag-FOXP3ΔE250 were treated with HDACi or co-transfected with p300. Both FOXP3 and FOXP3ΔE250 protein levels were increased by HDACi and p300 to a similar extent (**Figure S1B**), indicating that acetylation and modulation of protein expression is not dependent on dimerization *per se*.

Since we observed that FOXP3 protein levels are modulated by p300 mediated acetylation, we also wished to examine the molecular mechanism regulating deacetylation. To further pinpoint which HDAC is responsible for the effects observed, cells were treated with either TSA or NAM and FOXP3 expression levels were analyzed. TSA is an inhibitor of multiple HDAC families, whereas NAM selectively inhibits the SIRT HDAC family. Both TSA and NAM treatment increased FOXP3 protein levels in a dose-dependent manner (**Figure 3A**) indicating that FOXP3 can be deacetylated by members of at least two independent HDAC families.

To verify that SIRT is involved in modulation of FOXP3 expression, interaction between FOXP3 and SIRT was confirmed by co-immuneprecipitation after co-transfection of cells with Flag-FOXP3 and myc-tagged SIRT1 (**Figure 3B**). We next assessed whether SIRT1 could also modulate FOXP3 expression by co-transfecting cells with Flag-FOXP3 and myc-SIRT1. Immunoblots revealed that FOXP3 protein levels were clearly reduced in a dose-dependent manner in cells co-transfected with SIRT1 (**Figure 3C**). Taken together, these data support a role for acetylation/deacetylation in regulating FOXP3 protein expression levels, a process that can be reciprocally regulated by p300 and SIRT.

Acetylation impairs proteasome-mediated FOXP3 degradation.

To further evaluate the molecular mechanism underlying acetylation-dependent modulation of FOXP3 expression, proteasomal degradation was abrogated using the inhibitor MG132. Cells transfected with equal amounts of Flag-FOXP3 were incubated with HDACi with or without MG132, and FOXP3 protein levels were analyzed. Treatment with MG132 alone dramatically increased FOXP3 levels, indicating that FOXP3 is rapidly degraded by the proteasome. Importantly, treatment with HDACi did not further increase FOXP3 protein levels when cells were simultaneously incubated with MG132 (**Figure 4A**). To validate these observations, we repeated the experiments using additional specific proteasome inhibitors epoxomycin and lactacystin (**Figure 4B**). Similar results were obtained when compared to MG132 indicating that FOXP3 expression levels are regulated in a proteasome-dependent manner.

These data suggest that FOXP3 proteasomal turnover is abrogated by HDAC inhibitors. To confirm this, we made use of cyclohexamide (CHX), a widely used inhibitor of protein translation (**Figure 4C**). Cells were transfected with Flag-FOXP3 and then treated with cyclohexamide before analyzing FOXP3 expression levels. Cyclohexamide treatment led to a decrease in FOXP3 protein levels within six hours. However, when the cells were also treated with HDACi, FOXP3 protein levels stabilized, confirming that FOXP3 protein stability is indeed increased through acetylation.

Proteasomal degradation is mediated by poly-ubiquitination which similar to acetylation occurs at lysine residues. This suggests that acetylation of lysine residues may prevent ubiquitination and thereby inhibit FOXP3 degradation. To test this hypothesis, we generated a FOXP3 mutant in which all 22 lysines were mutated into arginines (FOXP3 K22xR). If direct FOXP3 ubiquitination is indeed responsible for increased protein turnover then this mutant should exhibit enhanced stability, and, importantly, FOXP3 protein levels should no longer be modulated by HDACi. Cells were transfected with equal amounts of Flag-FOXP3 or Flag-FOXP3 K22xR and incubated with HDACi (**Figure 4D**). As previously demonstrated, hyperacetylation increased the levels of wild-type FOXP3. In contrast, FOXP3 K22xR protein levels were not altered by treatment with

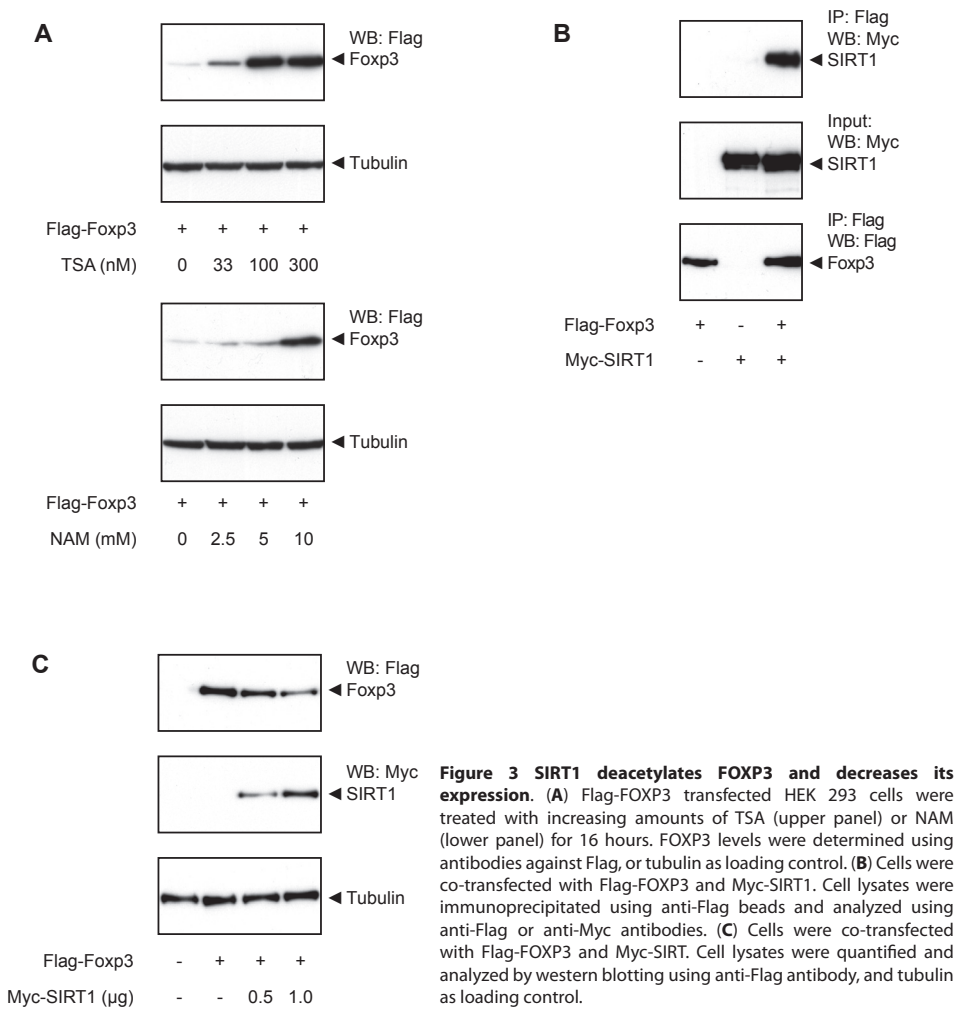

Figure 3 SIRT1 deacetylates FOXP3 and decreases its expression. (**A**) Flag-FOXP3 transfected HEK 293 cells were treated with increasing amounts of TSA (upper panel) or NAM (lower panel) for 16 hours. FOXP3 levels were determined using antibodies against Flag, or tubulin as loading control. (**B**) Cells were co-transfected with Flag-FOXP3 and Myc-SIRT1. Cell lysates were immunoprecipitated using anti-Flag beads and analyzed using anti-Flag or anti-Myc antibodies. (**C**) Cells were co-transfected with Flag-FOXP3 and Myc-SIRT. Cell lysates were quantified and analyzed by western blotting using anti-Flag antibody, and tubulin as loading control.

HDACi. Importantly, we observed that the levels of FOXP3 K22xR were considerably increased compared to wild-type FOXP3. To confirm our hypothesis that there is competition for the same lysines by acetylation and ubiquitination, we analyzed the effect of HDACi on the level of FOXP3 ubiquitination. Cells were transfected with FOXP3 and treated with HDACi. Subsequently, FOXP3 was immunoprecipitated, blotted and evaluated using an anti-ubiquitin antibody. As shown in **Figure 4E** there is a basal level of poly-ubiquitinated FOXP3. Treatment with HDACi however, considerably reduced the amount of poly-ubiquitinated FOXP3.
Taken together, these data demonstrate that FOXP3 protein levels are rapidly turned-over in a proteasome-dependent manner, and that acetylation likely prevents entry of FOXP3 into this pathway by competing with poly-ubiquitination.

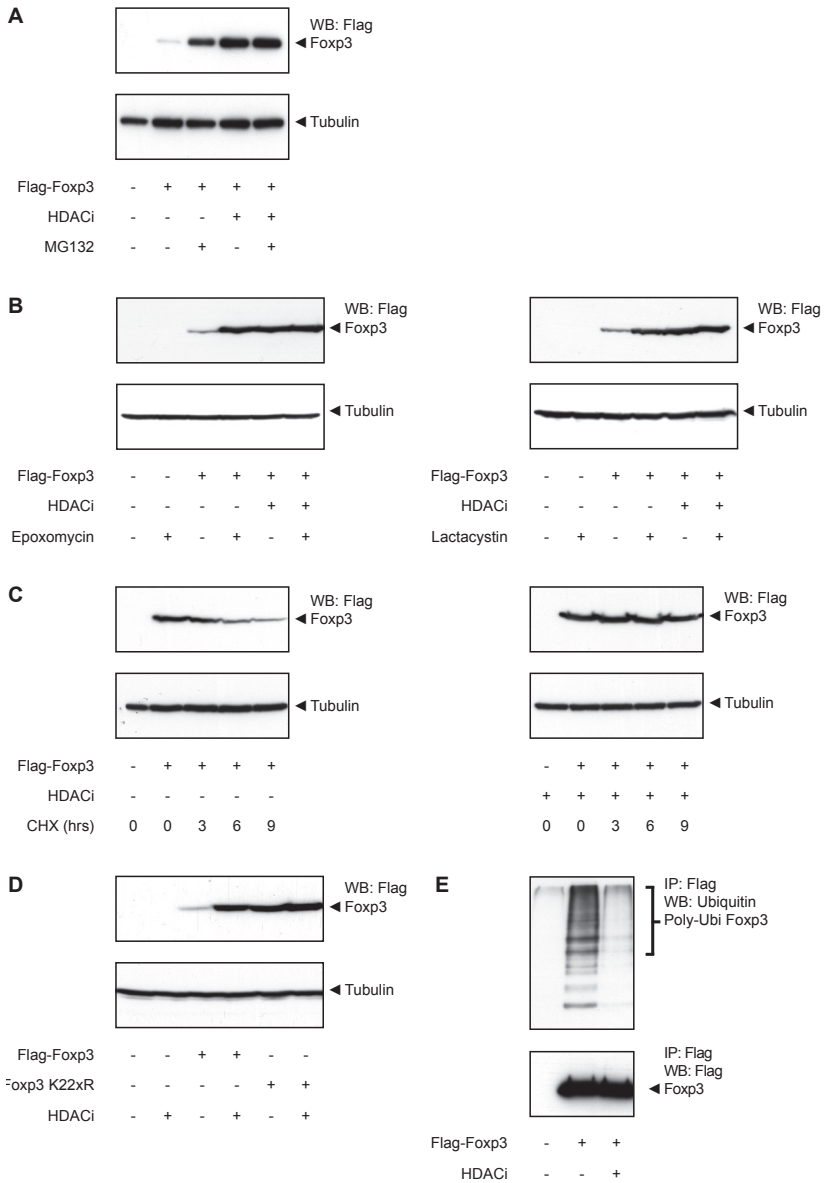

Figure 4 FOXP3 acetylation prevents proteasomal degradation. Flag-FOXP3 transfected HEK 293 cells were treated with 2 μM MG132 (**A**), 100 nM epoxomycin or 10 μM lactacystin (**B**) for 16 hours to inhibit proteasome function and/or HDAC inhibitors (HDACi) TSA 100 nM and NAM 2.5 mM also for 16 hours. FOXP3 expression was analyzed using a FOXP3 antibody, and equal loading was verified by analyzing tubulin expression. (**C**) Cells were transfected with equal amounts of Flag-FOXP3. Half of the cells were treated with or without TSA 100 nM and NAD 2.5 mM for 16 hours (right panel), and 5 μg/ml cylohexamide for indicated time points. FOXP3 expression was analyzed using a Flag antibody and tubulin expression was used as a loading control. All results depicted are representative of at least 3 independent experiments. (**D**) Flag-FOXP3 or a Flag tagged FOXP3 mutant in which all the lysines are mutated to arginines (Flag-FOXP3 K22xR) was transfected into HEK 293 cells. The cells were treated with the HDAC inhibitors TSA (100 nM) and NAM (2.5 mM) for 16 hours. Cell lysates were made and immunoblotted for Flag and tubulin as control. (**E**) HEK 293 cells were transfected with Flag-FOXP3, treated with or without TSA 100 nM and NAD 2.5 mM for 16 hours, cell lysates were immunoprecipitated using anti-Flag beads and analyzed using anti-ubiquitin and anti-Flag as transfection control.

Acetylation increases FOXP3 levels in primary T cells.

We next assessed the effect of acetylation on FOXP3 levels using primary T cells in four different models. T cells from four different sources were stimulated with IL-2, anti-CD3 and anti-CD28 to induce FOXP3, and FOXP3 positive cells were analyzed by flowcytometry. Firstly, $CD4^+$ cells from human PBMC's were treated with either the SIRT activator resveratrol(27), or the SIRT inhibitor nicotinamide (NAM) or control (**Figure 5A; S2**). We observed significantly decreased numbers of $FOXP3^+$ cells in the resveratrol treated cells, while the cells treated with nicotinamide showed a significantly higher percentage of FOXP3 positive cells compared to control. Secondly, since it has recently been reported that Tregs play a crucial role in immune homeostasis of the skin(28), T cells were isolated from human skin samples and treated with resveratrol or nicotinamide for 14 days (**Figure 5B**). Analysis of FOXP3 positive cells again revealed that treatment with resveratrol decreased the percentage of FOXP3 positive cells, while nicotinamide increased $FOXP3^+$ cell numbers. T cells were also isolated from healthy C57BL/6 mice spleens and cultured in the presence of resveratrol or nicotinamide (**Figure 5C**). Activation of SIRT resulted in a diminished percentage of FOXP3 positive cells. Treatment with nicotinamide however, increased the number of $FOXP3^+$ cells by up to ten-fold. We also generated a human T cell clones which were treated with resveratrol or nicotinamide (**Figure 5D**). Again, resveratrol treatment decreased the numbers of $FOXP3^+$ cells while inhibition of SIRT in these cells resulted in a significantly increased percentage of $FOXP3^+$ cells. Taken together, these data demonstrate that SIRT mediated regulation of acetylation influences FOXP3 expression levels in both mouse and human primary T cells from a variety of origins.

Hyperacetylation of FOXP3 increases Treg function.

Since we observed that acetylation impairs proteasomal degradation (**Figure 4**) we analyzed whether FOXP3 protein levels per cell were regulated by SIRT, as are the $FOXP3^+$ cell numbers. T cells from all four sources were treated with NAM or control and the mean fluorescence intensity (MFI) of $FOXP3^+$ cells was analyzed by FACS (**Figure 6A**). The MFI of the NAM treated cells was significantly higher compared to the control treated cells for all T cell types, indicating that SIRT inhibition not only increased the number of FOXP3 positive cells but also the FOXP3 levels per cell compared to control. To elucidate if the changes in FOXP3 expression are the result of increased protein stability, and not increased transcription, FOXP3 mRNA levels were analyzed. mRNA was isolated from control and NAM treated T cells from the human T cell clone that was previously used (**Figure 5D**). cDNA was prepared and analyzed using quantitative PCR for FOXP3 and β2 microglobulin (β2m) as reference. The ratio of FOXP3 versus β2m per FOXP3 positive cell is depicted in Figure 6b. No significant difference in FOXP3 mRNA levels per FOXP3 positive cell was observed, demonstrating that *FOXP3* transcription is not influenced by SIRT.

To confirm that acetylation not only regulates FOXP3 positive cell numbers and FOXP3 protein levels, but also Treg suppressor capacity, a suppression assay was performed. Primary T cells were stimulated and cultured with NAM for 5 days. Subsequently, the functionality of FOXP3 positive cells was analyzed in a CFSE based suppression assay (**Figure 6C**). NAM or control treated $CD4^+CD25^+$ T cells were co-cultured with effector T cells in a 1:5 ratio and proliferation of Teff cells was measured. As expected Tregs from the control treated sample were able to suppress the proliferation of effector T cells. Tregs from the NAM treated cultures, suppressed T cell proliferation compared to the control significantly better. These data clearly demonstrate that SIRT-mediated regulation of acetylation not only modulates $FOXP3^+$ cell numbers, but also FOXP3 protein levels independently of increased *FOXP3* transcription. Importantly, inhibition of SIRT also results in functionally improved Treg cells.

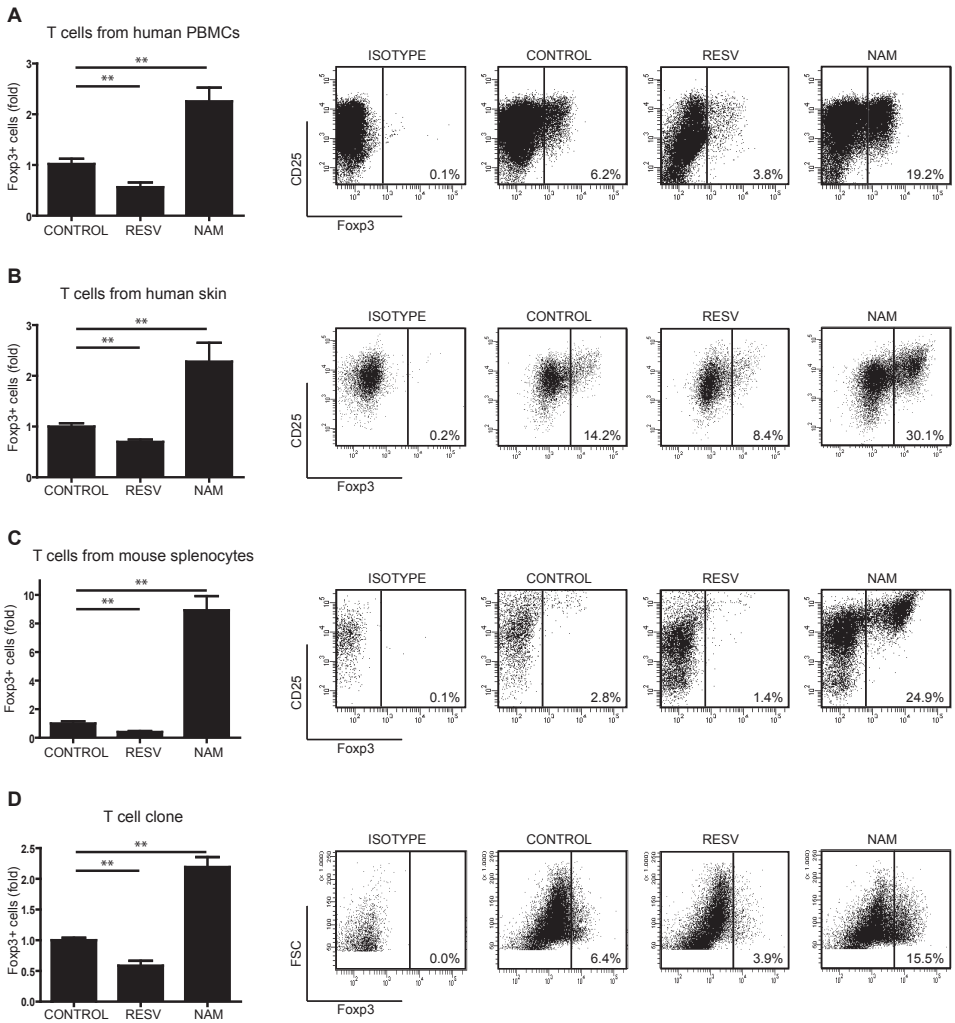

Figure 5 Treatment of primary T cells with a SIRT inhibitor results in increased FOXP3 positive cell numbers. Isolated primary T cells were cultured in the presence of IL-2, anti-CD3 and anti-CD28 (mouse splenocytes were also cultured in the presence of TGF-β). Cells were treated with the SIRT activator resveratrol, the SIRT inhibitor nicotinamide (NAM) or carrier as control. The percentage of FOXP3 positive cells was determined using FACS technology. T cells originated from human PBMC's (**A**), human skin (**B**), mouse spleen (**C**) and a human T cell clone (**D**). Results depicted are the means of at least 4 independent experiments. A double asterisk indicates $p<0.01$.

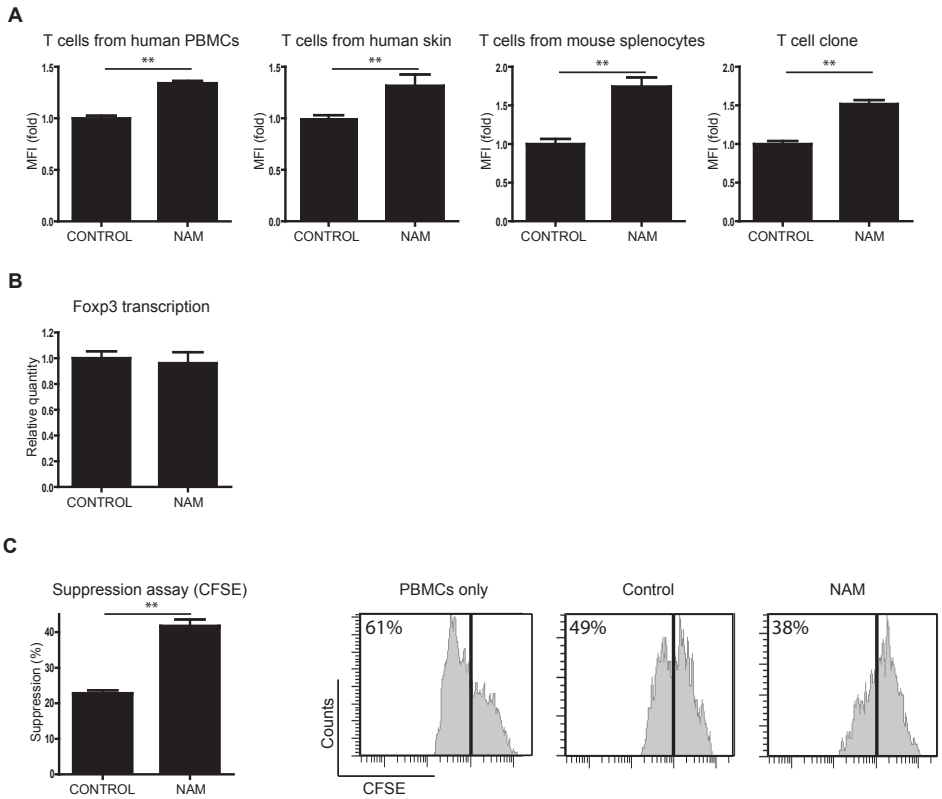

Figure 6 SIRT inhibition increases Treg functionality. Isolated primary T cells were cultured in the presence of IL-2, anti-CD3 and anti-CD28. Cells were treated with the SIRT inhibitor nicotinamide (NAM) or carrier as control. (**A**) FOXP3 levels per cells of different T cell sources were analyzed by FACS and data represents the mean fluorescence intensity of the FOXP3 positive population. (**B**) Cells from a human T cell clone were stimulated and treated with NAM or control. FOXP3 transcription was determined with quantitative PCR, results were corrected for the housekeeping gene β2-microglobulin and FOXP3 positive cell numbers. (**C**) CD4 T cells isolated from mouse splenocytes were stimulated using IL-2, anti-CD3 and anti-CD28 beads and treated with NAM or control. $CD25^+$ cells were sorted and the function of these induced Tregs was analyzed using a standard suppression assay. 10,000 effector T cells were co-cultured with 2,000 sorted $CD25^+$ cells for four days (without NAM). Proliferation of effector T cells on day 4 was measured by CFSE dilution within the $CD4^+$ effector T cell population. Data shown are representative of at least 3 independent experiments. An asterisk indicates $p<0.05$, a double asterisk indicates $p<0.01$.

Discussion

FOXP3 is a key transcription factor controlling immune homeostasis through regulating both the numbers and functionality of immunosuppressive Treg cells. While recent studies have focused on identifying FOXP3 transcriptional targets, or the molecular mechanisms underlying transcriptional regulation of FOXP3 expression, analysis of specific post-translational modifications regulating FOXP3 function have thus far not been critically addressed. Here we report for the first time that FOXP3 protein levels are directly controlled by acetylation, a process mediated by inhibition of proteasomal degradation. Moreover, we identified novel interaction partners that are key mediators of this process; the HAT p300 and the HDAC SIRT1. Furthermore, hyperacetylating FOXP3 in primary T cells resulted in more FOXP3$^+$ cells, higher FOXP3 protein levels per cell and better suppressive capabilities. Our data provide novel insights into post-translational mechanisms regulating FOXP3 function, and provide a model by which Treg numbers and functionality can be rapidly modulated by the extracellular milieu.

A recent study by Tao *et al* has demonstrated that *in vivo* HDAC inhibition results in enhanced Treg mediated suppression of homeostatic proliferation, decreased inflammatory bowel disease through Treg-dependent effects, and induction of permanent tolerance against islet and cardiac allografts(14). Since we have demonstrated that HDAC inhibitors increase FOXP3 expression through protein stabilization, this suggests a molecular mechanism explaining these *in vivo* observations. Importantly, we demonstrate that p300 increases FOXP3 expression levels, through inhibition of proteasomal degradation. This is a novel and unique observation for members of the forkhead transcription factor family(3). Degradation of other transcription factors is mediated by poly-ubiquitination of these proteins. Since lysine acetylation and ubiquitination are mutually exclusive, acetylation may thus prevent poly-ubiquitination through a competition-based mechanism. Indeed, in Figure 4 we demonstrate that by mutating all FOXP3 lysines into arginines we significantly increased FOXP3 protein levels, a process most likely mediated by inhibition of proteasomal degradation since FOXP3 K22xR cannot be poly-ubiquitinated. Inhibition of the proteosome-mediated degradation dramatically increased and stabilized FOXP3 protein levels, which could not be further increased by treatment with HDAC inhibitors. Furthermore, treatment with HDAC inhibitors did not further increase FOXP3 K22xR protein levels as observed in wild-type. In addition, we also show that treatment with HDACi dramatically decreased poly-ubiquitination of FOXP3. Taken together, we propose that poly-ubiquitination mediated FOXP3 degradation can be impaired by acetylation of lysines in a competition-based mechanism. A similar mechanism has been previously described for RUNX3, Smad7 and p53 where poly-ubiquitination of these transcription factors was significantly impaired by hyperacetylation(29-32).

Although p300 and TIP60 were both found to associate with FOXP3, we found that only p300 was able to acetylate FOXP3 (**Figures 1** and **2**). Li *et al* recently demonstrated that FOXP3 is part of a transcriptional complex containing both HDAC and HAT(13). This study reported that TIP60 over-expression promotes FOXP3 mediated transcriptional repression. However, as we did not observe TIP60-mediated FOXP3 acetylation, our data suggests that TIP60 predominantly acts indirectly on other (non-) histone proteins in the transcription/repression complex, rather than directly modulating FOXP3 transcriptional function.

We also examined which HDAC is responsible for regulating FOXP3 deacetylation. Treatment with either TSA or NAM alone was sufficient to result in increased FOXP3 expression (**Figure 3**). TSA has a broad specificity, inhibiting HDAC families I, II and IV. It has also recently been reported that both HDAC7 and HDAC9 associate with FOXP3 in a multimeric protein complex(13) and it is possible that FOXP3 deacetylation can also be mediated by one of these HDACs. Since NAM is a specific inhibitor of the SIRT HDAC III family, we conclude that there is also a role for SIRT in regulating FOXP3 activity, which we could subsequently further substantiate by co-immunoprecipitation experiments (**Figure 3**). Furthermore, *ex-vivo* treatment of CD4 positive T cells from four different sources with the SIRT activator resveratrol decreased the number

of FOXP3 positive cells. Interestingly, multiple studies have reported that resveratrol has anti-tumor activity although through an as yet undefined mechanism(33;34). Since Tregs repress immune function(35;36), it is interesting to speculate that resveratrol may act by reducing FOXP3 protein levels, thereby relieving Treg-mediated immune suppression, and ultimately resulting in enhanced immune activity towards tumors.
Although there has been a general paradigm that FOXP3 is exclusively expressed by regulatory T cells, it was recently shown that respiratory, thymic, prostate and mammary epithelium cells also express this transcription factor(8). FOXP3 function has also been directly linked with tumor suppressor activity, it was demonstrated that FOXP3 binds and represses the promoter of the HER-2/erbB2 and SKP2 oncogenes(37;38). In addition, somatic mutations and down-regulation of FOXP3 were found in human breast cancer samples and correlated significantly with HER-2/erbB2 and SKP2 expression. Since we have demonstrated that acetylation increases FOXP3 expression, it is plausible that treatment with HDAC inhibitors may enhance the tumor suppressor activity of FOXP3 and therefore have beneficial effects on tumor formation in the breast epithelium.
It has now become clear that $CD4^+$ effector T cells can also express FOXP3 after TCR stimulation *in vitro*, and FOXP3 expression has been strongly correlated with hyporesponsiveness of activated T cells(9-11). However, not all TCR stimulated FOXP3 positive cells have suppressive capabilities. In non-suppressive $FOXP3^+$ T cell populations FOXP3 expression was found to be transient, while stably expressing FOXP3 cells had true suppressive capabilities with a phenotype similar to that of naturally occurring Tregs(9;10). We speculate that this critical difference in transient versus stable FOXP3 expression is a key switch in the generation of "true" suppressor Tregs, and that this is directly regulated by acetylation. It is possible that normally antigen exposure activates T cells, resulting in transient FOXP3 expression levels which may act as an "immune brake" preventing T cell hyperactivation through repression of IL-2 and IFN-γ transcription(39). However, when T cells are chronically stimulated, for example by self-antigen, FOXP3 is acetylated stabilizing protein levels and allowing the initiation of a unique transcriptional program defining the Treg phenotype. Further work will be required to determine whether this is indeed the case.
Taken together, we have established that FOXP3 protein levels can be tightly regulated by acetylation. p300-mediated acetylation of FOXP3 plays a critical role in stabilizing FOXP3 protein levels. In contrast, we found that SIRT1 mediated deacetylation of FOXP3 results in reduced protein expression levels in cell lines as well as primary T cells. In addition we demonstrate that the mechanism by which FOXP3 protein levels are stabilized by acetylation, is by inhibition of proteasomal degradation. Our findings have important consequences for the development of novel molecular therapies regulating Treg numbers through pharmacological stabilization of FOXP3 protein levels.

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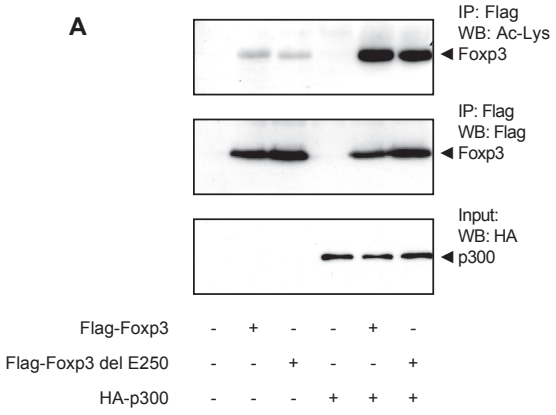
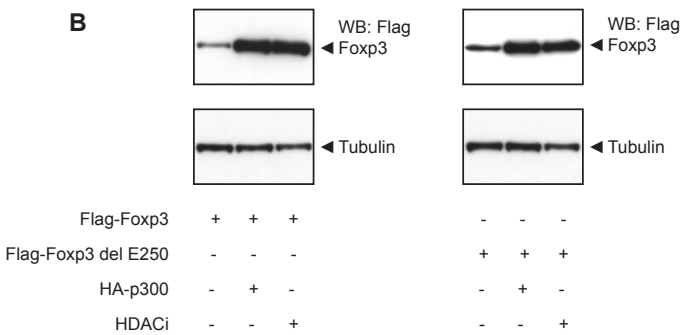
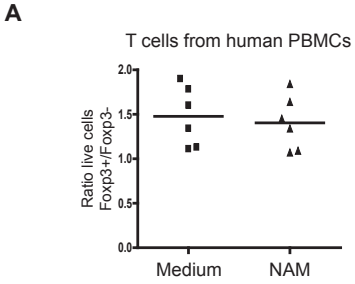
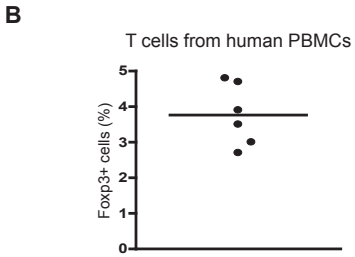
Supplementals



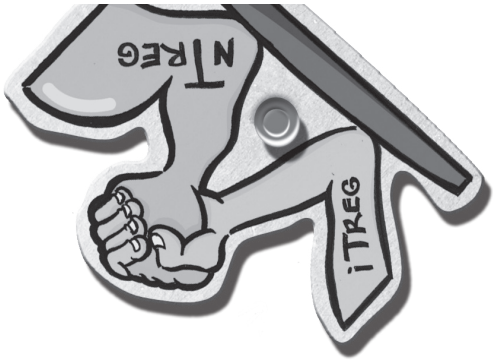


Supplementary Figure 1 FOXP3 acetylation and increased expression does not depend on homo-dimerization. (**A**) Flag-FOXP3 or Flag-FOXP3ΔE250 was co-transfected with p300 in HEK 293 cells. Using equal amounts of input protein, FOXP3 acetylation was analyzed using antibodies specific for acetyl-lysine (Ac-Lys), Flag or HA. (**B**) Cells were co-transfected with Flag-FOXP3 or Flag-FOXP3 ΔE250 and/or p300 and treated with 100 nm TSA and 2.5 mM NAM for 16 hours. Protein lysates were quantified and subsequently western blots were probed utilizing an antibody recognizing Flag, HA, or tubulin as loading control. All western blots are representative of at least 3 independent experiments.





Supplementary Figure 2 Increased FOXP3$^+$ cell numbers after HDACi treatment is not due to preferential cell death. (**A**) Human CD4$^+$ cells from six different donors were cultured in RPMI medium in the presence of IL2, anti CD3 and anti CD28 with or without NAM as described in figure 5 and 6. At day 7 cells were stained for FOXP3 to separate FOXP3$^-$ from FOXP3$^+$ cells and annexin V and 7-AAD to determine the percentage of apoptosis. The ratio of apoptotic cells in FOXP3$^+$ vs FOXP3$^-$ is depicted. (**B**) Analysis of FOXP3 positivity in CD4$^+$ compartment of human PBMCs at day 0 as the cells go into culture.



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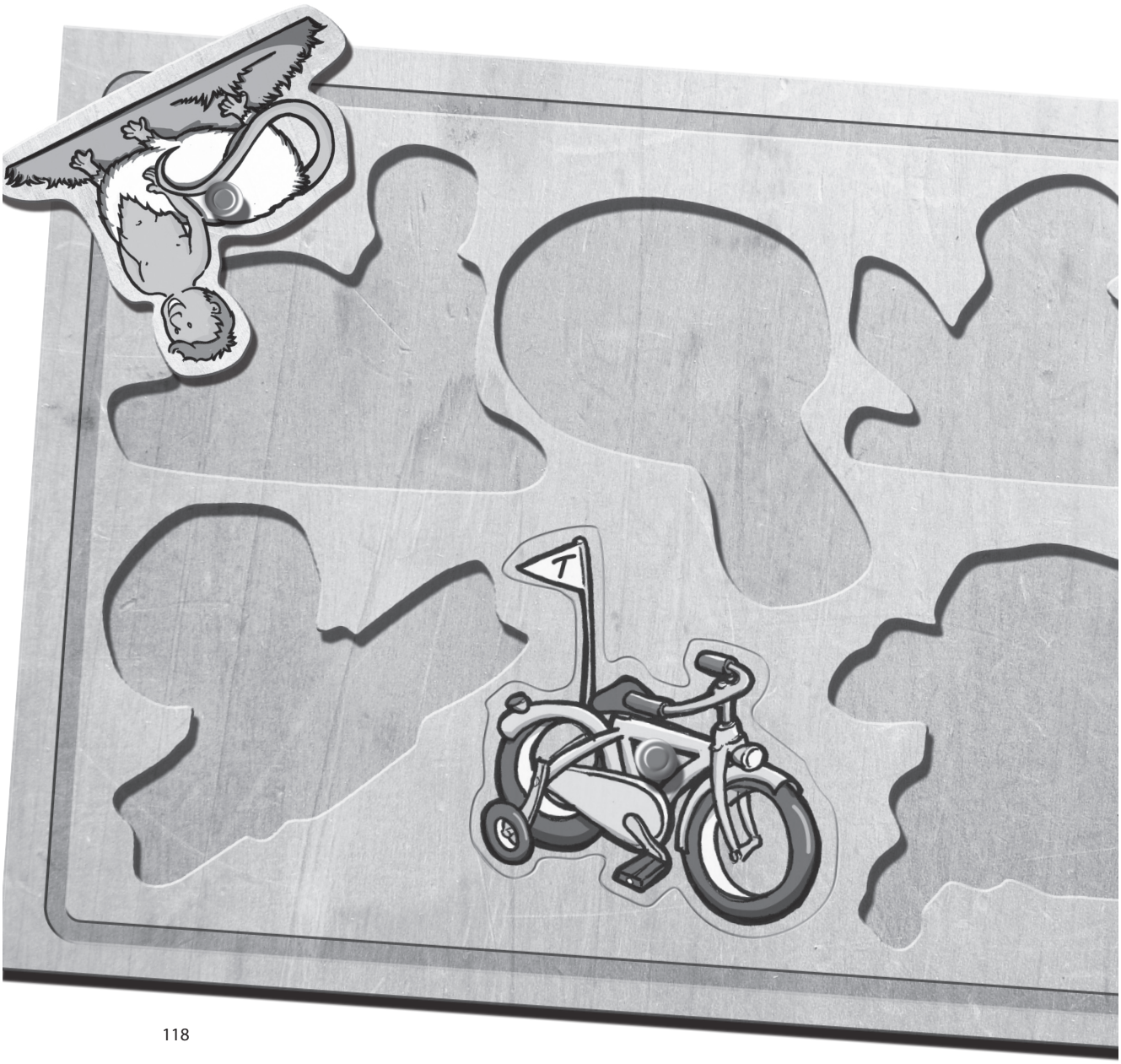

$CD4^+FOXP3^+$ Regulatory T Cells are abundantly present in inflamed muscle of patients with juvenile dermatomyositis

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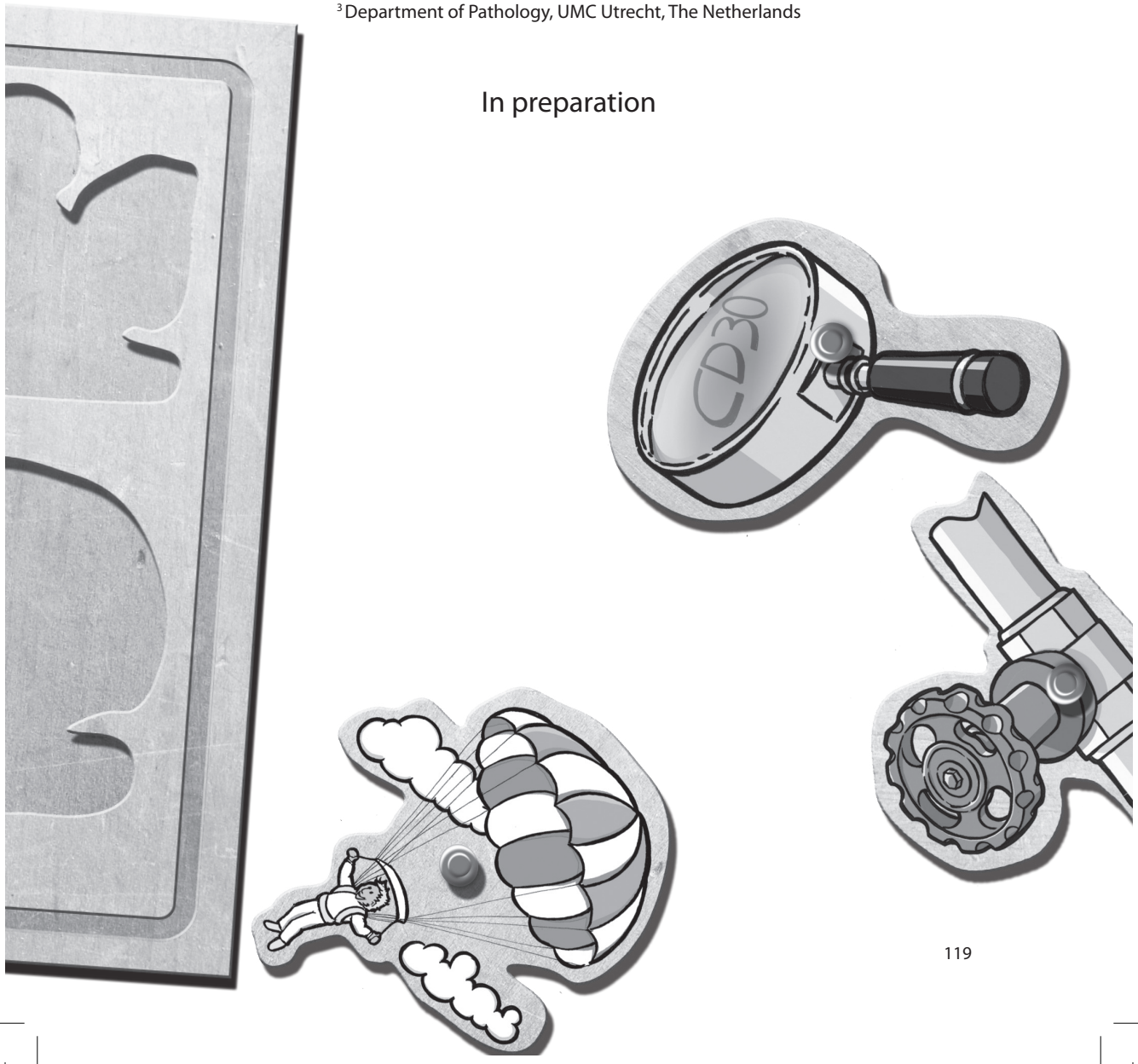
In preparation



Abstract

Juvenile dermatomyositis (JDM) is an autoimmune disease caused by inflammation of the microvasculature, resulting in skin and muscle damage. $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) are key regulators of immune homeostasis, and potential therapeutic targets in T cell-driven inflammatory disorders. However, a role for Treg in JDM pathogenesis has not yet been established. Here, we explored Treg presence and function in both peripheral blood mononuclear cells (PBMC) and muscle tissue of JDM patients. We analyzed expression of CD4, CD25, FOXP3, GITR and CTLA-4 in PBMC from JDM patients and age-matched controls by flow cytometry. Treg functionality was established in in vitro suppression assays. Infiltrated lymphocytes in muscle biopsies were analyzed for CD3, CD4, CD8 and FOXP3 expression by immunohistochemistry, qPCR (FOXP3) and flow cytometry (CD3 and CD4).

Overall, Treg number and phenotype of JDM patients was similar to age-matched controls. While JDM patients had increased numbers of Treg during active disease compared to remission, this increase was only found in JDM patients treated with high doses of corticosteroids. Treg of remitting JDM patients, but not of all active JDM patients were able to suppress effector T cell activation in vitro. Furthermore, in inflamed muscle tissue we detected high numbers of $CD4^+$ T cells, of which a large proportion expressed FOXP3.

Treg in peripheral blood of JDM patients had a similar phenotype and frequency as peripheral blood Treg from age-matched controls. Moreover, Treg were present in inflamed muscle tissue, and could therefore be targets for specific treatment of disease.

Introduction

Juvenile dermatomyositis (JDM) is a systemic autoimmune disorder in which the immune system targets the microvasculature of skeletal muscles, skin and other organs. In most patients this results in muscle weakness and a characteristic rash, consisting of heliotrope eyelid discoloration and Gottron's papules at extensor surfaces of joints. JDM is a rare disease, with an incidence rate of 3.2 per million in patients aged below 17 and affects mostly girls (1). Though the cause of JDM is still unknown, there is some evidence for genetic predisposition since JDM is strongly associated with HLA subtypes, such as HLA—DRB1 0301 (2, 3) , HLA-B8 (4), HLA-DQA1 0501 (5), and HLA-DRB1 1401 (6). Besides genetic predisposition, environmental factors such as viral infections may also contribute to JDM pathogenesis (7).

In muscle biopsies of JDM patients, of all infiltrated lymphocytes activated $CD4^+$ T cells are predominantly present, suggesting a pathogenic role for these T cells at the site of inflammation (8). $CD4^+CD25^+FOXP3^+$ regulatory T cells (Treg) are potent regulators of T cell mediated autoimmune responses and are suggested to play a role in both experimental and human autoimmune diseases. For instance, in patients with juvenile idiopathic arthritis (JIA), the presence of Treg correlates with improved disease outcome (9), while patients lacking functional FOXP3 due to a genetic mutation suffer from a lethal inflammatory disease, termed IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) (10, 11). Less is known about the presence and role of such cells in classical connective tissue diseases such as JDM. In patients suffering from systemic sclerosis, levels of $FOXP3^+$ Treg are low in both blood and inflamed skin, suggesting a lack of Treg (12). In contrast, in adults with poly- and dermatomyositis peripheral blood $CD4^+$ T cells express lower CD25 protein, but higher FOXP3 mRNA compared to healthy controls (13). However, it remains unclear as to whether FOXP3 expressing Treg are normally present and functional in peripheral blood or muscle tissue of JDM patients. As therapeutic modalities targeting Treg in autoimmune diseases are currently being developed (14-16) it becomes more critical to gain information on the functional role of FOXP3 expressing Treg in JDM.

Currently, treatment of JDM is mostly based on general immune suppression, with medication such as steroids, methotrexate (MTX), and in some cases the use of biologicals, such as anti-TNFα (17). All these approaches result in aspecific immune suppression with an increased risk of infections and other unwarranted side effects, but cannot cure the disease. Thus, the need for more specific treatment strategies is growing, and Treg have been suggested to be potential candidates for more specific treatment of human autoimmune diseases. For instance, in diabetes patients, manipulation of Treg by non-activating anti-CD3 treatment results in increased Treg numbers and suppresses disease (14-16)

In order to establish whether Treg could be a target for therapy in JDM, we investigated the presence and function of Treg in peripheral blood of JDM patients with active and remitting disease. Moreover, we studied whether Treg were present at the site of inflammation; in the muscle tissue of JDM patients. Our data suggests that percentages of $FOXP3^+$ Treg in peripheral blood of JDM patients were comparable to age-matched controls, and was only increased in patients treated with high doses of corticosteroids. While Treg from peripheral blood from patients with remitting JDM were suppressive, Treg from patients with active disease could not consistently suppress T cell proliferation in vitro. Furthermore, $FOXP3^+$ Treg were present in large numbers in inflamed muscle tissue, compared to activated T cells, suggesting that Treg were unable to suppress inflammation during active disease.

Patients and Methods

Participants

Peripheral blood samples from patients with Juvenile Dermatomyositis (JDM, n=22) or age-matched controls were included (C, n=6). For participant's characteristics and medication, see Table 1. Only patients diagnosed according to the Bohan & Peter criteria (18) were enrolled. All JDM patients were followed up at the department of Pediatric Immunology and Rheumatology of the University Medical Center Utrecht, the Netherlands. This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by the local institutional review board, and written consent was obtained from all parents of participants.

Cells and medium

PBMC were isolated using Ficoll Isopaque density gradient centrifugation (Amersham Biosciences, New Jersey, USA). PBMC were frozen in FCS containing 10% DMSO, and stored in liquid nitrogen before use. On the day of analysis cells were thawed and washed extensively to remove DMSO. Only cell samples with good viability, analyzed by trypan blue staining, were included. RPMI 1640 containing 10mM HEPES (Seromed), 2mM L-glutamine 100 U/ml penicillin-streptomycin and 10% human AB serum was used as culture medium (all Invitrogen, Carlsbad, USA).

Flow cytometry

PBMC were washed twice in FACS buffer (PBS containing 2% FCS) and adjusted to 0.5-1 x 10^6 cells/ml in staining buffer (FACS buffer containing 0.1% sodium azide) and blocked with mouse serum (30 min at 4°C). Subsequently, the cells were incubated in 50µl FACS buffer containing three or four appropriately diluted PE, FITC, PERCP-CY5.5, PE-Cy7, eFluor 450, Pacific Blue, APC labeled mAbs against human CD4 (clone RPA-T4), CD25 (clone 2A3), CD127 (clone hIL-7R-m21) and GITR (clone 110416). For intracellular staining of CTLA-4 (clone BN13) and FOXP3 (clone PCH101), the cells were first surface stained and subsequently treated with a FOXP3 staining kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. GITR mAb was obtained from R&D (Wiesbaden, Germany) and FOXP3 mAb from eBioscience (San Diego, CA, USA). All other mAbs were obtained from Becton Dickinson (San Jose, CA, USA). Stained mononuclear cells were diluted in FACSbuffer and run on a FACSCanto (BD). FacsDiva software (BD) was used for analysis.

Suppression assay

Suppressive function of sorted $CD4^+CD25^+CD127^{low}$ T cells was tested in co-culture experiments with fresh PBMC from the same donor. 10,000 PBMC were plated per well in plate-bound anti-CD3-coated wells (clone OKT-3, 1.5 µg/ml, eBioscience). $CD4^+CD25^+CD127^{low}$ T cells sorted by FACS Aria (BD) were directly sorted into the plate in different co-culture ratios. The cells were incubated at 37°C for 6 days, the last 18h in the presence of ^3H thymidine (1 µCi/well). The suppressive activity was determined by calculating the relative difference in proliferative response (mean ^3H thymidine incorporation (cpm) of triplicate, or sometimes duplicate wells) between $CD4^+CD25^-$ T cells cultured alone and $CD4^+CD25^-$ T cells cultured in the presence of Treg. Of the three patients with active JDM one patient received <0.5 mg/kg prednisone, one patient received 1mg/kg prednisone , and one patient received 3x15 mg/kg methyl-prednisolone pulses over 3 days.

mRNA analysis by quantitative PCR

Snap-frozen muscle tissue with infiltrates was thawed and cultured with 400,000 autologous CD3 depleted irradiated PBMC in medium for 4 days. Total RNA from cells migrating from the muscle tissue into the medium was isolated using Tripure isolation reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. RNA concentration was

determined by spectrophotometer and adjusted to a concentration of 500 ng/ml. First-strand cDNA was synthesized from total RNA using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV, Promega, Madison WI, USA) with 1 µg/µl Oligo (dT) and 10 mM dNTP's (both Amersham Pharmacia Biotech AB, Uppsala, Sweden). The reaction mixture was incubated at 40°C for 90 minutes followed by incubation at 70°C for 15 minutes. To ensure the fidelity of mRNA extraction and reverse transcription to first-strand cDNA all samples were subjected to real time PCR amplification with primers specific for the constitutively expressed gene beta-2 microglobulin (β2m). For FOXP3 and β2m transcripts real-time quantitative PCR was performed with a LightCycler (Roche Diagnostics, Mannheim, Germany) based on specific primers and general fluorescence detection with SYBR Green. The following primer combinations were used; FOXP3 Forward 5' TCAAGCACTGCCAGGCG 3', FOXP3 Reverse 5' CAGGAGCCCTTGTCGGAT 3' and β2m Forward 5' CCAGCAGAGAATGGAAAG TC 3', β2m Reverse 5' GATGCTGCTTACATGTCT CG 3'. All PCR reactions were performed using LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). Normalization to β2m was performed for each sample and a pool of cDNA from tetanus toxoid (TT) stimulated human PBMC was used as a standard. Semi-quantitative levels of mRNA were calculated, with the TT control set on 1.

Immunohistochemistry

Muscle biopsies were snap-frozen in liquid nitrogen. Tissues were obtained from the department of Pathology, UMC Utrecht, with local ethical committee approval. 7 µm thick acetone-fixed cryostat sections were prepared for immunohistochemical staining. The following antibodies were used: FITC conjugated mouse anti-human CD4, clone MT310 (DakoCytomation, Denmark), rat anti-human FOXP3, clone PCH101 (eBioscience, USA). For secondary staining a mixture of biotinylated rabbit anti-rat Ig and alkaline phophatase-conjugated sheep anti FITC (Roche, Germany), followed by avidin-biotin-peroxidase complex (Vactastain Elitekit, Vector Laboratories, USA). Alkaline phosphatase activity was visualized using naphtol-ASMX phosphatase (Sigma) as a substrate, and fast blue BB base (Sigma) as a chromogen, resulting in blue signal. Peroxidase activity was visualized using 3-amino-9-ethylcarbazole (AEC; Sigma), resulting in brownish-red signal. In order to analyze multiple T cell markers, single stainings were performed on different sections of one muscle biopsie (n=2). Single stainings were performed on 4 µm thick acetone-fixed cryostat sections with either mouse anti-human CD3 (clone SK7, BD, USA), CD4 (clone SK3, BD), or CD8 (clone SK1, BD), followed by horse-anti- mouse biotinylated (BA-2000, Vector Laboratories, USA), and streptavidin-HRP (IM0309, Immunotech), or with rat anti-human FOXP3 (clone PCH101, eBioscience) , followed by rabbit-anti-rat-HRP (DAKOCytomation), and Powervision goat-anti-rabbit IgG HRP (Klinipath, The Netherlands). Peroxidase activity was visualized using 3,3'-Diaminobenzidine (**DAB**) , resulting in brown signal. Muscle sections were examined by light microscopy.

Statistical analysis

Mann Whitney U test (comparing 2 groups), or one-way ANOVA analysis with Dunn's multiple comparison post test (comparing 3 or more groups) was used to analyze and compare protein expression FACS data, and suppression by Treg. $P<0.05$ was considered significant.

Results

Peripheral blood CD4 T cells from JDM patients and age-matched controls have similar expression of FOXP3 and CD25^{hi}

In order to establish Treg frequencies in peripheral blood from JDM patients, we first analyzed expression of CD25 on CD4$^+$ T cells. Since a high expression of CD25 (CD25^{hi}) is specifically indicative of Treg, we compared CD25^{hi} expression in JDM patients with age-matched controls. For participant's characteristics, see Table 1. In general, JDM patients and age-matched controls displayed similar percentages of CD25^{hi} expressing T cells (**Figure 1A**, left panel). However, when comparing patients in remission to patients with active disease, we found that patients with active disease had a higher level of CD25^{hi} expressing CD4$^+$ T cells compared to patients in remission (**Figure 1A**, middle panel). FOXP3 is indispensable for Treg development and function, and currently the best available Treg marker. In line with the increase of the percentage of CD25^{hi} expressing cells, increased percentages of FOXP3 expressing CD4$^+$ T cells were observed in active JDM patients. (**Figure 1A**, right panel, and **1B**). Thus, JDM patients with active disease had higher levels of Treg in their peripheral blood compared to patients in remission.

CTLA-4 and GITR are receptors expressed on Treg and involved in Treg suppressive function. In order to establish whether there were differences between age-matched controls and JDM patients, we analyzed expression of these receptors on CD4$^+$CD25^{hi} T cells (**Figure 1C**), and CD4$^+$FOXP3$^+$ T cells (**Figure 1D**). Percentages of CTLA-4 and GITR expressing CD4$^+$CD25^{hi} T cells were similar in all groups. However, analysis of CD4$^+$FOXP3$^+$ T cells demonstrated a significantly lower CTLA-4 expression in remitting JDM patients compared to age-matched controls, while expression of GITR was similar in all groups. Thus, percentages of GITR expressing Treg from JDM patients and age-matched controls were similar, and in remitting JDM patients percentages of CTLA-4 expressing FOXP3$^+$ Treg were decreased, compared with age-matched controls.

Only active JDM patients treated with high levels of corticosteroids have increased percentages of CD4$^+$ T cells expressing FOXP3 and CD25^{hi}

We observed that JDM patients with active disease had higher percentages of CD25^{hi} and FOXP3$^+$ CD4$^+$ T cells in peripheral blood, compared to patients in remission. Recent reports showed that high doses of corticosteroids increased numbers of Treg in patients with multiple sclerosis (MS) (19), and in a mouse model for experimental autoimmune encephalitis (EAE, equivalent for MS) (20). Since we also included JDM patients receiving high corticosteroid treatment (2 patients receiving 1mg/kg/day prednisone, 3 patients recently received methyl-prednisolone pulses), we analyzed these patients separately. Indeed, only the JDM patients with active disease on recent high-dose corticosteroid treatment had significantly higher percentages of both CD25^{hi} and FOXP3$^+$ CD4$^+$ T cells compared to patients in remission (**Figure 1E**). This suggests that corticosteroid treatment, rather than disease activity, influenced percentages of FOXP3 and CD25^{hi} expressing CD4$^+$ T cells.

Treg from patients with active JDM exhibit a compromised suppressive function

We observed normal percentages of FOXP3 and CD25 expressing CD4$^+$ T cells in both remitting and active JDM patients on low corticosteroids, while only patients on high corticosteroids had increased percentages of FOXP3 and CD25^{hi} cells. Since FOXP3 and CD25 can also be expressed on activated CD4$^+$ T cells, we also evaluated the suppressive function of Treg. To isolate FOXP3$^+$ Treg by FACS for in vitro suppression assays we used CD25$^+$ in combination with CD127^{low} expression which has been shown to be highly selective for FOXP3$^+$ Treg (21, 22). CD4$^+$CD25$^+$CD127^{low} T cells from either patients with active or remitting disease were sorted, and were indeed found

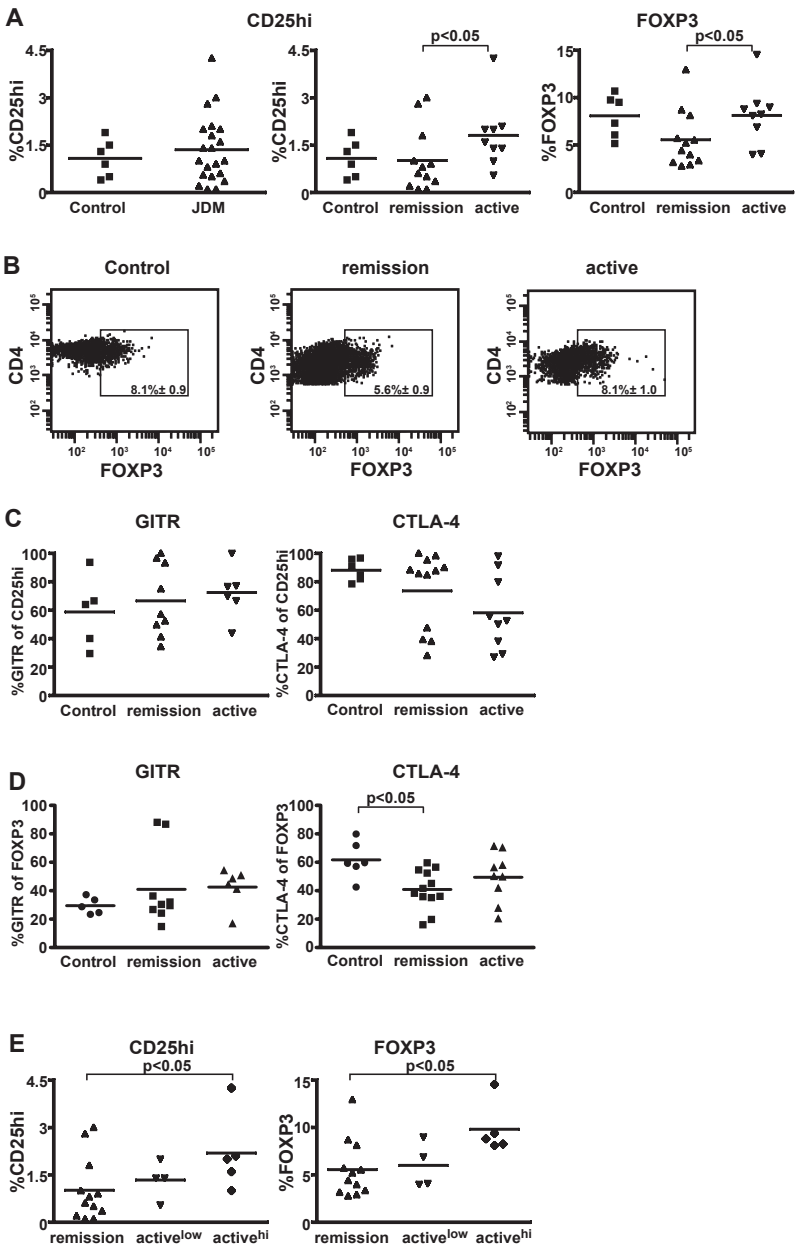


Figure 1. JDM patients have normal levels of CD4$^+$CD25^{hi}FOXP3$^+$ T cells in peripheral blood. PBMC from JDM patients (JDM) and age-matched controls (Control) were isolated and analyzed by flow cytometry. (A-C) The total group of JDM patients was analyzed (JDM), or a distinction was made between patients with active disease (active), and remitting disease (remission). (A) Percentage of CD25^{hi} (left and middle graph) and FOXP3 expression (right graph) on CD4$^+$ T cells. (B) Percentage of expression of GITR (left graph), and CTLA-4 (right graph), within CD4$^+$CD25^{hi} T cells, or (C) within CD4$^+$FOXP3$^+$ T cells. (D) Representative dot plots of FOXP3 expression in CD4$^+$ T cells, showing averages ± s.e.m. (E) Percentages of CD25^{hi} and FOXP3 expression on CD4$^+$ T cells from JDM patients. A distinction was made between patients with remitting disease (remission), active disease with <0.5 mg/kg prednisone (active^{low}), and 1mg/kg prednisone or methyl-prednisolone pulses (active^{hi}). P<0.05 was considered significant.

to express high percentages of FOXP3 (**Figure 2A**). Treg from patients in remission were able to suppress effector T cell activation (**Figure 2B,** each line representing 1 patient; up to 85%, 97%, or 78% suppression). Patients with active JDM treated with low prednisone (up to 97% suppression), or high methyl-prednisolone (up to 65% suppression) showed suppression of T cell activation similar to patients in remission. However, the patient receiving 1mg/kg prednisone only showed suppression up to 14 % (**Figure 2C**). The lack of suppressive function in this patient was independent of corticosteroid dose. Thus, peripheral blood Treg from patients with remitting JDM are potent suppressors of Teff proliferation, while Treg from patients with active JDM did not consistently suppress Teff proliferation.

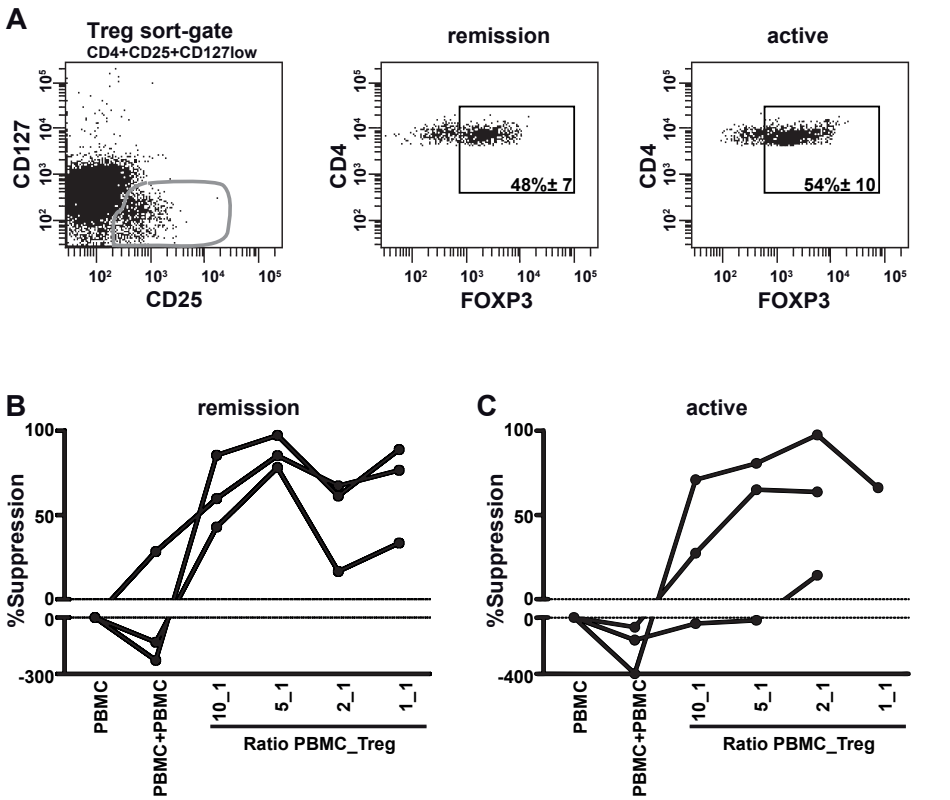


Figure 2. Treg from JDM patients show normal suppressive function. (**A**) Representative dot plots of FOXP3 expression of gated $CD4^+CD25^+CD127^{low}$ T cells (left dot plot) of remission (middle dot plot) and active disease (right dot plot) JDM patients, showing averages ± s.e.m. (**B,C**) $CD4^+CD25^+CD127^{low}$ T cells were sorted into co-cultures with anti-CD3 activated autologous PBMC in different ratio's. Proliferation was measured on day 6 by ^3H thymidine incorporation. Level of suppression of PBMC proliferation was calculated by comparison to proliferation of PBMC cultured alone (suppression=0%). (**B**) Suppression by $CD4^+CD25^+CD127^{low}$ T cells from 3 patients in remission and (**C**) 3 patients with active disease.

FOXP3^{+}CD4^{+} T cells are present in muscle tissue of patients with active JDM

To investigate whether Treg are localized at the site of inflammation, we analyzed muscle biopsies from new disease onset patients. Immunohistochemistry revealed large infiltrates of CD4 expressing cells in inflamed tissue (n=6), which were not present in tissue from controls (n=2) (**Figure 3A**). This was confirmed by single staining of CD3, CD4, CD8 and FOXP3 in separate biopsies (N=2) (**Figure 3B**). Furthermore, a large proportion of CD4^{+} T cells expressed FOXP3 (**Figure 3A, B**). Thus, Treg were present in muscle tissue in large numbers compared to activated T cells during active inflammation.

Lymphocytes in muscle tissue express high levels of FOXP3 mRNA

We showed that Treg are present at inflammatory sites. In order to analyze these Treg in more detail, we isolated cells from muscle tissue of 3 active, new onset, JDM patients. Inflamed muscle tissue was cultured as described in materials and methods, and the lymphocytes isolated from the tissue were analyzed by flow cytometry and RT-PCR (n=2). We found that a variable proportion (28%, or 91%) of the infiltrated lymphocytes were CD3^{+}CD4^{+} T lymphocytes (**Figure 3C**). Furthermore, we analyzed the isolated cells from the tissues by quantitative PCR, and compared them with PBMC from peripheral blood of a healthy adult donor, stimulated with tetanus toxoid (TT) as a standard control. T cells in the muscle expressed high levels of FOXP3 mRNA; 96-fold (± 50) higher compared to TT control (**Figure 3D**), while freshly isolated CD25^{bright} Treg from peripheral blood of healthy donors have been demonstrated to express a 6-fold (±1.6) of FOXP3 mRNA compared to TT control (23). Taken together, FOXP3 expressing T cells were present in large numbers in inflamed muscle tissue from JDM patients.

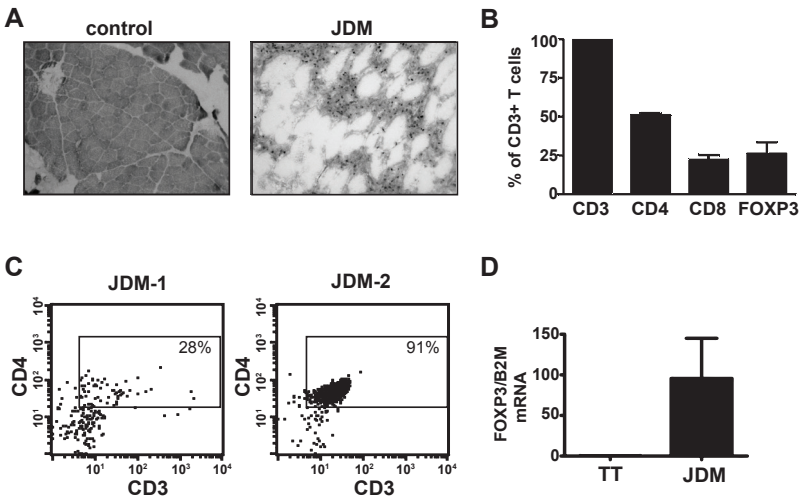


Figure 3. FOXP3^{+} Treg are abundantly present in inflamed muscle tissue. (**A**) Representative pictures from immunohistochemistry staining on muscle biopsies of controls (left, 1 example of n=2) and active JDM patients (right, 1 example of n=6). Blue staining indicates CD4 expression, brown-red staining indicates FOXP3 expression. (**B**) Percentages of CD4, CD8 and FOXP3 expressing cells of infiltrated CD3^{+} T cells (=100%) in muscle biopsies of 2 JDM patients with active disease. Displayed are means ± s.e.m.. (**C**) Dot plots show expression of CD3 and CD4 on lymphocytes from muscle tissue from 2 patients with active JDM. Percentages of CD3^{+}CD4^{+} T cells are presented for each donor. (**D**) Levels of FOXP3 mRNA expression (± s.e.m.) in lymphocytes from muscle tissue of 2 patients with active JDM. mRNA levels were normalized to TT control (=1).

Discussion

When it was demonstrated that FOXP3+ Treg could prevent autoimmune disease in several experimental models for colitis (24), type 1 diabetes mellitus (25, 26), EAE (27), and arthritis (28, 29), this raised the expectation that targeting Treg could provide a new generation of more specific therapies for human autoimmune diseases. This was underscored by the finding that IPEX patients, lacking functional FOXP3, suffered from non-remitting inflammation affecting multiple organs (10, 11). However, translation of the successful targeting of Treg in experimental models into new therapies for human autoimmune diseases has so far been slow, with a few notable exceptions; anti-CD3 and DiaPep277 in diabetes (14, 30, 31), and dnaJP1 in rheumatoid arthritis (32). There are several reasons for this. Firstly, there are subtle but important differences between human and murine Treg (33). For instance, the mechanism of Treg-mediated suppression is different between species. Human Treg , in contrast to murine Treg (34, 35), do not induce apoptosis in effector T cells due to Treg IL-2 consumption (36), and do not produce the suppressive cytokine IL-35 (37). Furthermore, the in vivo properties of human Treg are difficult to study, and, therefore, it is hard to determine the role of Treg in the regulation of human autoimmune disease. However, there is circumstantial evidence for a regulatory role of Treg in human diseases that is similar to that observed in murine models. For instance, in diabetes treatment with a peptide DiaPep277(31, 38), or anti-CD3 (14, 26) in both the murine model and in patients induces tolerogenic T cell responses and clinical improvement. The first step towards a better understanding of in vivo Treg properties, lies in ex vivo studies of Treg isolated from peripheral blood and, if possible, sites of autoimmune inflammation such as the muscle tissue of JDM patients. JDM is a rare autoimmune disease that affects skin and striated muscle of patients. Although T cells are clearly involved in the immune pathogenesis of JDM, still little is known about their pathogenic role in the observed inflammation, let alone whether Tregs may be involved in this process. In this study we show that Treg are present in both peripheral blood and inflamed tissue of JDM patients. The next question is whether these cells directly or indirectly influence the disease course of JDM. Interestingly, we found that active patients had significantly higher percentages of $CD25^{hi}$ $CD4^+$ T cells, in which CTLA-4 and GITR expression was similar to remitting patients. Accordingly, the percentages of peripheral blood $FOXP3^+$ $CD4^+$ T cells were higher in active patients. It has to be noted, that this was not significantly different from age-matched controls. Previous reports showed that corticosteroid treatment increases Treg numbers in MS patients (19) and in an experimental model for EAE (20). Indeed, we found that the increase of Treg percentages was only detected in active JDM patients treated with high levels of corticosteroids, and thus independently of disease activity. JDM patients receiving no or low corticosteroids had normal percentages of Treg in the peripheral blood. Moreover, Treg isolated from patients in remission showed suppressive capacity, while Treg from active patients were not consistently able to sufficiently suppress effector T cell proliferation. The clinical course of JDM can be either monophasic, cyclic or chronic (39) and so far little is known concerning the immune regulatory effects that may influence the outcome. Here we have shown that Treg may be involved in the regulation of JDM inflammation. Future prospective studies should determine whether Treg are able to actively influence the differential disease course in JDM.

Treg were present in muscle of patients with active JDM, but were unable to prevent tissue inflammation. Our findings are in line with reports in JIA patients; JIA synovial fluid contains large numbers of $FOXP3^+$ Treg (23, 40). In JIA synovial fluid these cells are accessible in large numbers. Suppression assays show that Treg from JIA synovial fluid are efficient suppressors of effector T cells from PBMC, however, effector T cells from synovial fluid are resistant to Treg mediated suppression (EJ Wehrens et al., manuscript in preparation). Resistance of effector T cells towards suppression has also been shown in T1D and SLE patients (41, 42). This could also explain why the abundantly present Treg cannot suppress inflammation in muscles of JDM patients with active disease.

Taken together, we show that Treg may play a role in JDM pathogenesis, since Treg from peripheral blood from remitting patients are potent suppressors, while Treg in muscle tissue seem unable to suppress active inflammation. Therefore, targeting of Treg alone will likely not induce disease remission, and immune suppression of effector T cells, which may create a window of opportunity for Treg, followed by specific stimulation of Treg-mediated regulation is more likely to successfully suppress disease for a long period of time.

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Supplementals

Table S1. Participant's characteristics.

	JDM remission	JDM active	HC
Number	12	9	6
Male	67%	50 %	50%
Mean age (yrs, min-max)	(13, 9-17)	(10, 6-14)	7 (2-13)
Therapy	8	6	0
Prednison use	8	6	-
Prednison low dose (< 0.5 mg/kg/day)	8	1	-
Prednison high dose (1 mg/kg/day)	-	2	-
Prednison high dose: methylprednisolon pulses (>1mg/kg/day)	-	3	-
MTX	4	-	-
IVIG	1	-	-

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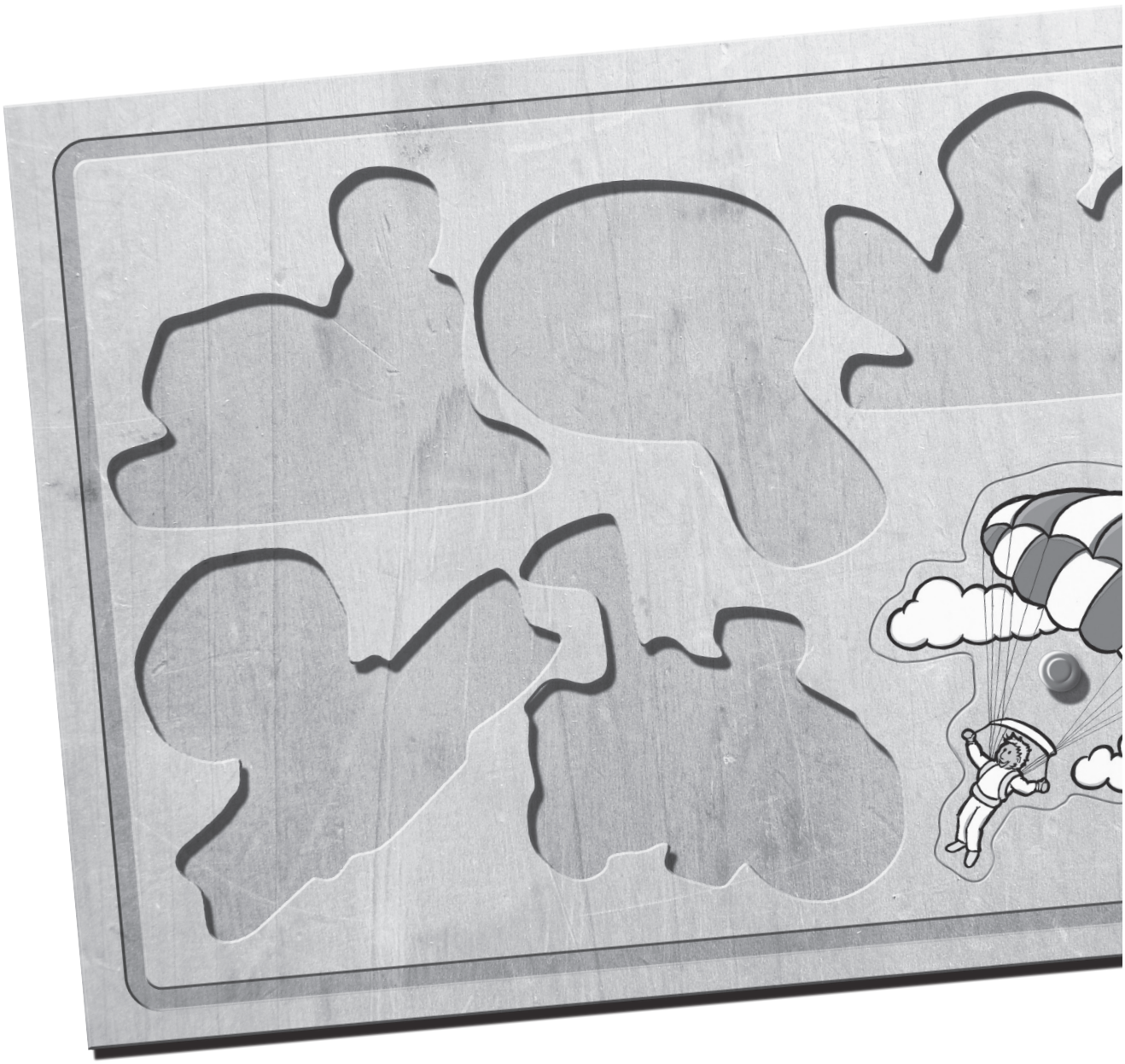

Heat Shock Protein 60 reactive T cells in juvenile idiopathic arthritis: what is new?

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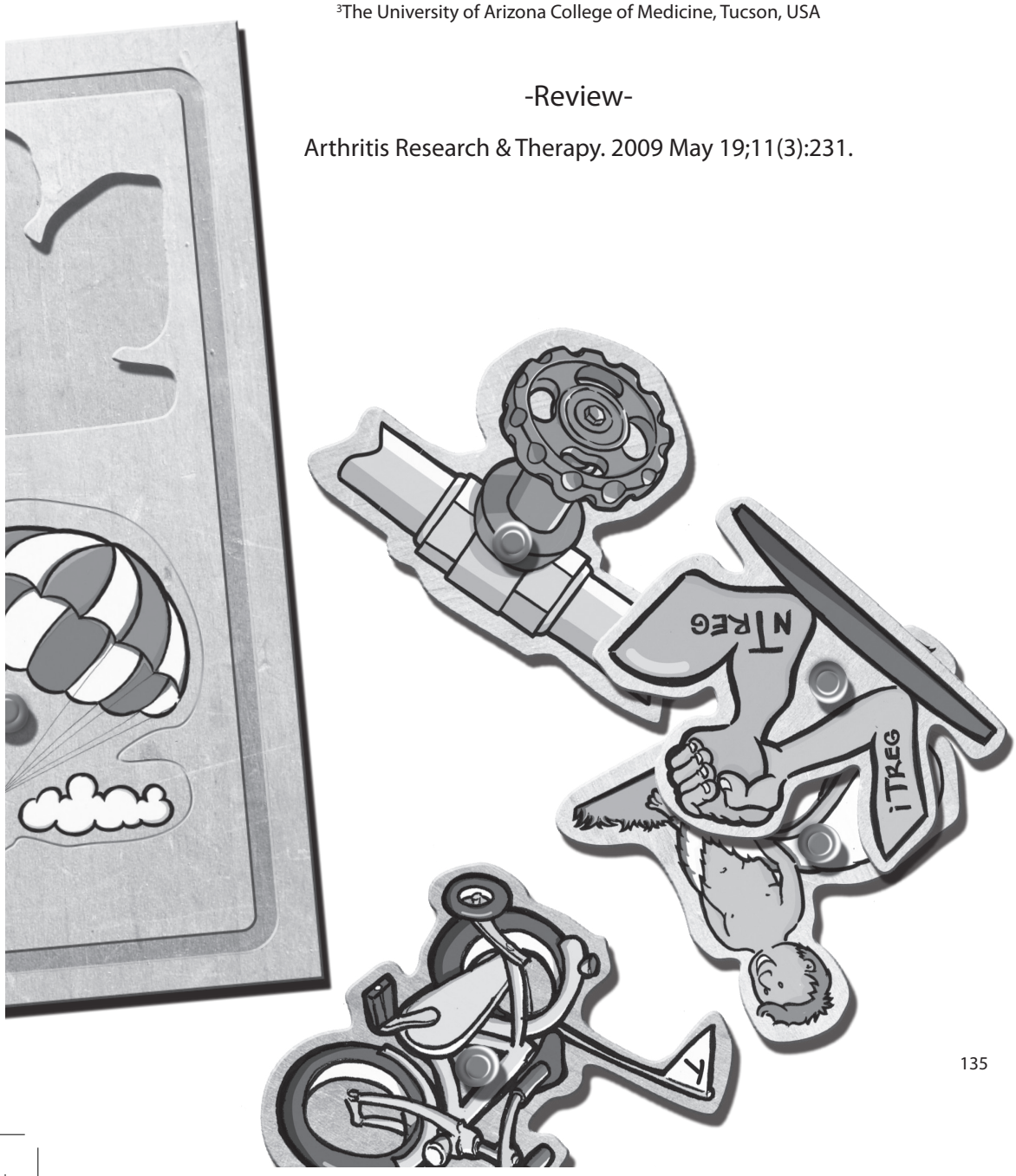

Abstract

Juvenile Idiopathic Arthritis (JIA) is a disease characterized by chronic joint inflammation, caused by a deregulated immune response. In patients with JIA, Heat Shock Proteins (HSPs) are highly expressed in the synovial lining tissues of inflamed joints. Heat shock proteins (HSPs) are endogenous proteins, which are expressed upon cellular stress, and are able to modulate immune responses. In this review, we will concentrate on the role of HSPs, especially HSP60, in modulating immune responses in both experimental and human arthritis, with a focus on JIA. We will mainly discuss the tolerogenic immune responses induced by HSPs, which could have a beneficial effect in JIA. Overall, we will discuss the immune modulatory capacity of HSPs, and the underlying mechanisms of HSP60-mediated immune regulation in JIA, and how this can be translated into therapy.

Introduction

Juvenile Idiopathic Arthritis (JIA) is an autoimmune disease of unknown cause, characterized by a deregulated immune response in synovial lining tissues of the joints, leading to chronic arthritis in children. According to the latest classification provided by the International League of Associations for Rheumatology, seven categories of JIA can be distinguished, based on features present in the first 6 months of disease (1). All forms of JIA are extensively described in a recent review by Ravelli and Martini (2). Most of the literature we will discuss in this review involves 2 of the most common forms: oligoarticular and polyarticular JIA. In the first 6 months, in polyarticular JIA five or more joints are inflamed, whereas in oligoarticular JIA only up to four joints are inflamed. Oligoarticular JIA can either be persistent, or extended to five or more joints. The fact that persistent oligoarticular JIA is self limiting and in about half of the cases even self-remitting, suggests an endogenous regulation of the immune response, sometimes resulting in disease stabilization.

Heat shock proteins (HSPs) are endogenous proteins, which are expressed upon cellular stress, and are able to modulate immune responses. HSPs are highly present at sites of inflammation, like the inflamed joints of JIA patients (3) (**Figure 1A**).

Previously, we reviewed the options for specific immunotherapy in JIA, by immune modulatory fragments of proteins, called peptides. This included some peptides derived from HSPs: HSP60 and the bacterial HSP dnaJ. These peptides were designed to be presented in MHC II molecules, and are recognized by T cells. Thereby, these peptides enhance a specific immune response. The mechanisms and advantages of specific immunotherapy in JIA, compared to currently used immunosuppressive therapies, were extensively discussed (4).

In this review, we will focus on the immune regulatory mechanisms of HSPs in arthritis, and, most importantly, JIA. Although we will shortly discuss other members of the family of HSP proteins, we will concentrate on HSP60. First, we will discuss the role of HSP60 in immune regulation. Second, we will continue with immune regulation by HSP60 in experimental models of arthritis and Rheumatoid Arthritis. Following, we will discuss the role of HSP60 in JIA. Last, we will hypothesize on how the immune regulatory properties of HSP60 can be translated into therapy.

Heat Shock Proteins

Heat shock proteins (HSPs) are evolutionary highly conserved proteins, which are either present constitutively, functioning as chaperones (5), or induced upon cell stress caused by, for instance, heat, oxidative stress, and hypoxia (6, 7). Several HSPs have been identified and, according to their size, organized into 6 families: HSP100, HSP90, HSP70, HSP60, HSP40, and HSP10.

In 1994 the 'danger model' was proposed by Polly Matzinger (8). According to this model, the reaction of the immune response is not aimed towards 'self' or 'non-self', but towards 'danger'. Immune cells can sense danger when tissues which are stressed due to, for instance, infection, release danger signals, such as HSPs. Therefore, an immune reaction is only elicited when necessary. As was discussed in a previous review, these danger signals also play a role in chronic inflammation (4). As an example, in the inflamed joints of JIA patients, HSPs are released from the injured or dying cells in the synovial lining tissue and act as 'danger signals', which alert the immune system (3, 4). This does not mean that HSPs cause autoimmunity, but the increased expression and release of HSPs can have major impact on the resulting immune response, as will be discussed below.

Indeed, different laboratories have documented variable immune responses towards HSPs in humans. *In vitro* experiments show that immune responses modulated by HSPs can be pro-inflammatory, for instance, by eliciting an Interferon γ (IFNγ) response by T cells, or TNFα production by macrophages (9-11). However, HSPs can also induce an anti-inflammatory response, for example, production of interleukin 10 (IL-10) (12, 13). HSPs are strong immune

modulators and are able to influence the impact and direction of immune responses. Not only complete HSPs, but also small immunogenic peptides derived from HSPs are discussed. In this review we will elaborate on the tolerogenic (anti-inflammatory) response, which, in different experimental settings, is attributed to an induction of IL-10 production. In the last decade, IL-10 producing T cells became more and more of interest. These cells belong to a population of T cells, called regulatory T cells (Treg), which are key players in immune regulation (14, 15). HSPs may be able to influence this population of Treg, and thereby induce tolerogenic responses.

HSP mediated induction of tolerance: depending on regulatory T cells?

Regulatory T cells

The involvement of HSPs in immune modulation, specifically in immune suppression, and T cell reactivity towards HSPs leading to suppression, raises the question whether regulatory T cells (Treg) are involved in this process.

In the last decade, Treg have been extensively investigated in several diseases. Their importance shows clearly in patients suffering from IPEX (Immune dysregulation polyendocrinopathy, enteropathy, X chromosome-linked syndrome), who lack functional Treg, due to a deficiency in Forkhead box transcription factor P3 (FOXP3) (15, 16). As a consequence, these patients suffer from severe autoimmunity and allergy. The relevance of Treg in regulation of autoimmunity has been confirmed by extensive research in several mouse models for Diabetes and Rheumatoid arthritis, in which the transfer of Treg to sick animals diminished disease (17, 18). Furthermore, the presence of Treg correlated with a mild disease course in JIA, implicating a role for Treg in controlling inflammation in JIA patients (19).

Subclasses of Treg can be distinguished by their mechanism of suppression and phenotype. In general, suppression by regulatory T cells is mediated by cell-cell contact and production of suppressive cytokines. Naturally occurring Treg (nTreg), for instance, are present from birth and are distinguished by the expression of interleukin 2 receptor α (IL-2Rα, also CD25) and FOXP3 (14). Tr1 (T regulatory 1) cells, however, are induced upon activation in peripheral tissues, and are characterized by production of IL-10 and IFNγ (14). It has been suggested that the population of activation-induced Treg is the most important for immune regulation in humans (20, 21). In ex vivo and in vitro studies in humans it is difficult to differentiate between natural Tregs and induced Tregs. It is safe to assume that all HSP60 induced Tregs are such activation induced Tregs, and thus in the following, when using the term Tregs, we are referring to these cells.

Treg induction by HSP60

There is indeed evidence that HSPs can contribute to an increase of Treg number and function. First of all, in animals with Adjuvant Arthritis treatment with HSP60 peptide and low dose anti-TNFα induced an increase in FOXP3 expressing T cells, compared to treatment with high dose anti-TNFα (22). More *in vitro* data are available from patients with JIA, which suggests that Treg are influenced by HSP60. In these patients the response of T cells to HSP60, which correlated with a beneficial outcome of disease, was characterized by a high production of IL-10, and expression of the cell surface receptor CD30 (13). CD30 is a member of the Tumor necrosis factor receptor (TNFR) family, and, in *in vivo* mouse experiments, was shown to be important for Treg function (23-25). CD30 expression on T cells correlated with the production of T helper 2 cytokines and IL-10, but also with the expression of CD25, which is a hallmark of regulatory T cells (23, 24) (**Figure 1B**). These CD30 expressing T cells could, therefore, represent a population of T cells which is able to regulate the immune response. A role for CD30 expressing $CD4^+$ T cells in regulation of human T helper 1 cell-driven diseases, like RA, has also been suggested (26, 27). We hypothesize that these $CD4^+CD30^+$ T cells can be important in regulation of inflammation in JIA joints as

well (**Figure 1B**). Future studies may elucidate whether in inflamed joints CD30 positive cells represent just activated cells, or cells with a possible more regulatory phenotype.
Direct evidence, showing an influence of HSPs on Treg, has been provided by *in vitro* experiments, in which HSP60 and the HSP60 peptide p277 were able to enhance the regulatory function of the CD25 expressing Treg population from human peripheral blood, probably by increasing Treg migratory capacity (28, 29)

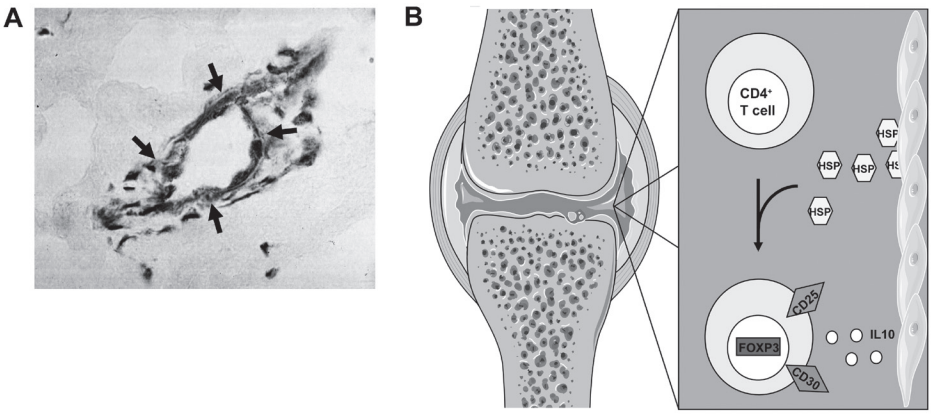

Figure 1. HSP60 specific T cells in the synovium of JIA patient are IL-10 producing CD30⁺ Treg-like cells. (**A**) HSP60 (stained brown and marked by red arrows) is highly expressed in synovial lining membranes in the inflamed joints of JIA patients. (**B**) HSP60 is released by the synovial tissues in the inflamed joint. In the synovial fluid, $CD4^+$ T cells are present. T cells that react to the self-HSP60, or HSP60 epitopes, produce IL-10(13, 68) and express CD30. Presence of these HSP60 reactive T cells correlates with mild disease course(13). Therefore, we hypothesize that these T cells could be CD25 and FOXP3 expressing naturally occurring Treg (19), or IL-10 producing Tr1 cells. Altogether, HSP60 may induce Treg in the joints of JIA patients and hereby regulate the inflammation of these JIA patients, as is seen in oligoarticular JIA.

HSP mediated immune modulation: a combination of innate and adaptive immunity

Thus, as discussed above, HSP60 and HSP60-derived peptides seem to be able to enhance (Treg mediated) immune suppression. The next important step would be to unravel the mechanism of Treg enhancement by HSP60. In this context, it is important to mention that HSP60 can trigger both adaptive and innate immune responses. Several times HSP60 has been suggested to be a ligand for the Toll-like receptors 4 and 2 (11, 30). The Toll-like receptors (TLRs) belong to a family of highly conserved receptors that recognize patterns of bacterial and viral origin, and form an important part of the innate defense.
The production of some HSPs used for *in vitro* experiments takes place in bacteria. This means that there are always bacterial components contaminating the HSP. For instance, microbial lipopolysaccharide (LPS), a ligand for TLR4, or microbial proteins, like peptidoglycan (PGN), a ligand for TLR2, are often detected in HSP preparations (31). Therefore, to prevent interference of the effects caused by bacterial products or HSPs, it is best to focus on either highly purified HSPs, or on HSP peptides which are synthesized *de novo* .
HSP peptides may be expected to induce a specific T cell mediated response, as their selection is based on T cell recognition. However, interestingly, Cohen and co-workers discovered that some HSP-derived peptides can also induce an innate immune response. They showed, as discussed before, that HSP60 peptide p277, which was originally identified as a peptide specifically

recognized by T cell clones (32), could enhance the population of Treg. This was, however, mediated by TLR2 expressed on T cells (28). Thus, apparently, similar to the whole protein, HSP60-derived peptides are capable of activating both innate and adaptive immunity.

Dendritic cells: linking innate and adaptive immunity

The most direct link between the innate and adaptive immune system is formed by the professional antigen presenting cells; dendritic cells (DC). DC express high levels of innate receptors, such as TLRs. Stimulation of these TLRs causes maturation of DC. Conflicting data have been published regarding the maturity of DC and their influence on Treg; some data support that mature DC are able to induce Treg (33), whilst other publications suggest that specifically immature DC can induce Treg (34) (**Figure 2A**). The role of TLRs, expressed on the DC, in induction of Treg has also been demonstrated to be controversial. On one hand, TLR stimulation on DC can enhance IL-10 production by DC, and thereby induction of Treg (**Figure 2A**). On the other hand, stimulation of TLRs on DC can lead to inhibition of Treg and inflammation (35). These data are mostly obtained from experiments in mice and *in vitro* experiments with human cells. Consistently, TLR-agonists, tested in clinical trials as treatment for tumors, do not always induce inflammation (35, 36). The direction of the immune response may depend on the intracellular signaling pathways that are induced (37). Altogether, innate receptors can both directly and indirectly, via DC modulation, influence adaptive immunity and are able, in some cases, to contribute to the induction of tolerance through Treg (**Figure 2A**). It seems likely that DC with their dual capacity to activate both through the innate and adaptive receptors could play an essential role in the regulation of the HSP60 mediated immunity. This is however not yet studied in humans.

Pathogen-associated molecular patterns enhance HSP mediated tolerance

In humans, memory T cells normally do not show a strong immune response to self-HSP60, but only to bacterial HSP60, whereas naïve T cells may readily respond to both (38). In addition, in cord blood, which comprises mainly naïve T cells, responses to both human and bacterial HSP60 have been detected (39). Low T cell responses to self-HSP60 and self-HSP60 peptides form an obstacle regarding their therapeutic applicability. For an optimal therapeutic effect, it seems logical to either enhance the self HSP mediated effect, or to use the bacterial homologues of the human HSP60 peptides, in order to induce a strong memory T cell response.

Thus, enhancement of this response can be achieved by combining the innate and adaptive immune system. The same principle is used in vaccinations: an adjuvant, like bacterial toxin, is used, to elicit an immune response, in many cases via TLRs, that is sufficient to achieve immunological memory (40, 41). Furthermore, the innate immune response can be used to steer the antigen specific immune response; TLRs can induce either a pro- or an anti- inflammatory reaction, as discussed above.

Altogether, as for vaccination, the combination of enhancing both adaptive and innate immunity may be attractive for immune therapy of arthritis. As discussed before, HSP60 may be able to do this on its own, as it can activate both the innate and adaptive immune system (**Figure 2B**). Bacterial patterns may enhance the effect of HSPs; for instance, a complex of LPS and HSP60, via TLR4 signaling in DCs, is able to enhance the IL-12 production by DCs and IFNγ production by T cells (9). In these experiments, the inflammatory response was enhanced via TLR4 on DCs. In contrast, there is evidence that bacterial patterns, signaling via TLR2, TLR4 and TLR5, are able to enhance Treg function as well (42-44). However, in this setting, the combination of bacterial patterns with HSPs or HSP peptides has not been investigated.

We hypothesize that the immune response can be modulated when T cell receptor (TCR) signaling is combined with TLR signaling: a synergy of innate and adaptive immunity. We propose that a combination of HSP peptides, signaling through the TCR and possibly also TLRs, with pathogenic patterns, signaling via TLRs, could induce a stronger tolerogenic response

(**Figure 2C**). Since HSPs can bind TLR2 and TLR4, their ligands, LPS and PGN, would be interesting targets. However, to achieve tolerance, the pathogenic patterns should be carefully tested in combination with HSP peptides, to ensure that the immune response is skewed towards tolerance, not inflammation.

Heat shock proteins in arthritis

Several HSP families have been studied in relation to arthritis, either in animal or *in vitro* JIA and RA models, and some were even tested *in vivo* in clinical trials for RA. Although RA and JIA differ in, for instance, disease presentation and age of onset -RA onset occurs in adulthood, whereas JIA begins before 16 years of age-, both are autoimmune diseases caused by a deregulated

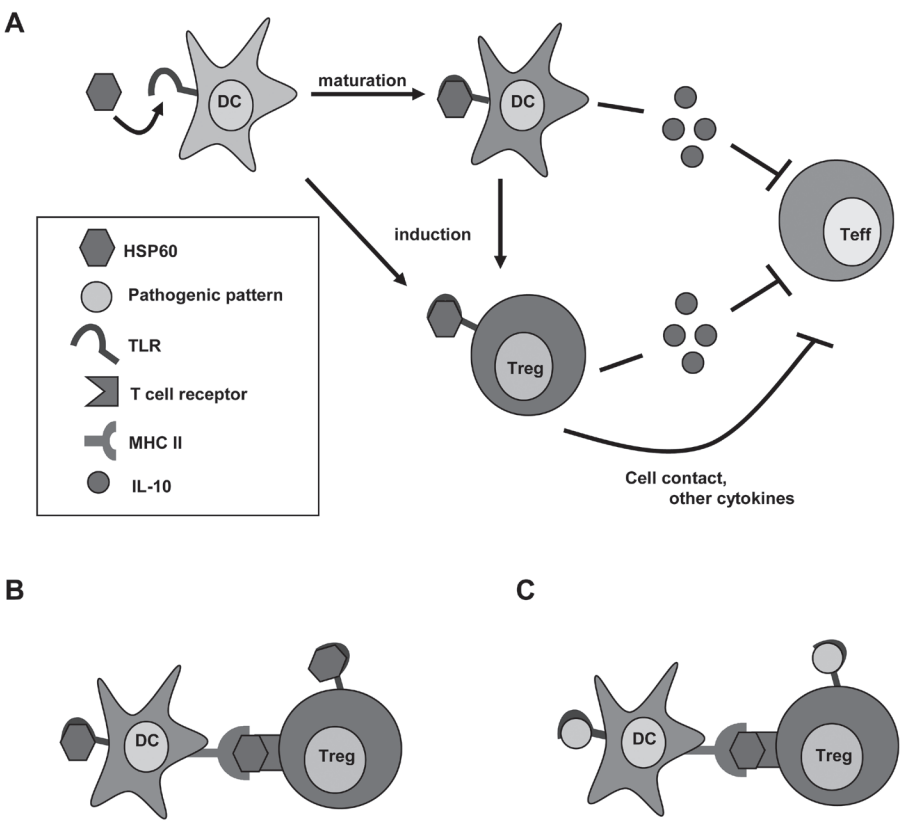

Figure 2. HSP60 uses both innate and adaptive immune pathways to enhance Treg function. (**A**) HSP60 and HSP60-derived epitopes are able to bind to Toll- like receptors (TLRs). By binding to TLRs present on Dendritic Cells (DC), HSP60 can induce maturation of DC, or cytokine production by DC (9, 11), which could possibly cause induction of Treg. Both DC and Treg are able to produce IL-10, which inhibits activation of effector T cells. To suppress the ongoing immune response, HSP60- induced Treg could inhibit activation of effector T cells by cell-cell contact and/or production of IL-10, or other cytokines, like TGFβ(14). (**B**) Pan-DR binding HSP60 epitopes are either presented to the Treg in an MHC II molecule by DC, which could stimulate Treg, or they are able to bind to TLRs on the Treg (28), and hereby enhance Treg function. We hypothesize that HSP60 and HSP60-derived epitopes can enhance or induce Treg by signaling through both the T cell receptor (TCR) and Toll-like receptor (TLR) at the same time. HSP60 epitopes may bind directly to TLRs on the T cells, or to TLRs on the DC, which could indirectly enhance the immune response. (**C**)**.** We hypothesize that a combination of a TCR signal by a HSP60 pan-DR binding epitope and a TLR signal by a pathogenic pattern could enhance the HSP60 induced, tolerogenic response, causing a stronger and longer lasting immune regulatory effect.

immune system (2, 45, 46). Therefore, experimental findings in RA can also be useful for developing potential treatments in JIA.
The tolerogenic effect of HSPs on immune responses was discovered in experiments studying reactivity of T cells towards bacterial and self HSP in relation to arthritis. Several members of the HSP family are immunogenic in both experimental and human arthritis. Moreover, modulation of the HSP-specific response can restore immune tolerance in experimental arthritis; immunization with HSP10, HSP60, HSP70 or HSP90 can suppress experimental arthritis in a rat model of adjuvant arthritis (AA) (47-50). In humans, immune reactivity towards several HSPs is found in both peripheral blood and synovial fluid from patients with RA (51), and JIA (52, 53). In JIA by far the most data are available on immune reactivity to human HSP60, which is discussed in depth below. Reactivity to other HSPs is described as well; for example, human homologues of peptides derived from the bacterial HSP dnaJ can induce tolerogenic IL-10 responses in synovial fluid cells of JIA patients (54).
In the context of RA, BiP, a member of the human HSP70 family, has drawn considerable attention. BiP is expressed in the synovium of RA patients, and is recognized by T cells from RA patients(55). These BiP-reactive T cells showed a low proliferative response and IFNγ production, but produced significant amounts of T helper 2 (Th2) cytokines, like interleukin 5 (IL-5), and IL-10. Furthermore, an anti-inflammatory gene profile was detected in monocytes after stimulation with BiP. These data suggest an immune modulatory role for BiP in RA. In a collagen-induced arthritis mouse model, similar results were found and, importantly, BiP was able to both prevent and treat arthritis (56). Currently, the first clinical trials with BiP in RA are being planned.
The first HSP that was tested in the clinic as a treatment for RA is dnaJP1, a peptide derived from dnaJ. In a Phase I clinical trial in patients with early RA mucosal administration of dnaJP1 (52), led to a shift in reactivity towards this peptide, from predominant pro-inflammatory, to a more tolerogenic immune response. A recent placebo controlled Phase II/III clinical trial reported clinical improvement in RA patients after oral treatment with dnaJP1 (E.C. Koffeman and S. Albani, submitted for publication). In this clinical trial, HSP peptides were administered orally. Mucosal tissues are highly tolerant tissues, as they need to be able to accept exogenous antigens, like gut microbes and food. Therefore, encounters of the mucosal immune system with antigens often leads to 'oral tolerance' (57, 58). Oral tolerance can be achieved by induction of Treg; for instance, mucosal administration of antigen can lead to the induction of IL-10 producing Tr1 cells, which eventually leads to tolerance towards the antigen (59). This suggests that mucosal administration of antigens, like HSPs, could be beneficial for the induction of a tolerogenic response in arthritis. However, in both animal models and one clinical trial in type 1 diabetes other ways of HSP administration induced tolerance as well (reviewed by van Eden et al. (60)).
So far, one HSP60 epitope was tested in a human autoimmune disease, namely in diabetes. In a phase Ib/II clinical trial, patients suffering from recent-onset type 1 diabetes were treated by subcutaneous injections with epitope p277; the same epitope that enhances Treg function *in vitro* (61). Interestingly, these patients showed mainly IL-10 production in response to the HSP60 peptide, and clinical improvement was correlated with a high IL-10 production before start of the therapy (62). Although this is not the subject of this review, it underscores the potential of HSP60 peptides for treatment. It is, however, crucial to better understand the mechanism of HSP60-induced immune regulation before initiating clinical trials in arthritis.
Thus, altogether, HSPs are immune modulators in both experimental and human arthritis. Their common qualities are their increased expression in inflamed tissue, like the synovial tissue, and their ability to induce a tolerogenic response, which may have therapeutic applicability. From now on, this review will concentrate on the most extensively studied HSP in arthritis; HSP60. First, we will discuss results obtained from animal models, than we will continue with the potential immune modulatory role of HSP60 in JIA.

HSP60 in arthritis

HSP60 in experimental arthritis

The studies on HSP60 in human arthritis originate from findings in the rat model of Adjuvant Arthritis (AA) in the late eighties (63). AA, an arthritis model with a close immunological and histopathological resemblance to RA and JIA, is induced by a single injection with heat-killed *Mycobacterium tuberculosis* in adjuvant.

First, it was found that T cell clone A2b, the arthritogenic T cell clone in AA was reactive to an epitope derived from mycobacterial HSP65. Immunization with whole (myco-) bacterial HSP65, however, not only failed to induce arthritis, but also protected against the induction of AA (63).

Next, Anderton demonstrated that T cells which were reactive towards bacterial HSP65 were responsible for the observed protection against the development of arthritis. Remarkably, protection from arthritis only occurred when peptides of bacterial HSP65 with a high degree of homology to self-HSP60 were used for immunization. This led to the induction of cross-reactive T cells, recognizing both bacterial and human HSP (64).

This was, at the time, a stunning finding: it showed that, even in an autoimmune model of arthritis, T cell reactivity to self proteins did not necessarily lead to autoimmune inflammation. Moreover, T cell reactivity to self-HSP60 could lead to a tolerogenic response, contributing to protection from arthritis (64), as well as interference in ongoing arthritis (12). The next obvious question is how this immune reactivity in experimental arthritis may relate to arthritis in humans.

HSP60 in JIA

It has turned out that the results obtained in AA are remarkably complementary to observations in a human autoimmune disease, namely JIA. In inflamed joints from JIA patients, high levels of self-HSP60 are expressed in the synovial membranes (3) (**Figure 1A**). Furthermore, in these patients self-HSP60 is recognized by T cells from the peripheral blood. Interestingly, when T cell reactivity in synovial fluid from JIA patients was analyzed, especially those patients suffering from the mild and self-limiting oligo-articular form of JIA had a clear T cell response towards self-HSP60, while poly-articular patients had not (65). This finding suggests a correlation between disease severity and HSP60 reactivity of T cells in these patients, in the sense that reactivity to self-HSP60 – like in the model of AA - may be beneficial. This has been further underscored in a prospective follow up study in new patients with JIA, that demonstrated that T cell reactivity to self-HSP60 at the onset of disease was associated with a benign disease course (66). Furthermore, self-HSP60 reactive T cells from synovial fluid express CD30 and produce IL-10 (13). As discussed before, we suggest that these T cells are Treg (**Figure 1B**).

HSP60 epitopes: specific immune modulators in JIA

Most of the discussed research has been performed using whole heat shock proteins. As HSPs are immune-modulators, it is important to achieve the wished tolerogenic effect by selecting the appropriate stimulus. This may depend on which part of the protein is recognized by T cells and elicits the immune response. Therefore, working with selected peptides derived from the whole protein increases specificity, and it probably reduces the chance on unwanted side effects. In experimental models T cell peptides from HSP60 are capable of interfering in arthritis with an efficacy that is similar to the whole protein. The problem, however, lies in the identification of such peptides in humans.

Humans, other than the inbred animals used in experimental models, have a very heterogenic HLA background. Different HLA alleles have different binding affinities for peptides (epitopes) that can fit in their HLA peptide binding site. As a consequence, it is very difficult to determine which epitopes of HSP60 can bind HLA from an individual patient. Thus, predicting which epitopes are immunogenic in a heterogenic disease such as JIA is extremely complicated. This problem was overcome when – in collaboration with Alex Sette from the La Jolla Institute of Allergy and Immunology – a PanDR binding motif predicting computer algorithm, which

predicts binding to all HLA-DR genotypes, was used (67). We succeeded in selecting a set of HSP60-derived epitopes, both from human and bacterial origin, that were immunogenic in JIA, irrespective of the HLA background of the individual patients(68).
In further studies, it has been shown that human HSP60-derived T cell epitopes, which were selected for their multiple MHC-genotype binding capacity, could both induce inflammatory cytokines, but also regulatory cytokines like IL-10, depending on the specific epitope. Similar results have been demonstrated for some selected homologous bacterial HSP peptides. The induction of a tolerogenic response was detected in patients with several MHC-genotypes, which means that these peptides are able to induce beneficial responses in a large population of patients, independent of HLA background (68).
In the model of Adjuvant Arthritis, the response to HSP60 has a dampening effect on chronic inflammation. As was shown *in vitro* for the HSP60 epitopes, this could also be the case for the response to HSP60 and HSP60 epitopes in JIA patients. Thus, HSP60 may contribute to the dampening of inflammation in the joints of JIA patients with a remitting course of disease. The identification of the HSP60 epitopes with a broad recognition in patients with JIA is major step forward towards therapeutic application of HSP60 peptides in JIA.

Treg in JIA joints: unable to suppress inflammation?

In the joints of JIA patients, many inflammatory cells are present. Due to inflammation, a high amount of self-HSP60 is released and, in addition, many FOXP3 and CD30 expressing T cells are present in the synovial fluid (Vercoulen, publication in preparation), (**Figure 1B**). Somehow, the presence of these T cells is not sufficient to completely diminish inflammation. The 'cytokine signature' in synovial fluid from oligoarticular and polyarticular JIA patients confirmed these observations: IL-10 levels were higher in synovial fluid compared to plasma from JIA patients. However, even higher levels of pro-inflammatory cytokines were present in synovial fluid; for instance, IFNγ and IL-6 (69).
The deficient immune regulation in the joints of JIA patients could be attributed to the Treg themselves: the amount of Treg may be insufficient, or the supposed Treg may actually be activated effector T cells that express FOXP3 transiently (70). However, in *in vitro* assays the FOXP3$^+$ Treg, isolated from the synovial fluid of JIA patients, can suppress activated T cells (Vercoulen, publication in preparation). Furthermore, the frequency of FOXP3 and CD30 expressing T cells is higher than in peripheral blood, whereas the suppressive capacity *in vitro* is comparable (Vercoulen, publication in preparation). Therefore, it is more likely that the inflammatory environment in the joint locally influences Treg functionality, or makes activated T cells insusceptible to Treg mediated suppression. Obviously, this local highly inflammatory state needs to be overcome, before HSP60-mediated therapy, which may target the Treg population, can be applied.

Conclusions

In this review we gave an overview of the relevance of HSP60, and a few other HSPs, in JIA and immune modulation. We discussed that HSPs in general can induce a tolerogenic immune response, not only *in vitro*, in human blood and synovial fluid cells from inflamed joints, and *in vivo*, in experimental models, but also in clinical trials in RA and type 1 diabetes. This tolerogenic response is marked by the induction of IL-10, an immune suppressive cytokine. Previous attempts of treatment with recombinant IL-10 in clinical trials have not led to improvements in RA patients (71). It has been suggested that IL-10, as immune suppressor, lacks efficacy (72). We suggest that administration of a single cytokine is not representative for skewing of an immune response, in which multiple cytokines are involved. HSPs probably not only increase IL-10 production by T cells, but also promote interactions of these cells with other immune cells, eventually leading to a tolerogenic response. Evidence for these suggestions has been provided in a recent publication, in which transgenic IL-10 producing T cells, specific for auto-

antigen, were transferred into mice suffering from proteoglycan-induced arthritis. Although this is a very artificial setting, these T cells reduced arthritis, which was achieved by enhancing the endogenous tolerogenic response (73). Another advantage of inducing antigen specific, IL-10 producing T cells, is that they continue to produce IL-10 as long as they are activated by their specific antigen (74); when inflammation decreases, less tissue proteins, like HSPs are released, and the regulatory response decreases as well.

HSP60 is able to modulate a suppressive subset of cells, the regulatory T cells (28). The tolerogenic IL-10 responses that have been found in several experimental settings, indicate that HSP60 may be able to induce IL-10 producing Tr1 cells. In JIA patients, HSP60 induces IL-10 producing $CD4^+$ T cells , which express CD30, and presence of these cells correlates with a mild course of disease (13). Therefore, we hypothesize that $CD4^+CD30^+$ T cells are able to regulate the immune response in JIA. The suggested capacity of HSP60 to induce Tregs emphasizes the applicability of HSP60 (or peptides derived from HSP60) for immune therapy in arthritis.

HSP60 peptides influence Treg via the T cell receptor (68), or via Toll-like receptors (28), which are present on both T cells and antigen presenting cells, like DC. This opens an intriguing possibility for immune therapy; to use one single peptide that may trigger antigen-specific T cells and may enhance this response by inducing innate immunity through TLR triggering at the same time.

Preliminary data suggest that a combination of both TCR and TLR activation causes induction of Treg (I. De Kleer et al., manuscript in preparation). We hypothesize that Treg induction could even be enhanced by further augmenting innate immunity, through TLR triggering by, for instance, pathogen-associated molecular patterns.

In humans, peripheral induction of Treg is thought to be of high importance in regulation of inflammation (20, 21). However, in JIA joints, many $CD4^+CD30^+FOXP3^+$ T cells are present, which are functional Treg in a neutral environment *in vitro* (Y. Vercoulen, manuscript in preparation). It is likely that the chronic inflammatory environment in the joints causes either local dysfunction of Treg, or makes activated cells insusceptible to Treg suppression. Therefore, suppression of inflammation is necessary, before Treg targeted therapy can be applied.

Towards therapy

Thus, to create a therapeutic window for HSPs, it may be important to first dampen chronic inflammation in the joints, for instance, by using anti-TNFα therapy (22). Second, the combination of HSPs and modulators of innate immunity, like pathogenic patterns, should be investigated more thoroughly. The combination of these two immune modulators may induce a stronger and longer lasting effect on the immune system by HSPs. However, both pathogenic patterns and HSPs can induce either tolerance or inflammation. Therefore, it is important to carefully select HSP epitopes and pathogenic patterns, to ensure that they only enhance the tolerogenic effect and do not tip the delicate immune balance towards inflammation.

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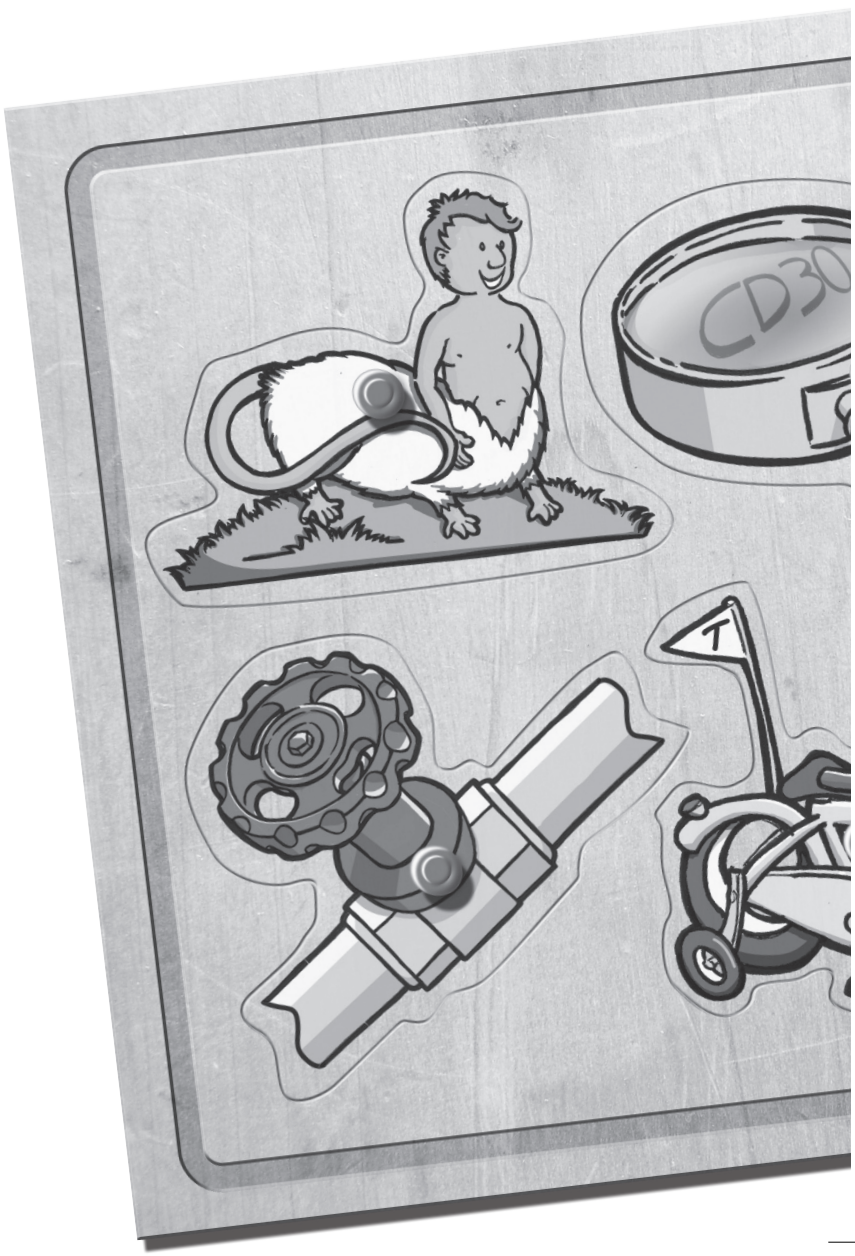

Summary & Discussion

Summary

In this thesis, we investigated human regulatory T cells as targets for therapeutic intervention in autoimmune diseases. We have extended findings from murine to human Treg, from protein modifications to in vitro Treg function, but also from 'in vitro' to 'in vivo' Treg properties. Our aim has been to establish a clear overview of the human Treg phenotype, mechanism of suppression, and suppressive function in vivo. Also, we evaluated the clinical significance of Treg in peripheral blood and muscle tissue of Juvenile dermatomyositis patients. Moreover, we investigated whether self-antigen stimulation or post-translational modification of FOXP3 increases human Treg frequency and function.

Human Treg do not induce effector T cell apoptosis.

We have clearly demonstrated that that there are important differences between human and murine Treg function. In **Chapter 2** we showed that human Treg mediated suppression of effector T cell responses is not mediated by induction of apoptosis due to cytokine consumption. Both peripheral blood Treg, and Treg from synovial fluid of JIA patients suppressed effector T cell proliferation and cytokine production, without inducing effector T cell apoptosis (1). This mechanism of Treg mediated suppression was shown in one study in mice (2), but was later demonstrated not to be involved in effector T cell suppression in mice infected with coronavirus (3). Furthermore, it was demonstrated that IL-2 depletion is not necessarily required for human Treg mediated suppression (4). Since we did not detect any apoptotic effector T cells in co-cultures with Treg, this shows that neither IL-2 consumption, nor apoptosis induction is required for human Treg mediated suppression. Our study shows that it is important to carefully evaluate findings from murine studies in human cells, in order to identify targets for intervention studies.

In vivo models are required to identify functional human Treg

In **Chapter 3**, we explored the differences between in vitro expanded Treg (nTreg) and induced Treg (iTreg) in both in vitro assays and to evaluate the in vitro results, we also analyzed Treg function in an in vivo model for xenogeneic Graft versus Host Disease (x-GvHD). In vitro assays demonstrated that both populations of Treg exhibited suppressive capacity. However, in vivo, only the expanded nTreg suppressed x-GvHD. We could not detect large differences between polyclonally expanded nTreg and iTreg that could explain these differences in Treg in vivo function. However, iTreg lost FOXP3 expression and, thereby, suppressive function shortly after being administered in vivo. In conclusion, in vitro suppression assays, which have so far been considered the golden standard for determining Treg suppressive function, are not sufficient to predict Treg suppressive function in vivo. Therefore, human Treg suppressive capacity should be analyzed in vivo in humanized mouse models.

HSP60 induces functional FOXP3^{+} Treg, marked by CD30 expression

To increase Treg numbers, we have explored the possibility of using HSP60; a self protein recognized by T cells, highly expressed at inflammatory sites (5, 6), and able to induce tolerogenic responses in murine models (7, 8). We showed that HSP60 (both complete protein and selected epitopes) induced Treg from human peripheral blood CD4^{+}CD25^{-} T cells. Furthermore, we observed that CD30 expression on HSP60 induced Treg distinguished functional Treg from non-suppressive cells (**Chapters 4 and 7**). Thus, HSP60 has the potential to increase Treg frequency by Treg induction, and is therefore a suitable candidate for Treg modulation in vivo.

FOXP3 acetylation increases FOXP3 expression and Treg suppressive function

While we have shown induction of functional Treg with HSP60, it is also important to establish how FOXP3 expression is regulated, since loss of FOXP3 causes a loss of Treg suppressive

capacity (**Chapter 3**). We show in **Chapter 5** that FOXP3 expression is regulated by protein acetylation. FOXP3 was acetylated by the HAT p300 and de-acetylated by the HDAC SIRT1. Acetylation of FOXP3 promoted protein stability by preventing proteasomal degradation. Accordingly, treatment of anti-CD3/ anti-CD28 induced human Treg with HDAC inhibitor nicotinamide (NAM), promoting acetylation, caused an increase of FOXP3 expression. Both the frequency of FOXP3$^+$ Treg, as well as expression of FOXP3 per cell was increased. Furthermore, we demonstrated that murine iTreg treated with NAM exhibited increased suppressive function in in vitro assays. Thus, HDAC inhibitors promote stable FOXP3 expression in induced Treg and increase suppressive function.

Treg involvement in the pathogenesis of JDM

Before investigating Treg targeting as a treatment for a disease, it is important to establish whether Treg are actually involved in the pathogenesis of an inflammatory disorder. In **Chapter 6** we showed that patients with Juvenile dermatomyositis (JDM) had normal Treg percentages in peripheral blood compared to age-matched controls, independent of disease activity, while in patients treated with high doses of corticosteroids increased Treg percentages were observed. Furthermore, Treg from JDM patients with remitting disease exhibited suppressive function in vitro, while Treg from patients with active disease were not consistently suppressive. In muscle tissue of JDM patients with active disease many FOXP3$^+$ Treg were present. Thus, Treg were able to migrate towards the inflamed tissue, but somehow were unable to suppress inflammation. In conclusion, Treg are probably involved in JDM pathogenesis, but unfortunately unable to suppress inflammation. Therefore, promotion of Treg function and frequency could improve disease outcome.

Discussion

In this thesis, we discussed several properties of human Treg, which were initially described in murine models. We showed that one suppressive mechanism of murine Treg, the induction of effector T cell apoptosis by cytokine consumption, is not utilized by human Treg (1). On the other hand, we found that CD30, which was important for Treg function in murine models, was exclusively expressed on in vitro suppressive human Treg (9). Thus, by validating studies utilizing murine models in human cells, we found both differences and similarities. Unfortunately, in high impact scientific journals, a majority of published research is restricted to murine models, without including data in human cells or tissues which could facilitate translation of results (10). For obvious reasons, research in human cells or tissues is mostly limited to in vitro experiments and therefore may not represent the true in vivo situation. However, restricting research to a small number of murine models, and thereby ruling out genetic differences, will likely lead to an exaggeration of findings which may not be relevant in humans (10, 11). Thus, evaluation in human cells or tissues is required for clinically relevant research, which may uncover leads to targets for clinical intervention in immune disorders, such as autoimmune diseases.

CD30 function in human Treg

Although we showed that CD30 is an important surface marker for functional Treg, the function of CD30 on human Treg is unknown. In mouse models CD30 has been extensively studied, and has been suggested to be important for early suppression of GvHD development (12). Already early in the course of disease Treg show increased expression of CD30, and DC have increased CD30 ligand expression (CD30L) (12). Moreover, blocking of CD30-CD30L interaction, or infusion of $CD30^{-/-}$ Treg abrogates suppression of GvHD (12). In a model for graft rejection CD30-CD30L interaction was also required for Treg mediated suppression, possibly through CD30-CD30L induced apoptosis in $CD8^{+}$ effector T cells (13). In human cells, however, only few reports have been published showing that CD30 expressing Hodgkin Reed-Sternberg tumour cells and plate-bound CD30 inhibited effector T cell proliferation and cytokine production through an interaction of CD30 with CD30L on effector T cells (14). We showed that only CD30 expressing HSP60-induced Treg suppressed effector T cell proliferation in vitro. However, how CD30 actually influences human Treg or effector T cells is unknown.

Since we observed that CD30 expression correlates with FOXP3, it may somehow be involved in regulation of FOXP3 expression. Alternatively, FOXP3 may either directly or indirectly induce CD30 expression. Intervention with CD30 signaling by either knockdown of CD30 by siRNA's or stimulation with CD30L, followed by analysis of FOXP3 expression and Treg function could shed more light on the function of CD30 on human Treg. Moreover, this could provide novel insights into CD30 as a target for Treg mediated intervention in autoimmune diseases.

Humanized mouse models: present and future prospects

We observed that iTreg were suppressive in vitro, but not in vivo in a murine model of xenogeneic Graft versus Host disease (x-GvHD). X-GvHD was induced by intravenous administration of human PBMC into the tail veins of adult $RAG2^{-/-}\gamma$-$chain^{-/-}$ mice. Following, co-injection of functional nTreg, but not iTreg prevented x-GvHD development. Although this model is helpful for determining Treg suppressive function, and is a major improvement compared to in vitro assays, some drawbacks remain. Firstly, a large number of effector T cells are necessary to induce disease, and accordingly a large number of Treg are required to suppress disease. Therefore, it is impossible to investigate Treg from patients with autoimmune disease without further expansion, and we have shown that results obtained in in vitro suppression assays are not always representative for the in vivo situation. In order to make the model more convenient for analysis of Treg function, research is required to down-scale cell numbers, but it is unclear whether less Treg will be able to suppress x-GvHD. Another problem is that reduction of PBMC numbers injected to induce x-GvHD influences disease course and mice seem to develop a chronic form of x-GvHD, which develops much slower (unpublished data). Thus, down-scaling of

PBMC alters both the timing and strength of the immune response, which hinders comparison to our previous experiments. Furthermore, it is unknown whether injection of Treg will efficiently suppress this disease. Secondly, xenogeneic inflammation results in a severe systemic disease, marked by a cytokine storm (Tuna Mutis & Anton Martens, unpublished data), such that T cells may respond differently than in non-xenogeneic models. Therefore, this model may not be truly representative for autoimmune disorders.

Currently, several comparable experimental models, based on immunodeficient mice are available. Since the 1970s development of these models has led to two different human immune system (HIS) mouse models. In one model, $RAG^{-/-}\gamma\text{-chain}^{-/-}$ mice are transplanted with human $CD34^+$ haematopoietic stem cells. These mice have normal immune responses towards viral infection, suggesting a functional immune system (15). In another model, immunodeficient NOD-SCID mice are transplanted with human embryonic bone marrow, liver and thymus, and these are termed BLT mice. These mice are transplanted with $CD34^+$ haematopoietic stem cells from the same donor, which develop into mature T and B cells (16). BLT mice also have normal responses towards viral infection.

Initially these mice were only used to study viral infections, but now several experimental modifications have been made, allowing the study of autoimmunity. Autoimmunity is often linked to expression of certain HLA alleles. For example, HLA-DR4 and HLA-DR1 alleles are risk factors for development of arthritis and transgenic mice with human HLA-DR4 or DR1 have been developed to study arthritis (17-19). Currently, a group of human genes involved in immunity, such as HLA genes, are introduced into HIS mice by replacement of endogenous loci in order to more accurately mimic the human immune system ((20),Personal communication, R Flavell). Particularly in autoimmune diseases, it is important to have similar HLA alleles, but also similar epitopes processed from tissue proteins for antigen presentation on these HLA-molecules, in order to ensure an appropriate tolerogenic or inflammatory response comparable with the human immune response. This partially overcomes the differences between the human and murine immune system, and will provide a significant advance in the field of translational research. In the future, these experimental models may replace in vitro suppression assays, and improve studies on human Treg function in autoimmunity in vivo, allowing investigation of, for example, the therapeutic potential of human HSP60 epitopes in autoimmune diseases, such as arthritis.

Stability of Treg: T cell plasticity

In our experiments we intravenously administered $FOXP3^+$ Treg in the x-GvHD model, however Treg rapidly lost FOXP3 expression and could not suppress disease. Sustained FOXP3 expression establishes functional Treg, and while ectopic expression of FOXP3 in murine $CD4^+CD25^-$ T cells can induce a suppressive phenotype (21, 22), Treg can lose FOXP3 and suppressive capacity in an inflammatory environment (23-26). Over the last few years, several protocols have been established for differentiating T cells into specific T cell subtypes in vitro. IFNγ and IL-12 are required to develop Th1 cells, IL-4 induces Th2 cells, TGFβ, IL-1 and IL-23 induce Th17 cells (23), and TGFβ and IL-2 induce Treg (27). Each T cell subtype has its own master transcription factor, which drives T cell differentiation and maintains T cell subtypes. For example, Treg express FOXP3, Th17 express RORC2 (in mice RORγT), Th1 express T-bet (28). Th2 express GATA3 (29) and IRF-4 (Interferon regulatory factor 4)(30). It has been recently demonstrated that human $FOXP3^+$ Treg can co-express RORC2 and produce IL-17 ex vivo (31). In mice, $FOXP3^+$ Treg can also co-express T-bet (32) or IRF-4 (33), which enables them to specifically suppress Th1 or Th2 responses. We showed that both in vitro expanded human iTreg and nTreg expressed FOXP3 and were suppressive in vitro, but at the same time produced Th17 (IL-17), Th2 (IL-4, IL-5, and IL-13) and Th1 (IFNγ) cytokines.

However, Treg can also differentiate into effector T cells. Human $FOXP3^+$ Treg can, under inflammatory conditions, convert to IL-17 producing, RORC2 expressing Th17 cells (24-26). This is of particular interest, since IL-17 is a pathogenic cytokine and highly expressed at

inflammatory sites in several human autoimmune diseases, such as multiple sclerosis (MS) (34, 35), rheumatoid arthritis (36), Crohn's disease (37) and psoriasis (38). Furthermore, in murine models it has been shown that Treg can lose FOXP3 and convert to Th2 (39), or to effector T cells producing IL-17 and IFNγ (40). This phenomenon is called T cell 'plasticity' (41-43), and is also observed in T helper cells, for example Th2 can convert to Treg (44), and Th17 to Th1 (45). T cell plasticity provides flexibility in T cell responses, which may be required to generate an appropriate immune response upon different types of infection (43). Taken together, iTreg with transient FOXP3 expression may not only lose Treg phenotype, but may even differentiate into pathogenic effector T cells once they are in an inflammatory environment. Therefore, it is important to carefully evaluate Treg stability to ensure that Treg are safe for treatment, or to promote FOXP3 expression in order to prevent conversion of Treg to effector T cells, for example by utilizing HDAC inhibitors, which have been demonstrated to prevent conversion of Treg to Th17 under inflammatory conditions in vitro (24).

Stable FOXP3 expression through promotion of post-translational acetylation

We showed that treatment of human iTreg with HDAC inhibitor nicotinamide (NAM) stabilized FOXP3 expression levels, and in murine cells promoted Treg suppressive function. Furthermore, mice treated with HDAC inhibitor Trichostatin A (TSA) have increased numbers of functionally improved Tregs correlating with suppression of inflammation in an induced colitis model as well as transplantation models (46) . Another HDAC inhibitor, Valproic acid (VPA) inhibited disease in murine models for colitis (46) and collagen-induced arthritis (47). HDACs can be distinguished in 4 classes; class I, II and IV consisting of HDACs, and class III consisting of sirtuins (SIRTs) (48). TSA inhibits HDACs classes I,II and IV (48), and Valproic acid inhibits HDACs classes I and II (48). The nicotinamide adenine dinucleotide (NAD^+) dependendent class III SIRTs can be inhibited with nicotinamide (NAM) (49). Several different HDAC inhibitors have been tested as cancer therapeutics in phase I and II clinical trials, mostly in patients suffering from heamatological cancers, such as cutaneous T cell lymphoma (CTCL) (reviewed in (48)). HDAC inhibitors have been demonstrated to induce cell cycle arrest, by increase of p21 (50, 51) or p53 (52) in tumour cell lines. Furthermore, HDAC inhibitors have been shown to upregulate proapoptotic genes Bmf (53) and Bim (54), expression of death receptors (55), and decrease expression of pro-angiogenic genes (56). Most clinical trials with HDAC inhibitors showed promising results (48), and vorinostat (suberoylanilide hydroxamic acid (SAHA), inhibiting HDAC class I, II and IV), has been approved in the US for treatment of CTCL (57). Only few adverse effects of HDAC inhibitors were reported, of which the most severe was cardiac failure, however this was not reported for vorinostat (48). Thus, vorinostat could be a safe therapeutic for promotion of Treg function, but first it should be established whether it can actually promote FOXP3 expression and Treg function similar to TSA and VPA in in vivo models and human Treg. We showed that nicotinamide inhibition of SIRT1 is likely important in primary human and murine T cells for stable FOXP3 expression and suppressive function (58). NAM is the water-soluble, physiologically active amide of vitamin B3, and since NAM only targets one group of HDACs, it may have less adverse effects compared to vorinostat, but thus far, no inhibitors for SIRTs have been tested in clinical trials (48). Thus, whether NAM exerts a similar effect on FOXP3 expression in vivo remains to be further investigated.

Autoimmunity treatment regimens: influence on Treg

All Treg studies in patients with autoimmune diseases should include a careful evaluation of the treatment of these patients, since medication itself can have an effect on both Treg frequency and suppressive function. Anti-TNFα (infliximab) increases Treg numbers threefold in rheumatoid arthritis patients, probably by induction of new $FOXP3^+$ Treg from $CD4^+CD25^-$ T cells, which was shown in vitro (59, 60). Furthermore, membrane-bound TNFα diminishes Treg

function, and anti-TNFα treatment decreases membrane-bound TNFα on Treg both in vitro and in rheumatoid arthritis patients, thereby restoring Treg function (61).
Steroid-mediated immune suppression may be enhanced by increasing Treg frequency or function. For instance, methyl-prednisolone pulses can increase the frequency of Treg in patients with MS (62, 63) and myasthenia gravis (64, 65) . In patients with myasthenia gravis the reduced suppressive function of Treg was restored by prednisolone treatment (65). This suggests that glucocorticoids can induce tolerogenic responses through Treg and thereby diminish disease. A proposed mechanism of glucocorticoid induction of tolerance is through inhibition of DC maturation (65-68). Immature DC produce TGFβ and IL-10 (65) and are able to induce Treg (69). Another mechanism is induction of cell death by glucocorticoids, specifically in effector T cells but not in Treg. Dexamethasone treated mice demonstrated increased apoptosis of $CD4^+CD25^-$ T cells, but not of $CD4^+CD25^+$ T cells, thereby increasing the percentage of $CD4^+CD25^+$ T cells, which were suppressive in vitro (70).
In JDM patients high doses of prednisone or methyl-prednisolone are often used to control active disease (71). Indeed, we found that active JDM patients treated with high doses of corticosteroids had higher levels of Treg in peripheral blood compared to remitting patients. This increase of Treg due to corticosteroid or anti- TNFα treatment has to be taken into consideration when studying Treg in patients receiving these therapeutics. Although very effective in suppressing inflammation in JDM, these general immune suppressants cause considerable side effects and often have to be used continuously to control disease. Therefore a different, more specific way of promoting Treg numbers and/or function would be preferable.

Putting the pieces together: Towards therapy

In humans, peripheral induction of Tregs is probably important for regulation of inflammation (72, 73). This would suggest that Treg are either absent or not functional in patients with autoimmune disease. However, in both JDM muscle and JIA joints large numbers of Treg are found, and Treg from JIA synovial fluid are functional in ex-vivo assays (74). It is likely that the chronic inflammatory environment in vivo either causes local dysfunction of Tregs or makes activated cells no longer susceptible to Treg mediated suppression (75-77). Thus, to create a therapeutic window for Treg targeted therapies, it may be important to first dampen chronic inflammation, for instance by using anti-TNFα therapy for a short period of time (78, 79).
Thereafter, Treg function should be induced, and in this thesis we described several approaches to do so. Firstly, HSP60 epitopes can induce HSP60-specific Treg, which will suppress actively and exclusively at sites where HSP60 is present; such as the synovial lining tissue in joints of JIA patients (**Chapter 7**), or damaged muscle fibres of JDM patients (6). Furthermore, in order to maintain Treg function, and prevent conversion into effector T cells, HDAC inhibitors, such as nicotinamide, can enhance FOXP3 stability **(Chapter 5)**. However, this still has to be tested more extensively in in vivo model systems.
Thus, a combination of different approaches will be required to establish a more specific immune suppression of autoimmunity, only enhancing Treg function at sites of ongoing inflammation, such as JDM muscle tissue (**Figure 1**). Experiments in appropriate humanized mouse models are a critical step in the process of putting all of these pieces together into a safe and specific therapy to treat inflammatory disorders, such as JDM and JIA.

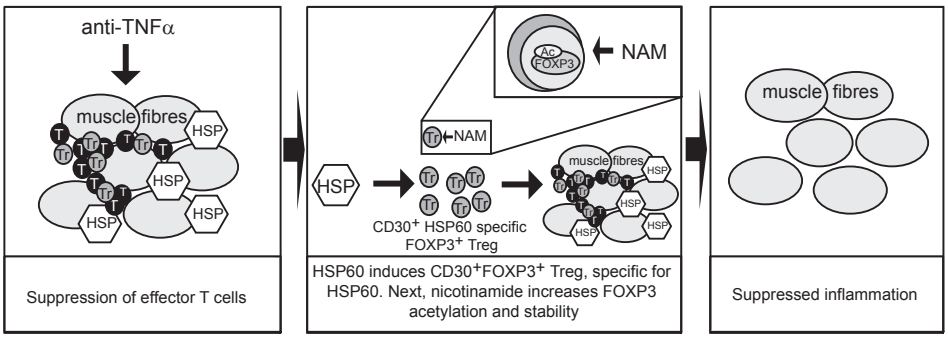

Figure 1. Proposed combination of strategies to achieve immune suppression by Treg in JDM. Inflammation of JDM muscle tissue is marked by infiltration of effector T cells (T) and HSP60 (HSP) expression by damaged muscle fibres. Since Treg (Tr) are present as well, but cannot suppress inflammation, it is likely that effector T cells are somehow resistant to Treg suppression. Therefore, a combination of stategies is required to achieve Treg mediated suppression of tissue inflammation. Firstly, the resistant effector T cells (Teff) in muscle tissue should be suppressed by, for instance, anti-TNFα. This creates a window of opportunity for Treg to prevent further inflammation (left panel). Following, peripheral Treg induction by oral administration of HSP60 epitopes (HSP) further increases Treg frequency , and results in functional CD30 and FOXP3 expressing Treg specific for HSP60. HSP60 is highly expressed in inflamed muscle tissue and, therefore, the newly induced Treg will only suppress at the inflammatory site (second panel). This increase of the Treg population with antigen specific potent Treg promotes rapid and efficient suppression of Teff. To stabilize FOXP3 expression in these induced Treg, HDAC inhibitor nicotinamide (NAM) increases FOXP3 acetylation, preventing degradation (second panel, enlarged). Accordingly, this prevents loss of Treg suppressive function and possible conversion of Treg into inflammatory effector T cells. Taken together, this could enable Treg to suppress inflammation specifically in inflamed muscle tissue (right panel).

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CD30
iTREG
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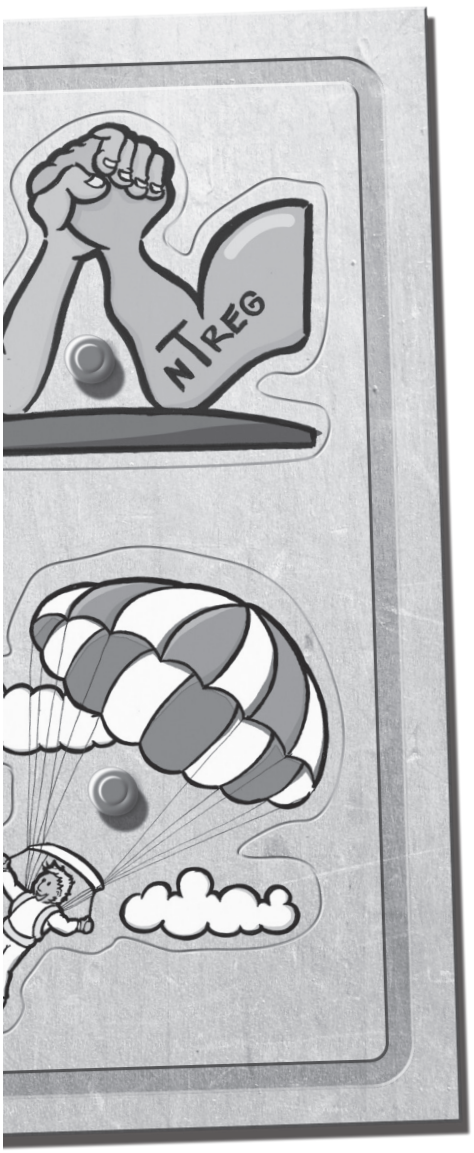
Nederlandse Samenvatting
(voor niet-wetenschappers)

Dankwoord

Curriculum Vitae

List of Publications

Colour Figures



Nederlandse Samenvatting (voor niet-wetenschappers)

Het immuunsysteem is onze verdediging tegen indringers, zoals bacteriën en virussen. Om deze indringers uit de weg te kunnen ruimen, moet het immuunsysteem ze eerst kunnen herkennen. Witte bloedcellen spelen een grote rol in zowel de herkenning als het opruimen van indringers. Bepaalde witte bloedcellen, de T cellen, hebben elk een karakteristieke receptor waarmee ze stukjes eiwit (peptiden) kunnen herkennen. Vervolgens kunnen de T cellen een ontstekingsreactie veroorzaken, waardoor indringers worden opgeruimd. Omdat de T cel receptor wordt gevormd door een willekeurig proces, kan het gebeuren dat T cellen soms peptiden uit het eigen lichaam herkennen. In gezonde mensen is dat geen probleem, omdat de T cellen dan geen ontstekingsreactie veroorzaken. Echter, in patiënten met auto-immuunziekten, zoals reuma en dermatomyositis, gebeurt dit wel, waardoor er een onnodige ontsteking optreedt. In kinderen met reuma raken de gewrichten van bijvoorbeeld knieën ontstoken, en in kinderen met dermatomyositis ontsteken de kleine bloedvaatjes in voornamelijk spieren en de huid, waardoor spierafbraak en huiduitslag optreedt. Een vermoedelijke oorzaak van deze onnodige ontstekingen, is het ontbreken van een ontstekingscontrole mechanisme. De controle wordt in gezonde mensen bewaakt door de regulatoire T cellen (Treg), die de ontstekingsreactie van andere T cellen kunnen onderdrukken. Echter, in patiënten met auto-immuunziekten zijn er te weinig functionele Treg. Dit betekent dat, als we een vermeerdering, of verbetering van functie van Treg willen bereiken in deze patiënten, we precies moeten weten hoe de Treg werken, hoe de Treg eruit zien, en wat er precies belangrijk is voor hun functie. Vervolgens kunnen we dan onderzoeken hoe we ze kunnen manipuleren zodat we een groter aantal functionele Treg kunnen bereiken. In dit proefschrift onderzoeken we de verschillende eigenschappen van de Treg en hebben we op verschillende manieren de Treg gemanipuleerd om meer functionele Treg te verkrijgen.

Hoe werken Treg?

Het is tot dusver niet precies bekend hoe Treg andere T cellen onderdrukken. Veel onderzoek gebeurt in muismodellen en aangezien het immuunsysteem van muizen en mensen niet precies gelijk is, moeten de bevindingen in muizen worden gecontroleerd in menselijke (humane) cellen. We beschrijven in **hoofdstuk 2** dat humane Treg, in tegenstelling tot muizen Treg, geen geprogrammeerde celdood (apoptose) veroorzaken in T cellen, maar toch de activiteit van andere T cellen kunnen onderdrukken.

Hoe zien Treg eruit?

Treg zijn te herkennen aan een eiwit, FOXP3 (forkhead box P3) dat ze continue nodig hebben voor hun onderdrukkende functie. Helaas zit dit eiwit in de cel en is daardoor niet zichtbaar, tenzij de cellen kapot worden gemaakt. Daarom is het beter om een eiwit op het oppervlak van de cel te gebruiken om Treg te herkennen. In **hoofdstuk 4** hebben we gevonden dat CD30 uitsluitend aanwezig is op het oppervlak van T cellen met FOXP3 en onderdrukkende functie. CD30 lijkt daarom een goede marker (kenmerkend molecuul) voor Treg te zijn.

Hoe kunnen we Treg manipuleren?

Om uiteindelijk de Treg te kunnen gebruiken voor therapeutische doeleinden in patiënten met auto-immuunziekten, hebben we de Treg functie en aantallen geprobeerd te manipuleren op 3 verschillende manieren, namelijk:

1- Maak grote hoeveelheden Treg door vermenigvuldiging.

Er zijn twee soorten Treg: Treg die er vanaf de geboorte al zijn (nTreg, ofwel natuurlijke Treg), of Treg die nieuw worden gevormd (iTreg, ofwel geïnduceerde Treg). Beiden zouden geschikt kunnen zijn als onderdrukkers van auto-immuunziekten. We hebben in **hoofdstuk 3** onderzocht

of nTreg en iTreg, na vermenigvuldiging door een algemene activering van de T cel receptoren, FOXP3 en goede onderdrukkende functie hebben. FOXP3 is in zowel vermenigvuldigde iTreg als nTreg aanwezig en de cellen hebben onderdrukkende functie in een kweeksysteem. Echter, in een muis die zelf geen immuunsysteem heeft, waarin je humane cellen kunt bestuderen (gehumaniseerde muis), onderdrukken de nTreg nog steeds, maar de iTreg niet, doordat ze FOXP3 verliezen. Hiermee laten we zien dat deze iTreg niet geschikt lijken voor therapeutische doeleinden. Verder zien we dat de onderdrukkende functie van Treg verschillend is tussen het kweeksysteem en het gehumaniseerd muismodel. Om potentieel gevaarlijke afwijkingen van de Treg aan te tonen, moeten ze eerst worden bekeken in een gehumaniseerd muismodel, voordat de Treg kunnen worden toegepast in patiënten.

2- Maak grote hoeveelheden Treg met lichaamseigen peptiden.
T cellen kunnen lichaamseigen peptiden herkennen. Het is opvallend dat juist vaak de Treg deze peptiden herkennen en dan geen ontsteking veroorzaken. De Treg kunnen na herkenning van deze peptiden zelfs beter andere T cellen onderdrukken, omdat ze na herkenning meer activiteit vertonen en zich kunnen vermenigvuldigen. Peptiden van het lichaamseigen eiwit HSP60 (heat shock protein 60) komen vrij op plekken van schade, bijvoorbeeld ontstekingsschade, zoals gewrichten van kinderen met reuma, of spieren van kinderen met dermatomyositis. Eerder is in ons lab aangetoond dat herkenning van HSP60 door Treg in kinderen met reuma samenhangt met een minder ernstig verloop van de ziekte. We hebben in **hoofdstuk 4** aangetoond, dat HSP60 de vorming van nieuwe, goed functionerende Treg bevordert. Dit zou kunnen betekenen, dat na toediening van HSP60 peptiden het aantal onderdrukkende Treg in patiënten stijgt, waardoor de ontsteking afneemt.

3- Voorkom dat Treg FOXP3 verliezen.
Treg die FOXP3 verliezen, kunnen een ontsteking niet onderdrukken. FOXP3 is dus erg belangrijk voor de functie van Treg. Een manier om het verlies van Treg functie te voorkomen, is het stabiliseren van FOXP3. In **hoofdstuk 5** laten we zien dat modificaties van de eiwitstructuur van FOXP3, door een verhoging van acetyl-groepen, de afbraak van FOXP3 voorkomen. Het verhogen van acetyl-groepen kan worden bereikt met deacetylase remmers (deacetylases verwijderen acetyl-groepen). Behandeling van Treg met deze deacetylase remmers zorgt ervoor dat FOXP3 niet wordt afgebroken, wat leidt tot een verbeterde onderdrukkende functie van Treg. Na een vermeerdering van de Treg, is het behoud van de onderdrukkende functie van belang om een ontsteking in patiënten te kunnen remmen.

De toepassing van bovenstaande bevindingen: ***'Putting the pieces together'***

In ontstoken spierweefsel van kinderen met dermatomyositis zijn grote aantallen Treg aanwezig (**hoofdstuk 6**), maar toch kunnen ze de ontsteking niet goed onderdrukken. Dit komt overeen met het beeld in de gewrichten van kinderen met reuma; ook hier zijn veel Treg aanwezig, maar de actieve T cellen lijken bestand tegen de onderdrukking van Treg. Dit soort ziekten zouden behandeld kunnen worden met de volgende strategie:
1- De actieve T cellen moeten worden onderdrukt met medicatie om de ontsteking te verminderen. **2-** Hierna kunnen de Treg aantallen of functie worden bevorderd om een nieuwe ontsteking te voorkomen. Hoge Treg aantallen zouden bereikt kunnen worden door de toediening van HSP60 peptiden. Hierna kunnen deacetylase remmers FOXP3 hoog houden en zorgen dat deze Treg hun onderdrukkende functie behouden.
De toediening van een bepaald peptide van HSP60 is al eens getest in patiënten met diabetes type 1 (alvleesklier ontsteking) en de ziekte verminderde hierdoor. Deacetylase remmers zijn nog niet getest in patiënten, maar in muismodellen voor colitis (darm ontsteking) werd de ontsteking onderdrukt na behandeling met deacetylase remmers. Kortom, manipulatie van Treg lijkt zeer geschikt voor de behandeling van auto-immuunziekten.

Dankwoord

Promoveren is als het beklimmen van Mont Ventoux:

Het begin gaat soepel, je hebt nog geen idee wat je te wachten staat,
maar vertrouwt erop dat deze weg de juiste is, en het wel goed gaat komen.

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Vervolgens, in de laatste fase een maanlandschap -verstoken van enig levend wezen en dan blijven doortrappen...

In de mist een monument, op de plek waar een voorganger het onderspit dolf,
blijven doortrappen...

Dan eindelijk op de top de overwinning vieren,
met vrienden zonder wie je het nooit had gehaald

Berent- Ook jij bent een fanatiek fietser als de agenda het toelaat. Een schitterend voorbeeld voor mij: naast de snelle tijd die jij op de Ventoux verschillende malen hebt neergezet, ben je ook tijdens voetbalpartijtjes en zelfs congressen uitermate fanatiek als het om de prijzen gaat. Met een echte winnaarmentaliteit, zonder die aan anderen op te leggen, maar slechts om die ander te inspireren. Veel dank voor alle vrijheid, maar ook voor al je adviezen en ideeën, waarmee je me ook soms het idee kon geven dat ik het helemaal zelf had bedacht. Je geeft al je mensen volledige credits voor wat ze doen en dat is een goede en bijzondere eigenschap, die lang niet iedereen in de wetenschap bezit.

Paul- Wat een uitdaging, een samenwerking tussen een moleculair bioloog en een kinderarts. Jorg en ik zouden de overbrugging moeten vormen, en dat is bijzonder goed gelukt. Jouw input heeft veel van het werk in dit boekje sterk verbeterd en het is verfrissend de zaken eens van een andere kant te bekijken. Ik heb er veel van geleerd, en vind het fantastisch dat zelfs jij tegenwoordig in de werkbespreking naar klinische relevantie vraagt. Veel dank voor alles.

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Nu is het tijd voor een heerlijk lange afdaling. Dit feest is voor jullie!
Proost!

Yvonne

Curriculum Vitae

Yvonne Vercoulen was born on May 26, 1982 in Tegelen, The Netherlands. In 2000 she graduated from the Valuascollege in Venlo. Following, she obtained her Bachelor's degree in Biomedical Sciences at the University of Utrecht.
During the master programme Biology of Disease, she worked for 9 months in the laboratory of Prof. Prakken under supervision of Dr. de Kleer at the Department of Pediatric Immunology at the Wilhelmina Children's Hospital in Utrecht. She wrote her thesis, entitled 'PARP-1 in the regulation of cell fate' under supervision of Dr. Jansen and Prof. Hoeijmakers at the Department of Genetics, Erasmus University, Rotterdam. After this, she worked for 7 months in the laboratory of Prof. Marshall, under supervision of Dr. Mavria at the Department Cell and Molecular Biology, Institute of Cancer Research, London, UK. Here, she investigated the influence of ERK and Rho kinase signalling in human endothelial cells on angiogenesis. In 2005, she graduated and returned to the laboratory of Prof. Prakken to start her PhD training at the Graduate school for Infection and Immunology, under supervision of Prof. Prakken and Prof. Coffer. The results obtained during her PhD training are described in this thesis.

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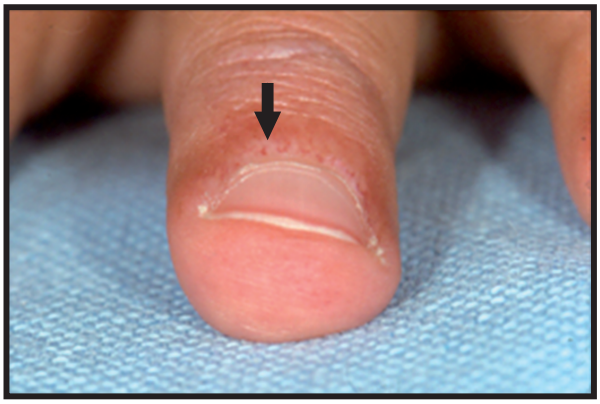
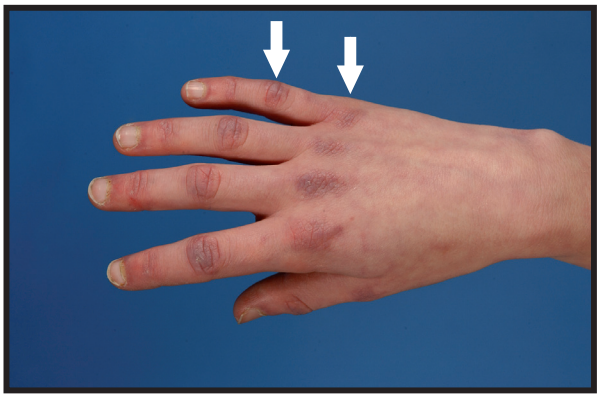
Colour Figures

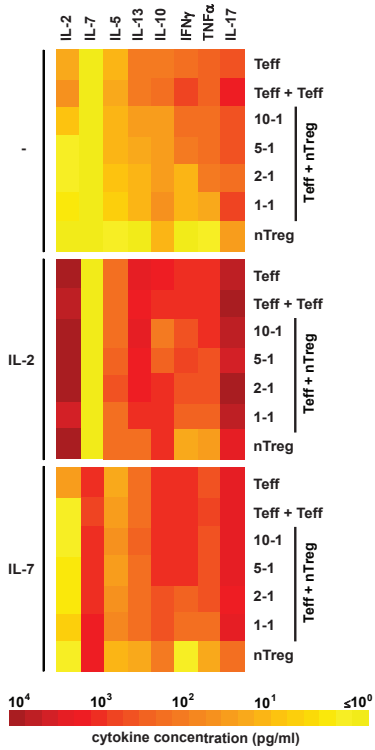
A



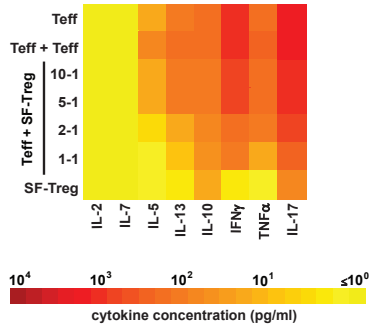
B



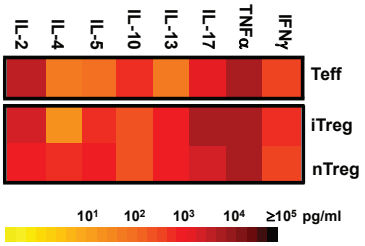
Chapter 1,Figure 4. JDM disease characteristics. In JDM patients inflammation of the microvasculature results in typical disease symptoms such as **(A)** abnormalities in nailfold capillaries, and **(B)** Gottron's papules on extensor surfaces of finger joints, indicated by arrows.



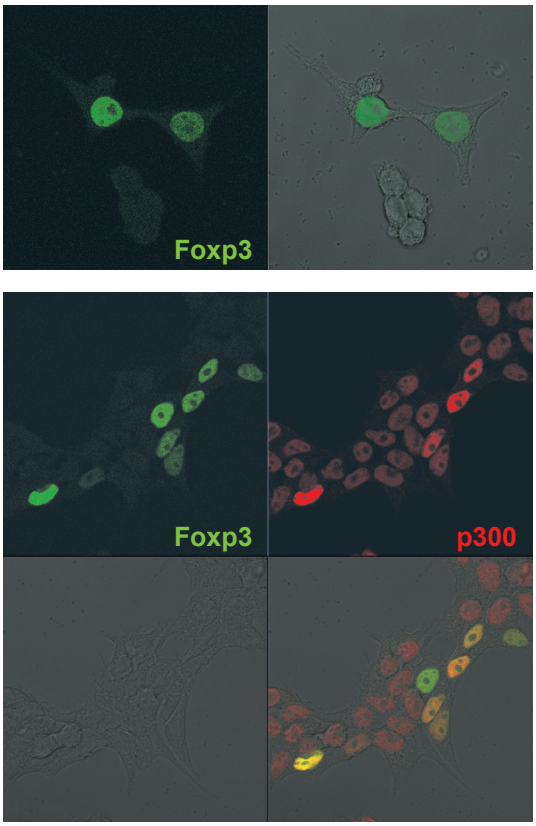
Chapter 2, Figure 2C. Exogenous IL-2 and IL-7 decrease suppression of Teff proliferation and cytokine production, but do not decrease apoptosis. Mean levels of cytokines, present in culture medium on day 5 of culture, in medium (-), with addition of IL-2 (IL-2) or IL-7 (IL-7). A color profile of the means was made to show the differences between culture conditions (see also Chapter 2, Table S1).



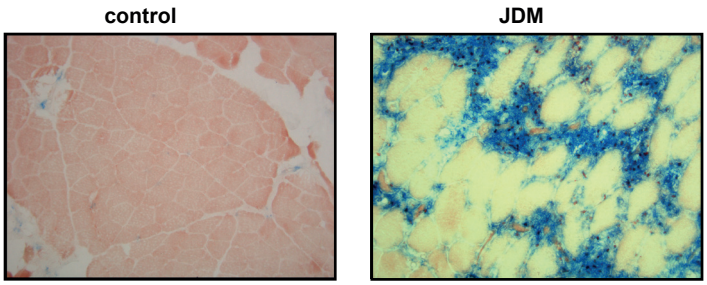
Chapter 2, Figure 3D. Synovial fluid-derived Treg suppress Teff proliferation and cytokine production, but do not induce apoptosis. Mean levels of cytokines, present in culture medium on day 5 of culture. A color profile of the means was made to show the differences between culture conditions (see also Table S2).



Chapter 3, Figure 2C. Phenotype and cytokine production profile of cultured nTreg and iTreg. Color profile of average cytokine excretion comparing nTreg, iTreg and CD4+CD25- effector T cells (Teff) (iTreg N=5, nTreg N=6, Teff N=6).

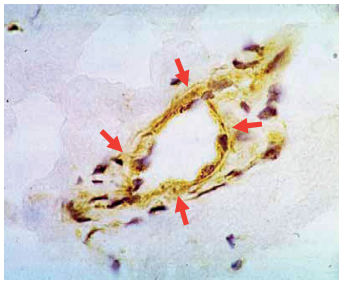


Chapter 5, Figure 2A Acetylation modulates FOXP3 protein levels. Representative examples of cells that were transfected with only FOXP3 (green; upper panel). Subcellular distribution of cells that were co-transfected with FOXP3 (green) and p300 (red) are shown in the lower panel. p300 was localized using an anti-p300 antibody that recognizes both endogenous and ectopically expressed p300. Co-localization of FOXP3 and p300 is indicated in yellow.



Chapter 6, Figure 3A. FOXP3$^+$ Treg are abundantly present in inflamed muscle tissue. Representative pictures from immunohistochemistry staining on muscle biopsies of controls (left, 1 example of n=2) and active JDM patients (right, 1 example of n=6). Blue staining indicates CD4 expression, brown-red staining indicates FOXP3 expression.

A



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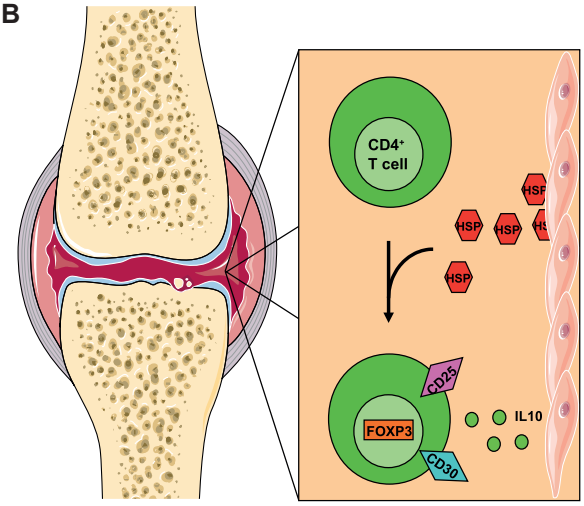
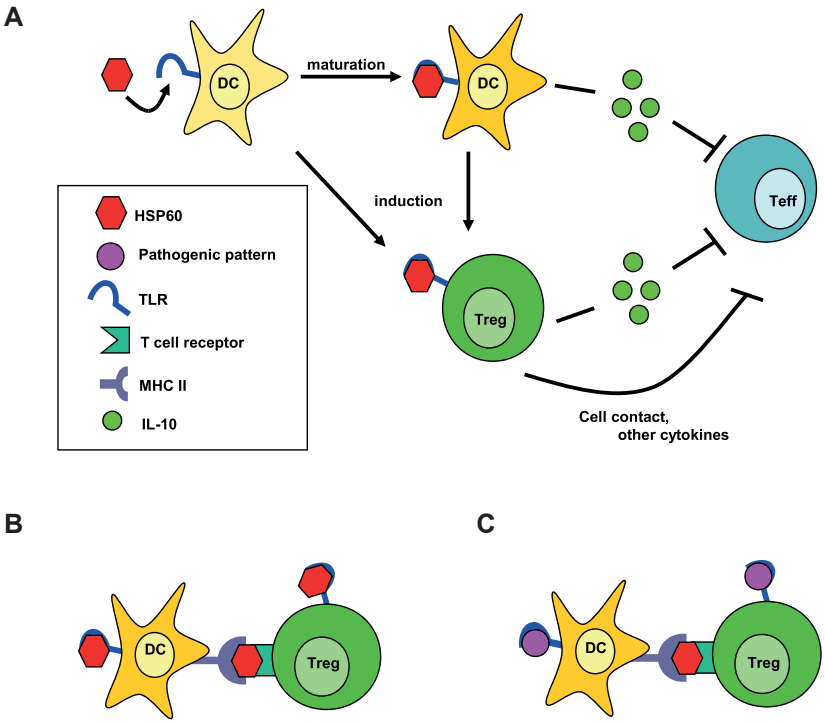


Chapter 7, Figure 1. HSP60 specific T cells in the synovium of JIA patient are IL-10 producing CD30⁺ Treg-like cells. (**A**) HSP60 (stained brown and marked by red arrows) is highly expressed in synovial lining membranes in the inflamed joints of JIA patients. (**B**) HSP60 is released by the synovial tissues in the inflamed joint. In the synovial fluid, $CD4^+$ T cells are present. T cells that react to the self-HSP60, or HSP60 epitopes, produce IL-10(13, 68) and express CD30. Presence of these HSP60 reactive T cells correlates with mild disease course(13). Therefore, we hypothesize that these T cells could be CD25 and FOXP3 expressing naturally occurring Treg (19), or IL-10 producing Tr1 cells. Altogether, HSP60 may induce Treg in the joints of JIA patients and hereby regulate the inflammation of these JIA patients, as is seen in oligoarticular JIA.



Chapter 7, Figure 2. HSP60 uses both innate and adaptive immune pathways to enhance Treg function. (**A**) HSP60 and HSP60- derived epitopes are able to bind to Toll- like receptors (TLRs). By binding to TLRs present on Dendritic Cells (DC), HSP60 can induce maturation of DC, or cytokine production by DC (9, 11), which could possibly cause induction of Treg. Both DC and Treg are able to produce IL-10, which inhibits activation of effector T cells. To suppress the ongoing immune response, HSP60- induced Treg could inhibit activation of effector T cells by cell-cell contact and/or production of IL-10, or other cytokines, like TGFβ(14). (**B**) Pan-DR binding HSP60 epitopes are either presented to the Treg in an MHC II molecule by DC, which could stimulate Treg, or they are able to bind to TLRs on the Treg (28), and hereby enhance Treg function. We hypothesize that HSP60 and HSP60-derived epitopes can enhance or induce Treg by signaling through both the T cell receptor (TCR) and Toll-like receptor (TLR) at the same time. HSP60 epitopes may bind directly to TLRs on the T cells, or to TLRs on the DC, which could indirectly enhance the immune response. (**C**)**.** We hypothesize that a combination of a TCR signal by a HSP60 pan-DR binding epitope and a TLR signal by a pathogenic pattern could enhance the HSP60 induced, tolerogenic response, causing a stronger and longer lasting immune regulatory effect.