Immune Regulation after Heart Transplantation

Dynamics of regulatory T cells in the transplanted heart and peripheral blood

Esmé Dijke

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Dynamiek van regulatoire T cellen
in het getransplanteerde hart en perifere bloed

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voor

papa en mama

[Ik knipper m'n ogen en zie hoe ze steeds
Weer een beetje veranderd is
Maar hoe groot ze ook mag zijn
In mijn ogen blijft ze altijd klein...][1]

en

Henno

[Too many things I have to do
But if all of these dreams might find their way
Into my day to day scene
I'd be under the impression
I was somewhere in between
With only two,
Just me and you,
Not so many things we got to do
or places we got to be
We'll sit beneath the mango tree, now,
It's always better when we're together...][2]

[1] Songtekst uit 'Dochters' van Marco Borsato
Album: 'Wit Licht' - Schrijver: John Ewbank -
Universal Music - 2008.
[2] Songtekst uit 'Better Together' van Jack Johnson
Album: 'In Between Dreams' - Schrijver: Jack
Johnson - Brushfire - 2005.

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Part I

Chapter 1

Introduction and outline of this thesis

based on:

Regulatory T cells after organ transplantation: where does their action take place? Dijke IE, Weimar W, Baan CC. Human Immunology 2008; 69:389-398

The control of anti-donor immune responses by regulatory T cells in organ transplant patients. Dijke IE, Weimar W, Baan CC. Transplantation Proceedings 2008; 40:1249-1252

Introduction

Transplantation of organs has become a rapidly evolving therapy for end-stage organ failure. Unfortunately, clinical success has been limited by the ongoing need for immunosuppressive therapy that reduces the risk of allograft rejection but also carries unwanted side effects, such as the increased susceptibility to infection and malignancy.[1] Moreover, although immunosuppressive drugs efficiently decrease the occurrence of acute allograft rejection, a substantial proportion of patients experience chronic rejection that ultimately leads to functional loss of the graft.[2] Thus immunosuppressive therapy is not sufficient to inhibit all immune responses directed to the transplanted organ. An alternative approach to avoid rejection and, subsequently, the need for life-long treatment with immunosuppressive drugs is the induction of tolerance to donor allografts. Tolerance may be achieved by a specialized subpopulation of T cells that control immune responses to self antigens and nonself antigens, the so-called regulatory T cells (or suppressor T cells). The identification and characterization of regulatory T cells that may play a relevant role in the induction and persistence of tolerance toward a transplanted organ has therefore become the focus of many studies.

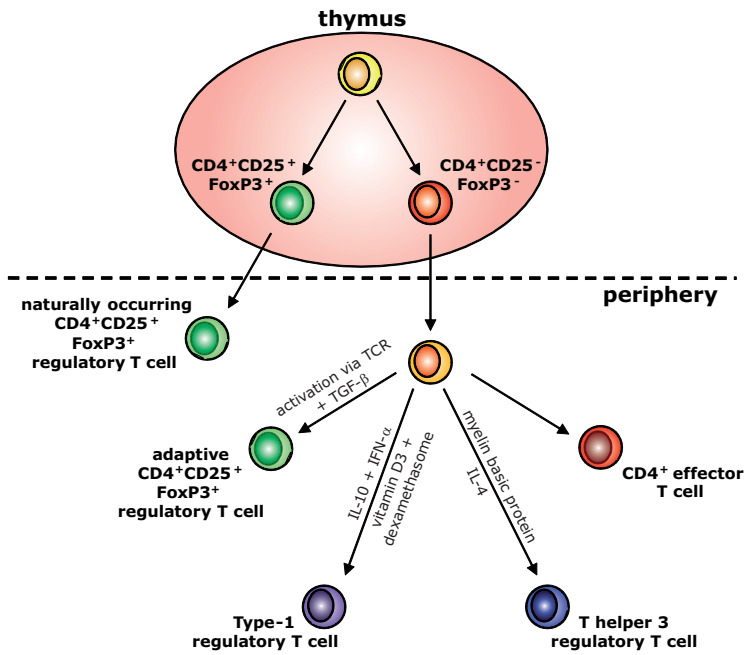

Figure 1. Developmental pathways of naturally occurring and adaptive $CD4^+$ regulatory T cells. Naturally occurring $CD4^+CD25^+$ regulatory T cells are derived from the thymus and comprise 5–10% of the $CD4^+$ T-cell population.[10] Stimulation of $CD4^+CD25^-FoxP3^-$ effector T cells through the TCR in the presence of TGF-β results in FoxP3 upregulation, generating adaptive $CD4^+CD25^+FoxP3^+$ T cells.[25,26] In addition, Type-1 regulatory (Tr1) T cells can be generated from naive $CD4^+$ T cells in the presence of IL-10 in combination with IFN-α or a combination of vitamin D3 and dexamethasone.[27,28] A further subset of regulatory $CD4^+$ T cells, T helper (Th)3 cells, are generated after oral administration of myelin basic protein or IL-4.[30]

Regulatory T-cell populations

One of the first studies that described a $CD4^+$ T-cell population with suppressive capacities was published in 1990. Hall et al. demonstrated in experimental animal models that $CD4^+$ suppressor cells that expressed the interleukin (IL)-2 receptor α chain (CD25) were able to inhibit cardiac allograft rejection.[3] Some years later, Sakaguchi et al. showed that a small subset of $CD4^+$ T cells that constitutively express CD25 was important for the prevention of autoimmune diseases.[4] Ever since, research on $CD4^+CD25^+$ T cells has experienced an enormous growth: studies showed that these regulators play an important role in autoimmune disorders, infections, tumors, allergy and transplantation.[5-9]

Naturally occurring $CD4^+CD25^+$ regulatory T cells are derived from the thymus and comprise 5–10% of the $CD4^+$ T-cell population (Figure 1).[10] Besides CD25, other surface markers that are expressed on these cells are cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4 or CD152), glucocorticoid-induced tumor necrosis factor (TNF) receptor family-related gene (GITR or TNFRSF18), CD62L (L-selectin), $\alpha E\beta 7$ integrin (CD103) and OX40 (CD134).[11-15] These markers, however, are not exclusive for the $CD4^+CD25^+$ regulatory T cells, as they are also expressed on other (activated) T-cell subsets, making them unreliable for precisely identifying this cell lineage, especially in humans. In 2003, Fontenot et al. and Hori et al. demonstrated that the transcription factor forkhead box P3 (FOXP3) is the key regulatory gene for the development and function of $CD4^+CD25^+$ regulatory T cells.[16,17] FOXP3 expression is restricted to $CD4^+CD25^+$ regulatory T cells in mice and to those in humans, although T-cell activation may also induce transient FOXP3 expression in nonregulatory human cells.[18] Recently, Liu et al. suggested that expression of the IL-7 receptor α chain (CD127) may be an additional marker for the identification of $CD4^+CD25^+FOXP3^+$ regulatory T cells, as CD127 inversely correlates with FOXP3 expression and regulatory T-cell activity.[19]

To exert their function, $CD4^+CD25^+$ regulatory T cells require antigen-specific activation via the T-cell receptor (TCR).[20] Yet, once activated, they suppress the proliferation and cytokine production of effector T cells in an antigen-nonspecific way. The mechanisms by which these regulators mediate their function include the secretion of cytokines such as transforming growth factor (TGF)-β and IL-10 and direct cell-cell contact through binding of cell-surface molecules such as CTLA-4.[11,21,22] Furthermore, $CD4^+CD25^+$ regulatory T cells may also downmodulate antigen-presenting-cell (APC) functions and thereby make APC unable to activate effector T cells.[23] In addition, Pandiyan et al. demonstrated that $CD4^+CD25^+$ regulatory T cells induced apoptosis of effector T cells by deprivation of cytokines.[24]

In addition to the thymically derived naturally occurring regulatory T cells, other $CD4^+$ regulatory T-cell populations are generated in the periphery (Figure 1). These regulators are often termed adaptive regulatory T cells. Several studies

showed that stimulation of $CD4^+CD25^-FoxP3^-$ effector T cells through the TCR in the presence of TGF-β results in FoxP3 upregulation, generating $CD4^+CD25^+FoxP3^+$ T cells with regulatory properties similar to naturally occurring regulatory T cells.[25,26] Another adaptive regulatory T-cell population is the Type-1 regulatory (Tr1) T-cell population. These cells can be generated from naive $CD4^+$ T cells by antigenic stimulation in the presence of IL-10 in combination with interferon (IFN)-α or a combination of vitamin D3 and dexamethasone.[27,28] Tr1 cells regulate immune responses by producing high levels of the immunosuppressive cytokines IL-10 and TGF-β.[29] A further subset of regulatory $CD4^+$ T cells, T helper (Th)3 cells, are generated after oral administration of myelin basic protein or IL-4 and produce high levels of TGF-β with various amounts of IL-4 and IL-10.[30]

Although attention has mainly focused on $CD4^+$ regulatory T cells, they were not the first T cells mentioned to exhibit suppressive capacities: $CD8^+$ T cells with regulatory activities were described 30 years ago.[31] Precise characterization of the specificity and functional phenotype of these suppressor cells, however, was impossible in those years, because of lack of monoclonal antibodies to the CD4 and CD8 subpopulations and of molecular genetics-based approaches. In addition, the nature of the TCR was not well characterized and the great majority of the immune regulatory cytokines had not yet been identified. Lack of this information caused interest in the models of T-cell suppression mediated by $CD8^+$ T cells to wane.[32] In 1992, the idea that $CD8^+$ T cells were able to suppress T-cell reactivity resurgenced: Koh et al. demonstrated that $CD8^+$ T cells participate in the regulation of experimental autoimmune encephalomyelitis.[33] At present, a well-described $CD8^+$ T-cell population with suppressive activities is the $CD8^+CD28^-$ regulatory T-cell population, also referred to as suppressor T cells.[34] These cells suppress the proliferation of $CD4^+$ Th cells by inhibiting the CD40 signaling pathway of APC.[35] Studies in heart transplant patients showed that $CD8^+CD28^-$ suppressor T cells upregulated immunoglobulin-like transcript 3 (ILT3) and ILT4 on dendritic cells (DC) rendering these APC tolerogenic.[36]

Additional subsets of regulatory T cells described are within the double-negative (DN; $CD4^-CD8^-TCR\alpha\beta^+$) population, within the γδ T-cell population, and the NKT cells. The suppressive effect of DN cells is attributed to Fas-mediated apoptosis of alloreactive T cells.[37] γδ T cells are present at high number in the mucosa and may have an immunosuppressive, antidiabetogenic effect that is probably mediated by IL-10.[38] NKT cells are characterized by expression of both NK and T-cell markers and are involved in autoimmune disorders, infectious diseases and cancer.[39] They are also considered to play a role in the induction of allograft tolerance.[40] The mechanism through which NKT cells mediate their suppressive function is unclear, but may be via the production of immunosuppressive cytokines.

Immune regulation: where does the action take place?

Close contact of regulatory T cells with effector cells, either directly or through an APC intermediate, is essential for exerting their regulatory function.[41] Thus, active suppression of anti-donor immune reactivity can only occur when migration and homing of regulatory T cells run parallel to that of donor-reactive cells, so that these cells can localize together at the sites of immune activity.

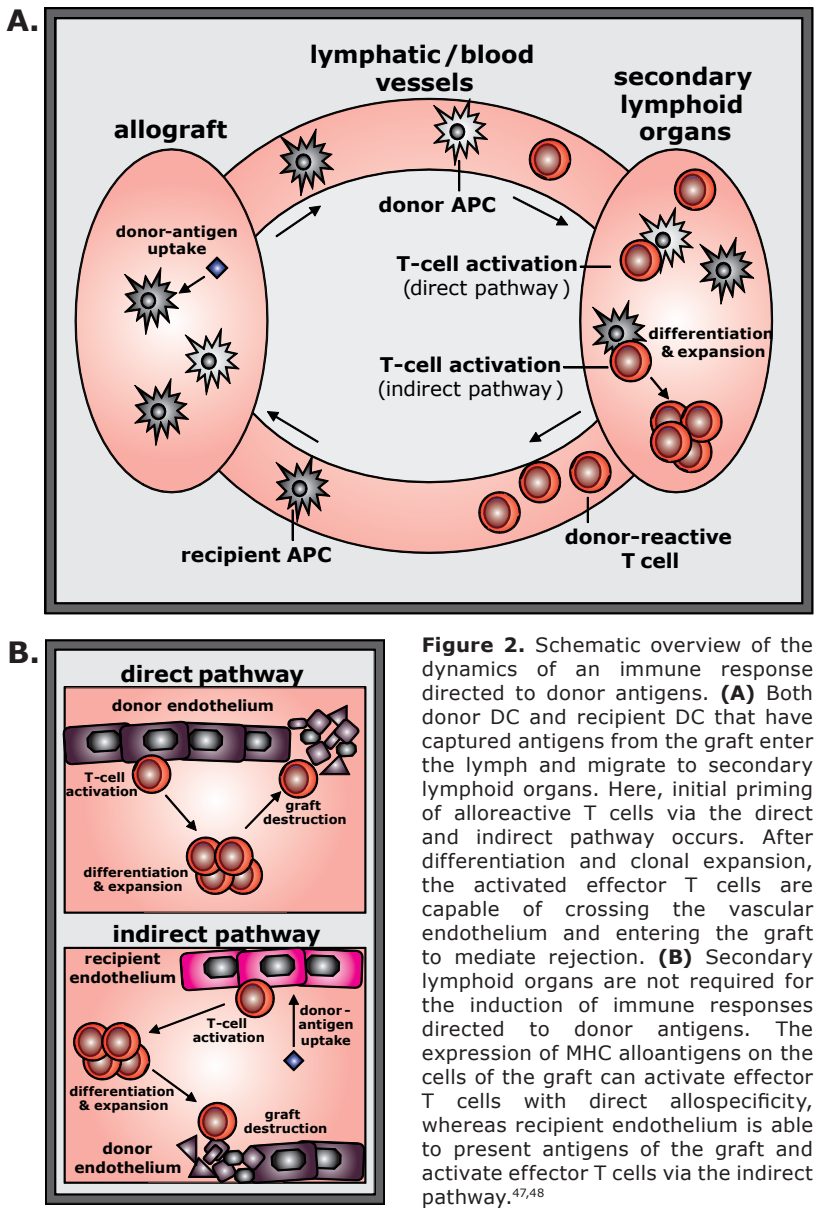

Figure 2. Schematic overview of the dynamics of an immune response directed to donor antigens. **(A)** Both donor DC and recipient DC that have captured antigens from the graft enter the lymph and migrate to secondary lymphoid organs. Here, initial priming of alloreactive T cells via the direct and indirect pathway occurs. After differentiation and clonal expansion, the activated effector T cells are capable of crossing the vascular endothelium and entering the graft to mediate rejection. **(B)** Secondary lymphoid organs are not required for the induction of immune responses directed to donor antigens. The expression of MHC alloantigens on the cells of the graft can activate effector T cells with direct allospecificity, whereas recipient endothelium is able to present antigens of the graft and activate effector T cells via the indirect pathway.[47,48]

The dynamics of donor-reactive cells are schematically depicted in Figure 2A. T cells can be sensitized against alloantigens via one of two distinct, but not mutually exclusive, pathways: direct and indirect.[42] Direct recognition requires that the recipient T cells recognize intact donor major histocompatibility complex (MHC) molecules complexed with peptide on donor APC; indirect recognition requires that the recipient APC process donor antigens before presenting it in a self-restricted manner. Both donor DC and recipient DC that have captured antigens from the graft enter the lymph and migrate to secondary lymphoid organs.[43] Here, initial priming of alloreactive T cells, that continuously circulate between blood and the secondary lymphoid organs, occurs.[44,45] After differentiation and clonal expansion, the activated effector T cells are capable of crossing the vascular endothelium and entering the graft to mediate rejection. So, secondary lymphoid organs are pivotal compartments for the induction of an immune response directed to donor antigens, and should therefore be important sites of action for regulatory T cells.

Zhou et al., however, showed that secondary lymphoid organs are not absolutely required for allograft responses, as skin and cardiac allograft rejection occurred in splenectomized mice that lack lymph nodes and Peyer's patches.[46] Kreisel et al. demonstrated that the expression of MHC alloantigens on the cells of the graft were sufficient to activate cytotoxic $CD8^+$ T cells with direct allospecificity and triggered acute cardiac allograft rejection.[47] In addition, the group of Heeger showed that recipient endothelium was able to present antigens of skin allograft in the context of recipient MHC class I molecules to $CD8^+$ T cells, and this indirect CD8 pathway could result in skin graft rejection.[48] Thus, immunological events capable of leading to allograft rejection may also occur at other compartments than the secondary lymphoid organs (Figure 2B). Regulatory T cells should therefore be able to home and suppress anti-donor immune responses not only in the secondary lymphoid organs, but also at other sites in the transplant recipient, such as the transplanted organ itself. The next section considers the different compartments in which regulatory T cells may localize to exert their suppressive function on immune responses directed to donor antigens.

Secondary lymphoid organs

To inhibit the priming of naive alloreactive T cells, regulatory T cells should be able to home into the secondary lymphoid organs. Indeed, $CD4^+CD25^+$ regulatory T cells express CD62L, a cell adhesion molecule required for constitutive migration of lymphocytes through secondary lymphoid organs, and CCR7, a chemokine that mediates homing into the secondary lymphoid tissues.[13] Moreover, it has been shown that homing of $CD4^+CD25^+$ regulatory T cells into the lymph nodes is required for the suppressive function of these cells: Schneider et al. demonstrated that $CD4^+CD25^+$ regulatory T cells of CCR7 KO mice were inefficient in homing to lymph nodes and did not inhibit the antigen-induced activation of T cells.[49] In addition, experiments in nonobese diabetic mice showed that only

regulatory T cells able to home to and proliferate in the draining lymph nodes were able to reverse diabetes.[50]

In humans, phenotypical characterization and functional analyses of lymphocytes isolated from lymph nodes revealed that these secondary lymphoid organs contain sizeable amounts of $CD4^+CD25^+$ regulatory T cells.[51] Moreover, studies in tumor-draining lymph nodes demonstrated that the number and suppressive activity of $CD4^+CD25^+$ regulatory T cells were increased in these compartments and these cells protected the tumor from tumor-directed immune responses.[52] In addition, Filaci et al. detected $CD8^+CD28^-$ suppressor T cells that were able to inhibit both T-cell proliferation and cytotoxicity in tumor-draining lymph nodes.[53]

Thus, the specific homing of regulatory T cells into secondary lymphoid tissues reflects features that are mandatory for effective suppression early during an immune response. Do these homing regulators, however, have the capacity to inhibit anti-donor immune responses? In experimental transplant models, Hara et al. showed that $CD4^+CD25^+$ regulatory T cells harvested from secondary lymphoid tissues (i.e., lymph nodes and spleen), prevented skin allograft rejection *in vivo*.[54] In addition, Lee et al. demonstrated that $CD4^+CD25^+$ regulatory T cells harvested from lymph nodes inhibited proliferation of graft-specific effector T cells *in vivo*, prolonging the survival of skin allografts.[55] Moreover, regulatory T cells that had not encountered the graft itself were capable of suppressing graft-specific effector T cells in the draining lymph nodes, suggesting that the lymph nodes are an independent site of suppression of the allograft response.[56] A study published by Ochando et al. stated that homing to lymph nodes is required for tolerance induction to alloantigens, as tolerance could not be induced in CD62L -/- mice.[57] Furthermore, $CD4^+CD25^+Foxp3^+CD62L^+$ regulatory T cells accumulated and expanded *in vivo* in the lymph nodes of tolerant animals. This localization was prevented by anti-CD62L monoclonal antibodies (mAb), and rejection rapidly ensued.[58] These observations suggest that regulatory T-cell occupancy of the lymph nodes is an important feature in the control of anti-donor immune reactivity.

Yet, the role of other secondary lymphoid tissues, such as the spleen, in allogeneic immune responses remains to be defined. Saiki et al. found that donor APC migrate preferentially to the spleen, whereas they were absent in lymph nodes.[43] In the spleen, these donor APC can interact with allogeneic effector T cells by the direct pathway, and induce their activation leading to acute rejection.[59] Thus, to actively suppress these immune responses, the spleen should also be an important site of action for regulatory T cells.

The studies about the suppression of immune responses directed to donor antigens in secondary lymphoid tissues, however, were all performed in experimental transplant models. In human organ transplantation, the role of the secondary lymphoid organs in the control of allogeneic immune reactivity is still unknown, as these tissues cannot be analyzed.

Peripheral blood

After activation and differentiation, effector T cells migrate from the secondary lymphoid tissues via the blood system to the site of inflammation, which, in the case of organ transplantation, is the transplanted allograft. From the viewpoint of immune tolerance, it would be beneficial for regulatory T cells to have the same migration features as those of donor-reactive effector T cells to exactly follow them from the secondary lymphoid tissues in the peripheral blood to the graft. In 2001, the presence of $CD4^+CD25^+$ regulatory T cells was described in the peripheral blood of healthy human individuals.[60,61] These peripheral $CD4^+CD25^+$ regulatory T cells display a broad usage of the T-cell receptor Vβ repetoire, indicating that they recognize a wide variety of antigens.[62]

Studies in human organ transplantation have investigated the number of peripheral regulators in relation to immunological quiescence (stable patients on immunosuppression), tolerance (stable patients off immunosuppression), and acute or chronic rejection. Some studies found an association between the number of peripheral regulatory T cells and the occurence of acute rejection: Demirkiran et al. described that liver transplant patients who experienced an acute rejection episode had a lower number of $CD4^+CD25^+$ T cells in the peripheral blood than patients who remained free from rejections.[63] Vlad et al. analyzed the messenger RNA (mRNA) expression levels of FOXP3 in T-cell populations to assess the presence of peripheral regulatory T cells in rejecting and stable heart transplant patients on immunosuppression.[64] They found a lower expression of FOXP3 in the $CD4^+$ T-cell population during rejection compared with those during immunological quiescence. A relationship between the frequency of peripheral $CD4^+CD25^+$ regulatory T cells and chronic rejection has been described in both lung and kidney transplant patients: patients with a chronic rejection had a lower number of these cells than stable patients on immunosuppression.[65,66] Interestingly, no differences were observed between stable patients on immunosuppression, those off immunosuppression and healthy individuals.

Besides the frequency of regulatory T cells in the peripheral blood, another parameter that may contribute in determining transplant outcome is the suppressive potency of peripheral $CD4^+CD25^+$ regulatory T cells. Our group analyzed the *in vitro* suppressive function of peripheral $CD4^+CD25^+$ regulatory T cells in kidney transplant patients shortly after transplantation. We observed that depletion of these cells from the peripheral blood mononuclear cells (PBMC) increased the proliferative response directed to donor antigens and the IL-2 production significantly, indicating that these cells suppressed the anti-donor immune reactivity.[67] Our findings are in line with Bestard et al., who demonstrated in kidney transplant patients that depletion of $CD4^+CD25^+$ T cells significantly recovered the anti-donor cytokine production of donor-specific hyporesponders.[68]

Functional $CD4^+CD25^+$ regulatory T cells specific for the direct pathway were also detected in clinically stable immunosuppressed kidney transplant patients some years after transplantation. These cells were at least partially responsible for the nonreactivity toward donor antigens.[69] These findings are supported by a study of Akl et al.[70] Game et al., however, detected no regulation of the direct immune reactivity by peripheral $CD4^+CD25^+$ regulatory T cells in stable kidney transplant patients some years after transplantation, and suggested that these cells rather regulate immune responses initiated by the indirect pathway.[71] A peripheral $CD25^+$ regulatory T-cell population that suppressed the indirect anti-donor immune response was indeed found in clinically stable kidney transplant patients.[72]

In patients with chronic rejection, the findings of the immune regulatory function of $CD4^+CD25^+$ regulatory T cells are contradictory: Akl et al. detected no regulatory activities of these cells in samples taken from chronic rejecting kidney transplant patients, whereas Braudeau et al. observed no differences in the suppressive capacity of peripheral $CD4^+CD25^+$ regulatory T cells from these patients compared with those from clinically stable kidney transplant patients.[70,73] This controversy, however, may be explained by the different stimuli used for the assays: Akl et al. analyzed the suppressive capacity of $CD4^+CD25^+$ T cells on the proliferative response directed to alloantigens, whereas Braudeau et al. examined the suppression of anti-CD3 stimulation. So far, no study has examined the immune regulatory function of $CD4^+CD25^+$ T cells in patients experiencing acute allograft rejection.

The presence of a $CD8^+CD28^-$ suppressor T-cell population in the peripheral blood of heart, kidney and liver transplant patients was described by the group of Suciu-Foca.[74] Moreover, they observed an inverse correlation between the presence of peripheral suppressor T cells and the incidence of acute cellular rejection in these patients. In addition, Sindhi et al. showed in pediatric transplant patients that $CD8^+CD28^-$ suppressor T cells were present in patients in whom immunosuppression was successfully discontinued, whereas these cells were absent in three of the four patients with acute cellular rejection.[75] Thus, the presence of these suppressor cells in the circulation seems to be associated with a good graft outcome. One study examined the immune regulatory function of $CD8^+CD28^-$ suppressor T cells in heart transplant patients. Circulating $CD8^+CD28^-$ T cells isolated from patients who did not experience any acute rejection episodes inhibited CD40L-induced upregulation of costimulatory molecules and enhanced the expression of the inhibitory receptors ILT3 and ILT4 on donor APC and, consequently, suppressed T-cell allorecognition via the direct pathway.[36] In contrast, $CD8^+CD28^-$ T cells from patients with at least one episode of acute rejection did not have this effect.

Transplanted organ

The main target of alloantigen-activated T cells is the transplanted organ, so as to eliminate the foreign antigens. Our group demonstrated in heart transplant patients that cytotoxic $CD8^+$ and $CD4^+$ T cells with high avidity for donor antigens home in the graft to induce transplant rejection.[76,77] For effector-cell recruitment, experimental data point to the chemokine receptors CXCR3 and CCR5, which are induced upon activation of T cells, as key mediators in response to the local elaboration of chemokines by graft endothelial and parenchymal cells.[78] Interestingly, lymphocytes may not only be attracted into the graft by inflammatory chemokines. Kerjaschki et al. observed in kidney transplant patients that the occurrence of massive lymphatic neoangiogenesis was associated with allograft rejection.[79] These investigators suggested that the lymphatic endothelial cells actively recruit lymphocytes, presumably by the production of the homeostatic chemokine CCL21, which attracts $CCR7^+$ cells.

We analyzed chemokine and chemokine receptor gene expression in cardiac allograft biopsy specimens from a 9-month follow-up of 21 patients after heart transplantation. The gene expression levels indicated that, apart from a strong association of intragraft levels of inflammatory chemokines (CCL5 and CXCL10) and their corresponding receptors (CCR5 and CXCR3) with the appearance of acute rejection, also levels of the homeostatic chemokine CCL19 and its receptor CCR7 were significantly elevated during acute cellular rejection (Figure 3A-B). These findings suggest that the chemokine microenvironment in the graft during acute rejection consist not only of inflammatory chemokines but also of homeostatic ones. So, besides recruiting effector T cells activated in the secondary lymphoid tissues, this microenvironment can also attract naive T cells and DC, which may lead to intragraft DC maturation and T-cell activation. In addition, the graft itself may also activate these infiltrating T cells by the expression of MHC alloantigens.[47,80]

To effectively control aggressive intragraft anti-donor immune reactivity, it is thus important that regulatory T cells are able to home in the transplanted organ. Indeed, studies in experimental mouse models have demonstrated that $Foxp3^+$ regulatory T cells accumulate in the transplant allograft.[81] Moreover, this accumulation was observed in tolerized skin and cardiac grafts, indicating that the presence of intragraft $Foxp3^+$ regulatory T cells is associated with the induction of tolerance.[82,83] Yang et al., however, measured higher levels of Foxp3 mRNA expression in islet allografts undergoing acute rejection than in allografts that were not acutely rejected, suggesting that $Foxp3^+$ regulatory cells may also be an integral component of acute allograft rejection.[84] The recruitment of the $Foxp3^+$ cells in the tolerized grafts was related to high intragraft mRNA expression of CCR4 and its ligand MDC, and not to CCR5 or CXCR3.[83] In contrast, in rejecting grafts, high Foxp3 mRNA expression was associated with high intragraft mRNA levels of CXCR3 and the chemokines CXCL10 and CCL5.[84] Thus regulatory T cells

may be attracted to the graft by different chemokine profiles depending on the intragraft immunological processes.

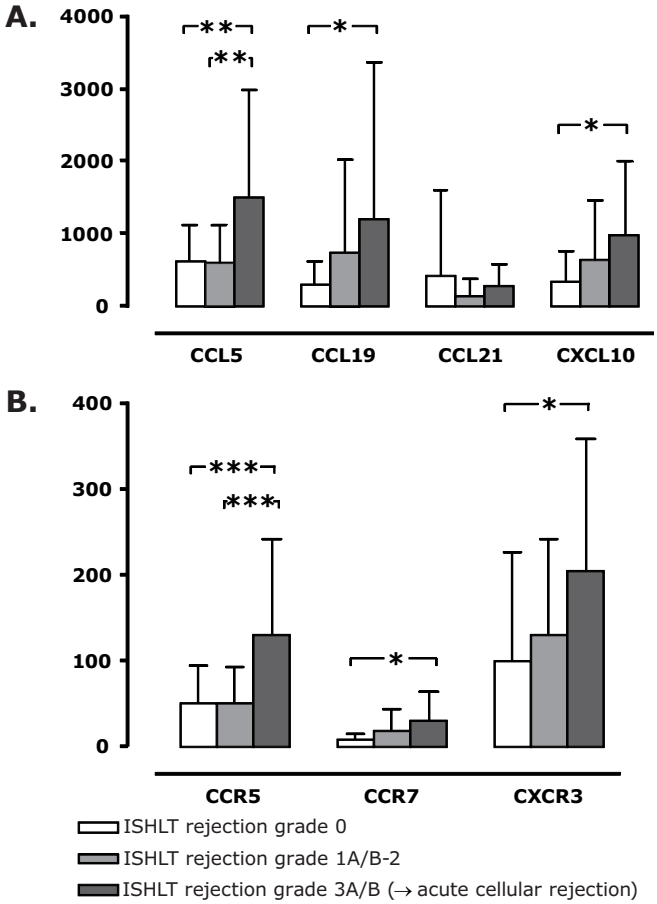

Figure 3. Gene expression levels in cardiac allograft biopsy specimens. **(A)** Chemokine and **(B)** chemokine receptor gene expression levels were compared with pathological grades of rejection of the 1990 International Society for Heart and Lung Transplantation (ISHLT) grading system[90] in 83 endomyocardial biopsies collected in the first 9 months posttransplantation from 21 heart transplant recipients (ISHLT 0, n=42; ISHLT 1A/B-2, n=28; ISHLT 3A/B, n=13). According to the definition of rejection, only patients with ISHLT rejection grade >2 were considered to have a clinically relevant acute cellular rejection (AR+) necessitating therapy. The expression levels of the inflammatory chemokine CCL5 as well as its corresponding receptor CCR5 were significantly higher in biopsy samples with histological signs of AR+ than in those with ISHLT rejection grades 0 and 1A/B-2 (one-way analysis of variance (ANOVA), $p<0.001$ and $p=0.001$, respectively). The same was observed for CXCL10 and its receptor CXCR3 when compared with grade 0 (one-way ANOVA, $p<0.001$ and $p=0.047$, respectively). With regard to homeostatic chemokines, greater expression of CCL19 and its receptor CCR7 were observed during AR+ compared with grade 0 (one-way ANOVA, $p=0.03$ and $p=0.02$, respectively). Expression levels of the other CCR7 ligand (CCL21) were not significantly different between any ISHLT rejection grades. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ represent the outcome of the Tukey multiple comparisons test.

The suggestion by Yang et al. that graft-infiltrating lymphocytes during acute rejection may also comprise cells with an immune regulatory function seems to hold for human organ transplantation. We recently demonstrated in heart transplant patients that the FOXP3 mRNA expression was highly expressed in endomyocardial biopsies (EMB) taken during an acute cellular rejection compared to those preceding this rejection.[85] These results are in line with Veronese et al., who found high FOXP3 mRNA levels in biopsies taken during acute cellular rejection after clinical kidney transplantation.[86] In addition, Muthukumar et al. observed higher FOXP3 mRNA expression levels in the urine of kidney transplant patients with an acute rejection than in that of patients with normal biopsy results.[87] Moreover, very high levels of FOXP3 during rejection was related with successful reversal of acute rejection, suggesting that the $FOXP3^+$ cells in the graft may be involved in "damage control". This was supported by findings of Strehlau et al.[88]

The studies in human organ transplantation demonstrated that $FOXP3^+$ T cells are present in the transplanted organ, suggesting a role for regulatory T cells in the control of immune responses directed to the donor graft. Some reports, however, indicated that FOXP3 might not always be associated to regulatory T-cell function, as FOXP3 can also be induced in activated $CD25^-$ cells.[18] Thus it cannot be excluded that the high FOXP3 mRNA levels found in the transplanted organ are partially caused by activated effector T cells. Besides, even if the increased FOXP3 mRNA expression were caused by the accumulation of regulatory T cells, this would not automatically mean that these cells would have the potential to suppress the anti-donor immune response. Other factors, such as the cytokine environment in the graft, may be of considerable importance.

Thus, to determine whether the transplanted organ is a site where anti-donor immune reactivity can be controlled, it is important to investigate if regulatory T cells that are present in the graft are able to suppress anti-donor immune responses. Therefore, functional analyses of the $FoxP3^+$ T cells are necessary. Recently, Eljaafari et al. showed that expanded skin-infiltrating lymphocytes isolated from a well-accepted human hand transplant were unresponsive to donor-antigens and inhibited donor-directed blood T-cell alloresponses.[89] This indicates that cells with the potential to suppress anti-donor immune responses are present in the graft of a stable hand transplant recipient. Yet, whether functional regulatory T cells are present in the graft during allograft rejection remains to be elucidated.

Aim and outline of this thesis

Although much has been written about the involvement of regulatory T cells in the control of alloreactivity in experimental transplant models, knowledge about the clinically relevant role of these cells in immunological processes, such as immunological quiescence and rejection, is still limited. Furthermore, the compartments where these cells interact with allogeneic effector T cells and APC are unclear. The central aim of the research described in this thesis is to investigate the role of regulatory T cells in the control of immune responses directed to the graft of heart transplant recipients. For this research, we focused on two compartments where functional regulatory T cells may be present, i.e. the transplanted heart and the peripheral blood.

Part II - Regulatory T cells in the transplanted heart

Part II of this thesis covers our studies on the presence of regulatory T cells in the transplanted heart. In **Chapter two** we address the question whether the presence of FOXP3 mRNA-expressing cells is associated with immunological processes, such as immunological quiescence and acute cellular rejection. **Chapter three** describes the expansion of FoxP3 protein-expressing lymphocytes from EMB with histological signs of acute cellular rejection. Subsequently, we investigated whether lymphocytes present in the graft during cardiac allograft rejection exhibit immune regulatory activities, of which the results are described in **Chapter four**.

Part III - Regulatory T cells in the peripheral blood

In **Part III** of this thesis we outline our research on the frequency and function of peripheral regulatory T cells in heart transplant patients. In **Chapter five** we discuss whether FOXP3 mRNA expression levels in PBMC correlate with the immunological processes in the graft. **Chapter six** describes the phenotypical characteristics and immune regulatory function of peripheral $CD4^{+}CD25^{bright+}$ T cells of heart transplant recipients who remain free from acute rejection and of those who experience an acute cellular rejection episode. Finally, in **Chapter seven** we focus on the donor-specific proliferative and cytotoxic T-cell responses and the mRNA expression levels of FOXP3, GITR and IL-10 of PBMC derived from patients some years after heart transplantation.

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Regulatory T cells in the transplanted heart

Part II

Chapter 2

Intragraft FOXP3 mRNA expression reflects anti-donor immune reactivity in heart transplant patients

Dijke IE, Velthuis JHL, Caliskan K, Korevaar SS, Maat APWM, Zondervan PE, Balk AHMM, Weimar W, Baan CC.

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Abstract

Regulatory FOXP3$^+$ T cells control immune responses of effector T cells. However, whether these cells regulate anti-donor responses in the graft of heart transplant recipients is unknown. Therefore, we investigated the gene expression profiles of regulatory and effector T-cell markers during immunological quiescence and acute rejection. Quantitative real-time PCR was used to analyze messenger RNA (mRNA) expression levels in time-zero specimens (n=24) and endomyocardial biopsies (EMB; n=72) of heart transplant patients who remained free from rejection (nonrejectors; n=12) and patients with at least one histologically proven acute rejection episode (rejectors; International Society for Heart and Lung Transplantation (ISHLT) rejection grade >2; n=12). For all analyzed regulatory and effector T-cell markers, mRNA expression levels were increased in biopsies taken after heart transplantation compared with those in time-zero specimens. Posttransplantation, the FOXP3 mRNA levels were higher in EMB assigned to a higher ISHLT rejection grade than the biopsies with grade 0: the highest mRNA levels were detected in the rejection biopsies (rejection grade >2; p=0.003). In addition, the mRNA levels of CD25, glucocorticoid-induced tumor necrosis factor (TNF) receptor family-related gene (GITR), cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), interleukin (IL)-2 and Granzyme B were also significantly higher in rejection EMB than in nonrejection EMB (rejection grade ≤2). This increase in expression levels in relation to the histological rejection grade was only observed in patients who developed an acute rejection episode; the mRNA levels of nonrejectors remained stable irrespective of ISHLT rejection grade. These observations suggest that, after clinical heart transplantation, FOXP3$^+$ T cells do not prevent acute rejection, but rather are a response to anti-donor effector T-cell activity.

Introduction

Regulatory T cells comprise several T-cell populations that control immune responses to self and nonself antigens. Attention has focused in particular on a specific subset of $CD4^+$ T cells that constitutively expresses the interleukin (IL)-2 receptor α chain (CD25) and the transcription factor FOXP3.[1,2] These $CD4^+CD25^+$ regulatory T cells (Treg) can be divided in the naturally occurring Treg derived from the thymus and in Treg that develop from activation and differentiation of peripheral $CD25^-$ T cells, and have been extensively studied for their roles in autoimmunity and tolerance.[3-5]

The mechanisms by which both naturally occurring and adaptive Treg mediate their function involve a discretionary requirement of the immunoregulatory cytokines IL-10 and transforming growth factor (TGF)-β and depend on cell-cell contact, possibly by signals through the CD28 homolog cytotoxic T lymphocyte-associated antigen 4 (CTLA-4 or CD152) as well as regulation coordinated by the glucocorticoid-induced tumor necrosis factor (TNF) receptor family-related gene (GITR or TNFRSF18).[6-8] None of these molecules, however, are exclusively expressed by $CD4^+CD25^+$ Treg as activated $CD4^+CD25^-$ T cells also express CD25, CTLA-4 and GITR, albeit at different levels than regulatory T cells.[8] In contrast, the expression of the transcription factor FOXP3 has been claimed to be a unique marker for regulatory T cells. FOXP3 is the key regulatory transcription factor for the development and function of cells with immunoregulatory activities.[2,9] FOXP3 interacts with the nuclear factor of activated T cells (NFAT) and NF-κB, two key transcription factors, to inhibit the expression of cytokine genes and, consequently, T-cell effector function.[10] The specificity of FOXP3 was proven by *ex vivo* gene transfer of FOXP3 into $CD4^+CD25^-CD45RO^-$ T cells, which converted these cells into regulatory T cells.[11]

In experimental transplantation, regulatory T cells are considered to play an important role in the induction of tolerance towards the transplanted allograft. In a number of different experimental animal models it has been demonstrated that the presence of $CD4^+CD25^+$ regulatory T cells was crucial for the prevention of acute allograft rejection and the maintenance of tolerance.[7,12,13] Furthermore, Nishimura et al. showed that permanent graft tolerance can be achieved by *in vivo* expansion and subsequent adoptive transfer of antigen-specific $CD4^+CD25^+$ regulatory T cells.[14] Other studies investigated whether regulatory T cells were present in the transplanted allograft and had the capacity to suppress anti-donor responses locally. The group of Waldmann showed that T-cell-mediated suppression of graft rejection is an active process and involves the persistent presence of regulatory T cells at the site of the tolerated transplant.[15] Furthermore, they found high levels of FOXP3 mRNA and $GITR^+CD25^+$ T cells within the tolerated skin grafts of long-term tolerant mice.[16] In addition, Lee et al. showed that FOXP3 mRNA expression was specifically upregulated within allografts of mice displaying donor-specific tolerance.[17]

Yet, it is unclear whether regulatory T cells are also present within the transplanted organ of human recipients to control immune responses directed at the donor allograft and contribute to graft acceptance. Unlike the experimental mice models in which long-term tolerance is induced by costimulation blockade or donor-specific transfusion (DST) protocols, human transplant recipients need life-long immunosuppressive treatment to prevent graft rejection. These immunosuppressive drugs may affect the development and function of regulatory FOXP3$^+$ T cells.[18]

To investigate whether regulatory FOXP3$^+$ T cells are involved in the immunological processes of quiescence in the graft of transplant patients biopsy specimens are required. Therefore, the best human model in which graft-infiltrating FOXP3$^+$ T cells may be analyzed are heart transplant recipients, as EMB are standard protocol after clinical heart transplantation. Previously, we demonstrated that FOXP3 mRNA levels were highly expressed in EMB taken during a histologically proven acute rejection compared with the levels in EMB preceding the rejection.[19] However, the involvement of FOXP3 expressing cells in anti-donor immune responses in patients who do not reject their allograft is unknown. Hence, we analyzed the gene expression profiles of regulatory T-cell (associated) markers, activation markers, effector T-cell markers as well as several cytokines in EMB taken from both heart transplant patients who remained free from rejection and those who experienced at least one rejection episode.

Materials and methods

Study group

Time-zero specimens and posttransplant EMB from 24 heart transplant patients transplanted between January 1999 and September 2004 were studied for gene expression of regulatory T-cell molecules. The biopsy specimens were obtained from each patient after informed consent and approval by the local medical ethical committee. The demographics of the patients are summarized in Table 1. All patients received induction therapy (anti-thymocyte globulin (ATG): 23/24 and OKT3: 1/24) for 5 to 7 days and triple maintenance immunosuppressive therapy consisting of cyclosporine A (CsA) or tacrolimus (Tacro), prednisone (Pred) and mycophenolate mofetil (MMF). MMF was withdrawn from one patient approximately one month prior to analysis. Cytomegalovirus (CMV) and toxoplasmosis seronegative recipient/seropositive donor combinations received prophylaxis for 3 months. CMV disease, as manifested by symptoms and a positive PCR, was treated with ganciclovir for 10 days.

Table 1. Demographics of the studied patients

Patient	Age at transplant	Gender	Primary disease	Ischemia time (min)	Maintenance immunosuppressive therapy	AR+ at 1 year posttransplant	Number of studied EMB
Nonrejectors							
1	48	M	IHD	160	CsA-MMF-Pred	0	4
2	60	M	IHD	176	Tacro-MMF-Pred	0	4
3	17	F	HCM	138	CsA-MMF-Pred	0	4
4	28	M	DCM	213	Tacro-MMF-Pred	0	4
5	66	M	IHD	191	CsA-MMF-Pred	0	4
6	15	M	DCM	153	CsA-MMF-Pred	0	4
7	68	M	IHD	195	CsA-MMF-Pred	0	4
8	71	M	IHD	95	CsA-MMF-Pred	0	4
9	65	M	IHD	173	CsA-MMF-Pred	0	3
10	38	M	DCM	190	CsA-MMF-Pred	0	4
11	55	M	DCM	168	CsA-MMF-Pred	0	3
12	64	F	DCM	260	CsA-MMF-Pred	0	5
Rejectors							
13	54	M	IHD	251	CsA-MMF-Pred	1	3
14	33	F	CHF	167	CsA-MMF-Pred	1	1
15	58	M	IHD	165	CsA-MMF-Pred	2	1
16	56	F	IHD	252	CsA-MMF-Pred	1	3
17	47	F	GCM	90	Tacro-MMF-Pred	2	2
18	18	M	DCM	174	CsA-MMF-Pred	1	2
19	57	M	DCM	197	CsA-MMF-Pred	2	3
20	53	F	DCM	NA	Tacro-Pred	1	2
21	57	F	DCM	97	CsA-MMF-Pred	1	2
22	47	M	DCM	206	CsA-MMF-Pred	1	2
23	58	M	IHD	159	CsA-MMF-Pred	2	2
24	39	F	DCM	139	CsA-MMF-Pred	1	2
Total							72

Acute rejection was defined as ISHLT rejection grade >2.[20] IHD, ischemic heart disease; HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; CHF, congenital heart failure; GCM, giant-cell myocarditis; NA, not available; CsA, cyclosporine A; MMF, mycophenolate mofetil; Pred, prednisone; Tacro, tacrolimus; EMB, endomyocardial biopsies.

Sample collection and processing

Endomyocardial biopsies

EMB taken within the first 8 months posttransplantation were studied. In this period, biopsies were performed weekly during the first 6 weeks, biweekly during the next month, monthly for the following 2 months, and every 6 weeks for the next 3 months. Biopsies were repeated more frequently after a rejection episode. Rejection was histologically diagnosed in surveillance EMB according to the 1990 International Society for Heart and Lung Transplantation (ISHLT) grading system by a pathologist who was blinded to the results of the molecular studies.[20] According to the definition of rejection, only patients with ISHLT rejection grade >2 were considered to experience acute cellular rejection. Upon biopsy-proven rejection episodes, patients received anti-rejection therapy consisting of 1 g methylprednisolone on three consecutive days.

Eight patients underwent one acute rejection (AR+) and four patients underwent two acute rejections within the first year posttransplantation (Table 1); we analyzed the first AR+ in three of these patients and the second AR+ in one of them, as no biopsy specimens of the first AR+ were available for mRNA analysis. All the investigated acute rejection episodes occurred within the first 6 months after transplantation (median: 44 days, range: 15–180 days). Of each rejector, EMB before AR+ and during AR+ (the first EMB of the AR+ episode) were studied. In 12 patients no histological signs of acute rejection were observed during the first year posttransplantation (nonrejectors). The analyzed EMB of these patients were matched in time with the EMB from the rejectors. In total, 72 EMB were available for analysis (Table 1). In addition, time-zero specimens sampled from the 24 patients during the transplantation procedure were analyzed.

Quantitative real-time polymerase chain reaction

Messenger RNA extraction from the time-zero specimens and EMB, cDNA transcription and DNA amplification was performed as described before.[19] We used quantitative real-time polymerase chain reaction (Q-PCR) to quantify the amount of CD3ε, FOXP3, CD25, GITR, CTLA-4, IL-2, Granzyme B, IL-10 and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression in the samples. Assay-on-Demand products for the detection and quantification of CD3ε (Hs00167894_m1), FOXP3 (Hs00203958_m1), CD25 (Hs00166229_m1), GITR (Hs00188346_m1), CTLA-4 (Hs00175480_m1), IL-2 (Hs00174114_m1) and IL-10 (Hs00174086_m1) mRNA were designed by Applied Biosystems (Applied Biosystems, Foster City, CA). A 5 μl sample of cDNA was added to 20 μl PCR mixture containing 12.5 μl Universal PCR Master Mix (Applied Biosytems), 0.625 μl of each specific Primer & Probe-on-Demand mix, and 6.875 μl H_2O. The choice of primers and probes for Granzyme B were defined using the primer express software (Applied Biosystems). Nucleotide sequences were

as follows: sense primer: 5' CCC TAC ATG GCT TAT CTT ATG ATC TG 3', anti-sense primer: 5' A ACC CCT TCG AGG TAT TTA CAG 3', probe: 5' TGA GCA GCT GTC AGC ACG AAG TCG T 3'. To determine Granzyme B we added 5 μl cDNA to 20 μl PCR mixture containing 12.5 μl Universal PCR Master Mix, 0.5 μl sense primer (25 pmol), 0.5 μl anti-sense primer (25 pmol), 0.5 μl FAM-labeled probe (5 pmol), and 6 μl H_2O. A Biosource kit (Biosource, Camarillo, CA) was used for the detection and quantification of GAPDH (primers: GHO0106, probe: GHO0105). A 5 μl sample of cDNA was added to 20 μl PCR mixture containing 12.5 μl Universal PCR Master Mix, 2.5 μl sense and anti-sense primers, 0.5 μl FAM-labeled probe and 4.5 μl H_2O.

The PCR reaction was performed after a first step of 2 minutes 50°C and 10 minutes 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C (CD3ε, FOXP3, CD25, GITR, CTLA-4, IL-2, IL-10 and GAPDH) or 58°C (Granzyme B) using the ABI Prism 7700 sequence detector (Applied Biosystems). Each run contained several negative controls (no template), and two positive reference samples to check intra- and inter-assay variations. The same reference samples were used in all the experiments. There were no significant differences in cycle threshold (Ct) values within and between the experiments (all <0.25 Ct). The amount of each target molecule was quantified by measuring the Ct, which was transformed to the number of cDNA copies $[2^{(40-Ct)}]$ on a TaqMan Real-Time PCR system.

The relative concentrations of the analyzed markers were normalized to the relative concentration of the housekeeping gene GAPDH present in each sample and multiplied by 10^3 due to the lower concentration of the target gene compared with the concentration of GAPDH. In two time-zero specimens and seven EMB from eight patients we measured GAPDH mRNA levels that were below the detection limit, and these samples were therefore excluded for analysis.

Statistical analysis

A log transformation was performed on the mRNA levels of all analyzed markers to reduce the positive skew of the distribution. We used the Kruskal-Wallis test to identify differences among >2 groups and the Mann-Whitney test to compare mRNA levels between two groups. The Student's *t* test was used to analyze differences of clinical data; the Mann-Whitney test was performed, when the values of the clinical data deviated from a normal distribution. P values ≤0.05 were considered to be significant. GraphPad Version 4.0 statistical program (San Diego, CA) was applied for analyses and graphics.

Results

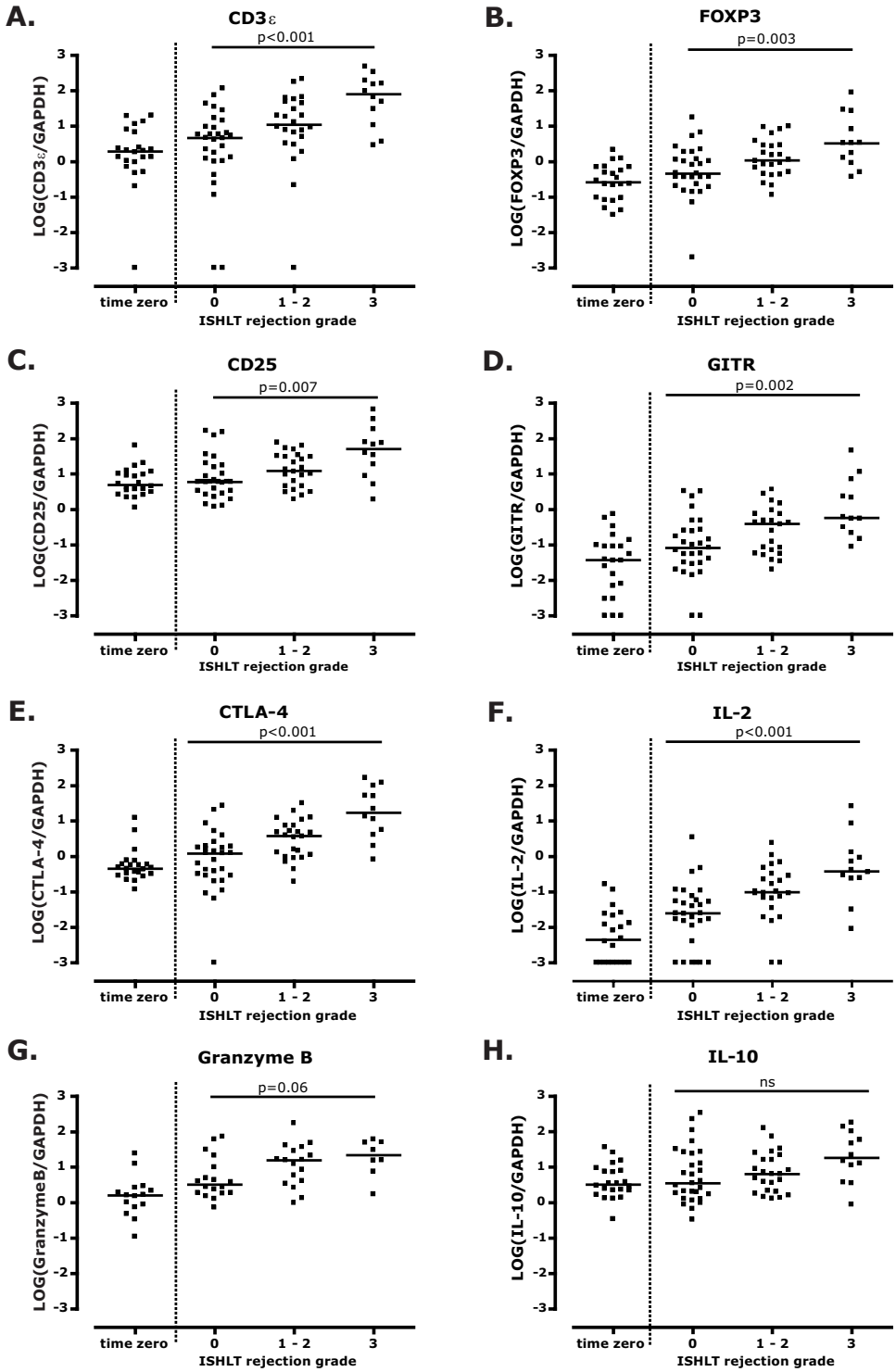
Clinical characteristics

We studied EMB of patients who remained free from rejection (nonrejectors; n=12) and of patients who experienced at least one biopsy-proven acute rejection episode during the first year posttransplantation (rejectors; n=12). The age, gender, primary disease, cold ischemia time and type of immunosuppression were not significantly different between the two groups (Table 1). In addition, no differences were observed for HLA-A/B mismatches (mean±SD for nonrejectors vs. rejectors: 3.2±0.8 vs. 3.3±0.8). In contrast, the rejectors had more HLA-DR mismatches than the nonrejectors (1.9±0.3 vs. 1.4±0.5, p=0.008). Neither the number of CMV or the number of toxoplasmosis seronegative recipient/seropositive donor combinations was significantly different between the nonrejector and rejector group (5/12 vs. 1/12 and 1/12 vs. 3/12, respectively). CMV disease was diagnosed in one patient with histological signs of rejection and in one nonrejecting patient.

Intragraft gene expression levels in relation to histological rejection grades

To examine the gene expression levels in relation to ISHLT rejection grades of the 1990 ISHLT grading system,[20] a total of 87 samples were analyzed: 22 time-zero specimens, 29 EMB with ISHLT rejection grade 0, 24 EMB with rejection grades 1–2 and 12 biopsies with rejection grade 3. The biopsy specimens with grade 1 and grade 2 were grouped, as no differences in mRNA expression levels of the analyzed markers between these EMB were found (data not shown). Moreover, these biopsies are also combined in the new revised 2004 ISHLT rejection grading system.[21] For all analyzed markers, the GAPDH-normalized, log-transformed mRNA levels measured in the time-zero specimens were lower than the levels measured in the biopsy specimens taken after transplantation. Posttransplantation, the mRNA expression levels of CD3ε were higher in the biopsies assigned to a higher ISHLT rejection grade than the biopsies with grade 0: the highest CD3ε mRNA levels were measured in the rejection biopsies ($p<0.001$; Figure 1A). Interestingly, the FOXP3 mRNA levels showed

Figure 1. Gene expression levels in relation to histological rejection grades. The GAPDH-normalized, log-transformed mRNA levels of CD3ε **(A)**, FOXP3 **(B)**, CD25 **(C)**, GITR **(D)**, CTLA-4 **(E)**, IL-2 **(F)**, Granzyme B **(G)** and IL-10 **(H)** measured in 22 time-zero specimens, 29 EMB with ISHLT rejection grade 0, 24 EMB with grades 1–2 and 12 biopsies with grade 3 are depicted.[20] The levels of CD3ε, FOXP3, CD25, GITR, CTLA-4, IL-2 and Granzyme B were elevated in the biopsies taken during an acute rejection episode (ISHLT rejection grade 3) compared with nonrejection EMB (ISHLT rejection grade ≤2). The mRNA levels of IL-10 were not differently expressed between any rejection grades. P values are based on the Kruskal-Wallis test. Due to shortage of material, the mRNA levels of Granzyme B could not be tested in 29 biopsies of eight heart transplant patients.


A.
CD3ε
p<0.001
LOG(CD3ε/GAPDH)
B.
FOXP3
p=0.003
LOG(FOXP3/GAPDH)
C.
CD25
p=0.007
LOG(CD25/GAPDH)
D.
GITR
p=0.002
LOG(GITR/GAPDH)
E.
CTLA-4
p<0.001
LOG(CTLA-4/GAPDH)
F.
IL-2
p<0.001
LOG(IL-2/GAPDH)
G.
Granzyme B
p=0.06
LOG(GranzymeB/GAPDH)
H.
IL-10
ns
LOG(IL-10/GAPDH)
time zero
0
1 - 2
3
ISHLT rejection grade

a similar pattern: not the ISHLT rejection grade 0 or grades 1-2, but the ISHLT rejection grade 3 expressed the highest FOXP3 mRNA levels (p=0.003; Figure 1B). In addition, the mRNA levels of CD25 (p=0.007), GITR (p=0.002), CTLA-4 (p<0.001), IL-2 (p<0.001) and Granzyme B (p=0.06) were higher in the rejection biopsies than the nonrejection biopsies (Figure 1C-G). The mRNA levels of IL-10 were not differently expressed between any rejection grades (Figure 1H).

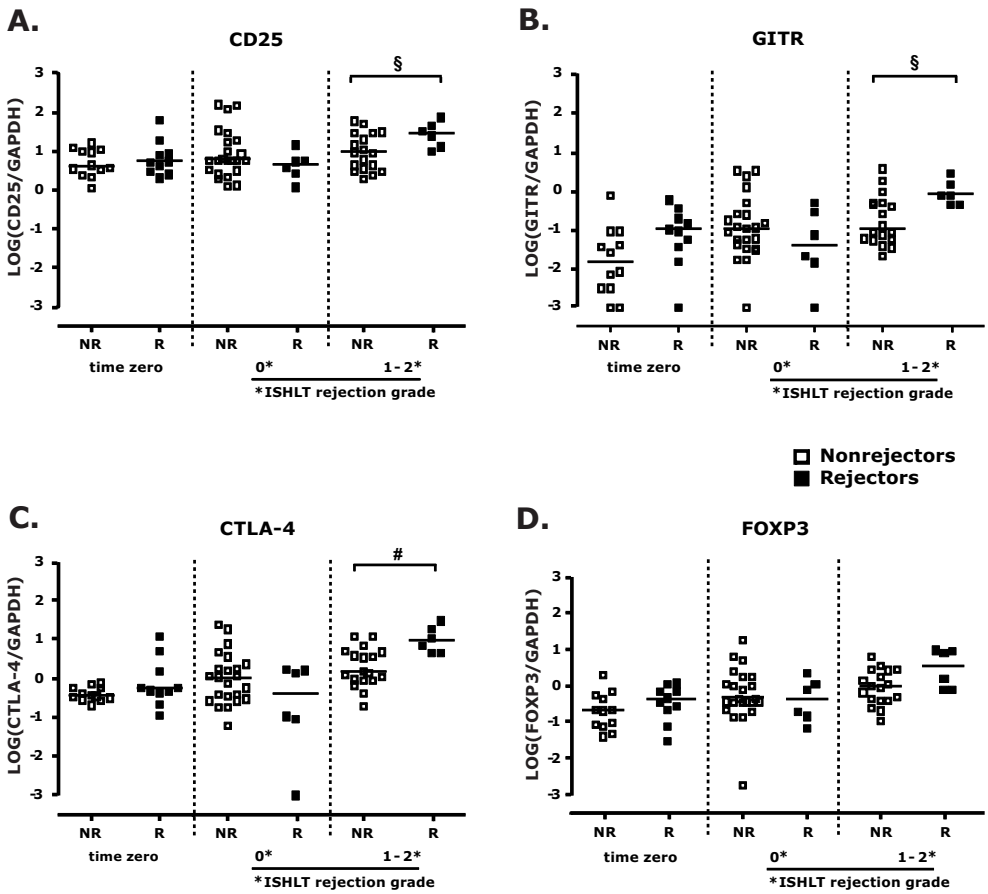


Figure 2. Differential expression levels between nonrejectors and rejectors. Comparison of the GAPDH-normalized, log-transformed mRNA expression levels of CD25 **(A)**, GITR **(B)**, CTLA-4 **(C)** and FOXP3 **(D)** between EMB of nonrejectors (NR; open boxes) and those of rejectors (R; filled boxes) stratified to ISHLT rejection grade.[20] In patients that were going to have an acute rejection, the mRNA levels of CD25, GITR and CTLA-4 were elevated in the EMB with rejection grades 1–2 compared with those of the nonrejectors. § p≤0.05 and # p≤0.01 represent the outcome of the Mann-Whitney test. *Rejection grade of the 1990 ISHLT grading system.

Differential intragraft gene expression levels between nonrejectors and rejectors

To analyze whether patients who remained free from rejection (nonrejectors) had different gene expression levels than patients that were going to have an acute rejection episode (rejectors), we compared the mRNA levels between both groups stratified to ISHLT rejection grade. All time-zero specimens (n=22), EMB with ISHLT rejection grade 0 (n=29) and EMB with grades 1–2 (n=24) of the 24 heart transplant patients were used in this analysis. The biopsies were divided in two groups: the EMB taken from rejectors (n=12) and the EMB from nonrejectors (n=12). Ten of the 22 time-zero specimens, six of the 29 EMB with ISHLT rejection grade 0 and six of the 24 EMB with grades 1-2 were from rejectors; the other biopsies were from nonrejectors. For all analyzed markers, we found no differences in mRNA expression levels in the time-zero specimens and in the EMB with rejection grade 0 between the nonrejectors and the rejectors. However, when we compared the mRNA levels of the EMB with ISHLT rejection grades 1–2 between nonrejectors and rejectors, different expression levels were measured for a number of markers: the mRNA levels of CD25, GITR and CTLA-4 were significantly higher in the biopsies taken from patients that were going to have a rejection than in those from patients free from rejection ($p=0.05$, $p=0.03$ and $p=0.009$, respectively; Figure 2A–C). In addition, the mRNA levels for FOXP3, IL-2 and Granzyme B were elevated in the EMB with grades 1–2 of the rejectors compared with those of nonrejectors, albeit not significantly (Figure 2D and data not shown).

Dynamics of intragraft gene expression are different between the non-rejector and rejector group

Next, we asked whether the dynamics of FOXP3 and the other markers associated with regulatory T cells are different between patients who remained free from rejection and those who developed rejection. Within the nonrejector group, the mRNA expression levels of all analyzed markers were comparable between the EMB with rejection grade 0 and those with grades 1–2 (Figure 2; open boxes). In contrast, within the rejector group, the mRNA expression levels of CD25, GITR and CTLA-4 were significantly higher in the biopsies with rejection grades 1–2 than in those with grade 0 ($p=0.009$, $p=0.009$ and $p=0.002$, respectively; Figure 3A–C). Interestingly, no differences were observed between grade 3 and grades 1–2, suggesting that the mRNA levels for CD25, GITR and CTLA-4 were already maximally expressed in the EMB with ISHLT grades 1–2 before an acute rejection. For FOXP3 mRNA expression, the highest levels were measured in biopsies with ISHLT rejection grade 3 (Figure 3D). This pattern was also observed for Granzyme B and IL-2 (data not shown).

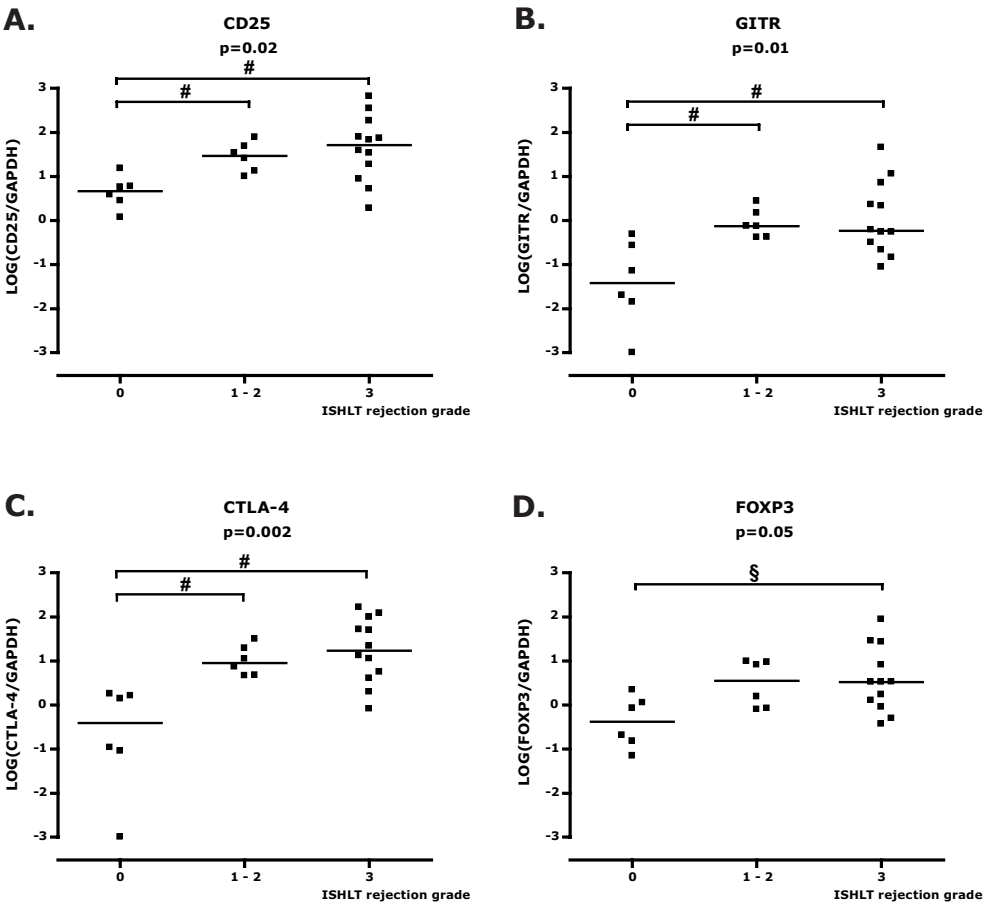


Figure 3. Gene expression patterns within the rejector group. Comparison of the GAPDH-normalized, log-transformed mRNA expression levels of CD25 **(A)**, GITR **(B)**, CTLA-4 **(C)** and FOXP3 **(D)** within the EMB of the rejectors stratified to ISHLT grade.[20] The mRNA levels of CD25, GITR and CTLA-4 were already abundantly expressed in EMB with rejection grades 1-2 taken before an acute rejection. Overall statistical results from Kruskal-Wallis analyses (p) are depicted accordingly; § $p \leq 0.05$ and # $p \leq 0.01$ represent the outcome of the Mann-Whitney test.

Discussion

The role of $FOXP3^+$ regulatory T cells (Treg) in organ transplantation is still under debate. Experimental animal models demonstrated that the presence of donor-specific $CD4^+CD25^+FOXP3^+$ Treg in the graft and the draining lymph nodes was crucial for the prevention of allograft rejection and the maintenance of tolerance.[7,13,15] Steger et al., however, recently showed that the presence of $CD4^+CD25^+$ Treg was not a unique feature of allograft acceptance, as Treg could also be isolated from mice with acutely rejecting cardiac allografts.[22] Previously, we demonstrated that after clinical heart transplantation the FOXP3 mRNA

levels in the transplanted organ were elevated during an acute rejection episode compared with the levels before the rejection and after successful anti-rejection treatment.[19] In the present clinical study, we investigated the FOXP3 mRNA levels in heart transplant patients without any histologically proven myocardial damage in the first year posttransplantation and compared these levels with those measured in rejecting patients. We demonstrated that high intragraft FOXP3 mRNA expression levels of heart transplant recipients were associated with anti-donor immune reactivity and not with immunological quiescence. The highest FOXP3 mRNA expression levels were measured during acute cellular rejection (ISHLT rejection grade >2). In addition, elevated mRNA expression levels of CD25, GITR, CTLA-4, Granzyme B and of the cytokine IL-2 were found in rejection biopsies compared with nonrejection biopsies.

As high intragraft FOXP3 mRNA levels in experimental mouse models were associated with graft acceptance,[16,17] it may be considered paradoxical that in patients high FOXP3 mRNA levels are especially found in the graft during acute rejection. However, recent reports describing how FOXP3 gene transcription is mediated may clarify why increased numbers and/or activities of $FOXP3^+$ Treg are present in the graft during immune reactivity, and not during immunological quiescence. Heart transplant patients are continuously treated with immunosuppressive drugs that may affect the function of $FOXP3^+$ regulatory T cells. Previously, we showed that the calcineurin inhibitors (CNI) tacrolimus and cyclosporine A (CsA) inhibited the FOXP3 gene transcription.[19] In addition, Mantel et al. demonstrated that CsA completely inhibited the mRNA induction of FOXP3 as well as the promotor activity by blocking the NFAT translocation into the nucleus.[23] Wu et al. showed that regulatory T-cell function is mediated by a complex of NFAT with FOXP3.[24] Thus, CNI may interfere with the complex formation of NFAT and FOXP3 and consequently have an inhibitory effect on the function of regulatory T cells. Hence, both the effector and regulatory T-cell population may be controlled by immunosuppressive medication. Nevertheless, in heart transplant patients acute rejections still occur. Thus, under certain conditions, effector T cells escape suppression by immunosuppressive drugs. It seems plausible that, at that moment, i.e., during rejection in the absence of effective suppression by immunosuppressants, the FOXP3 regulatory T-cell population is able to exert its suppressive function.

Also, the function of regulatory T cells may depend on activity of effector cells by their production of IL-2. As known, the presence of alloantigen and high dose of IL-2 are important factors for the induction of an immune response.[25] The presence of IL-2 in allografts has been associated with the occurrence of acute rejection.[26] Likewise, the mRNA levels of IL-2 were abundantly elevated in the rejection biopsies in this study. Recent studies, however, have shown that IL-2 is also critically required for the activation and the maintenance of $CD4^+CD25^+$ regulatory T cells.[27,28] The mechanism by which IL-2 supports Treg is related to the regulation and the maintenance of FOXP3 mRNA expression.[28,29]

In addition, Nishimura et al. showed that the antigen-specific expansion of Treg can be achieved by stimulating naturally occurring $CD4^+CD25^+$ T cells with alloantigens in the presence of IL-2.[14] These expanded Treg expressed high levels of FOXP3 mRNA and were potent suppressors of immune responses. Thus, the presence of both alloantigens and high doses of IL-2 during an acute rejection may activate the function and expansion of $FOXP3^+$ regulatory T cells.

The suggestion that initial infiltration and activation of effector cells may be needed for the infiltration or expansion of regulatory T cells is supported in this study. Our data indicates that the upregulation of activation markers preceded rejection: the mRNA levels of CD25, GITR and CTLA-4 were already abundantly expressed in the EMB with rejection grades 1–2 taken before the histological diagnosis of acute rejection. The initial activation of effector cells before induction of suppressive $FOXP3^+$ T cells may impose a time constraint on when suppression may become manifest. Hence, the regulatory T cells may not be able to suppress initial immune responses leading to rejection, but rather control the immunological processes in a later stage. This is in line with a study of Martin et al., who demonstrated that *in vivo* $CD4^+CD25^+$ regulatory T cells do not affect initial activation of naive T cells as well as their differentiation into Th1 effectors.[30] In addition, Mempel et al. suggested that regulatory T cells need to be activated and clonally expanded before they can confer suppressive signals and that regulation may only take effect late after CTL priming and effector differentiation.[31] These suggestions are in line with the findings of Muthukumar et al., who found higher FOXP3 mRNA levels in urine of kidney transplant patients undergoing rejection than in patients with normal biopsy results. Moreover, very high levels of FOXP3 during rejection was related with successful reversal of acute rejection, suggesting that regulatory T cells in the graft are involved in "damage control" rather than play a preventive role.[32]

Some reports indicated that FOXP3 might not always be associated to regulatory T-cell function, as FOXP3 can also be induced in activated human $CD25^-$ peripheral blood mononuclear cells.[23,33,34] In the present study, we cannot exclude that the elevated FOXP3 mRNA levels during cardiac allograft rejection is partially caused by activated effector T cells. Therefore, functional analysis of the $FOXP3^+$ T cells that are present in the graft during acute rejection are necessary to elucidate whether these cells are only regulatory T cells. However, only a few lymphocytes are present in the biopsies, rendering direct isolation of regulatory T cells impossible. Expansion of graft lymphocytes from rejection biopsies may provide a tool to functionally analyze these cells.

In conclusion, the mRNA levels of the regulatory T-cell marker FOXP3 as well as the levels of other regulatory T-cell associated markers, activation markers and effector markers were elevated during acute rejection, but not during immunological quiescence. These observations suggest that $FOXP3^+$ T cells do not prevent acute rejection, but rather are a response to anti-donor effector T-cell activity.

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Chapter 3

FoxP3^{+} T cells can be expanded from rejecting cardiac allografts

Dijke IE, Velthuis JHL, Balk AHMM, de Kuiper R, Klepper M, Caliskan K, Weimar W, Baan CC.

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Abstract

A specific subset of T cells, the FoxP3$^+$ regulatory T cells, control effector T-cell responses to self and foreign antigens. In transplant patients, we and others showed that high intragraft FOXP3 mRNA levels are associated with acute rejection, suggesting that immune regulation is dependent on immune activation. To study whether transplanted grafts harbor FoxP3$^+$ T cells and to functionally analyze them, graft lymphocytes (GL) must be propagated from the transplanted tissue. In the present study, we analyzed whether FoxP3$^+$ T cells can be grown from endomyocardial biopsies (EMB; n=5) of patients after heart transplantation during acute cellular rejection. After 18 to 21 days of culture, 0.5 to 1.0×10^6 GL were cultured from the EMB. Of these GL, 10.6% (median, range: 1.6%–17.1%) stained positive for FoxP3. Thus FoxP3$^+$ T cells can be grown from EMB, providing the tools to functionally characterize these cells in depth in forthcoming studies.

Introduction

Regulatory FoxP3+ T cells play a critical role in the control of immune responses and are required for the induction of unresponsiveness to alloantigens. In experimental transplant models, high intragraft FOXP3 mRNA expression levels were measured in tolerated transplants.[1,2] After clinical organ transplantation, however, FOXP3 transcripts were abundantly expressed during acute cellular rejection.[3-5] These findings support the hypothesis that FoxP3+ T cells control anti-donor responses in the graft itself, and suggest that the regulatory activities of FoxP3+ T cells or the generation of these cells are an intrinsic part of activation. The graft lymphocytes (GL) may also constitute a special local regulatory population different from the naturally occurring $CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells. Furthermore, at present it is not known whether these graft FoxP3+ T cells control local immune responses.

To study the regulatory capacities of graft FoxP3+ T cells, the cells should be propagated and expanded from the grafted tissue. Expansion of peripheral $CD4^+CD25^+FoxP3^+$ T cells was shown upon alloantigen stimulation with high-dose interleukin (IL)-2, circumstances that are present in the graft and during rejection in particular.[6,7] In the present study, we determined whether FoxP3+ T cells can be propagated from endomyocardial biopsies (EMB) with histological signs of acute rejection after clinical heart transplantation. The availability of large numbers of graft-derived FoxP3+ T cells from patients is advantageous, and provides the tools to study the mechanisms by which these suppressors control allogeneic responses in the transplanted organ.

Materials and methods

Study group

We analyzed EMB (n=5) obtained from 5 heart transplant patients during an acute cellular rejection episode (17–203 days posttransplantation; Table 1). Maintenance immunosuppressive therapy consisted of cyclosporine A and low-dose steroids. Rejection was histologically diagnosed in EMB according to the criteria of the 1990 International Society for Heart and Lung Transplantation (ISHLT) grading system.[8] According to the definition of rejection, only patients with ISHLT rejection grade >2 were considered to experience acute cellular rejection. Upon biopsy-proven rejection episodes, patients received anti-rejection therapy.

GL culture method

GL cultures were grown from EMB in round-bottomed wells of a 96-well culture plate (Corning Inc Costar, Acton, MA) as described previously.[6] Briefly, GL were

expanded in culture medium consisting of RPMI 1640-DM (Gibco BRL, Paisley, Scotland) supplemented with 10% heat-inactivated, pooled human serum, 4 mmol/l L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, in the presence of 30 U/ml exogenous IL-2 (lectin-free lymphocult T-LF, Biotest AG, Dreieich, Germany) and 10^5 irradiated (40 Gy) autologous peripheral blood mononuclear cells (PBMC). GL cultures were grown at 37°C/5% CO_2 for 2-3 weeks (Table 1). Half of the medium was refreshed every 2-3 days. After culturing, the GL were frozen in RPMI 1640-DM supplemented with 10% heat-inactivated, pooled human serum, 4 mmol/l L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% dimethyl sulfoxide (Merck Schuchardt, Hohenbrunn, Germany) and stored at –140°C until analysis.

Table 1. Proportion of FoxP3$^+$ T cells in cultures derived from EMB of rejecting heart transplant patients.

Patient	EMB (days after transplantation)	GL culture (days)	CD4$^+$ T cells (%)	FoxP3$^+$ of CD4$^+$ T cells (%)	FoxP3$^+$ of CD4$^-$ T cells (%)
I	17	18	50	2.2	1.2
II	42	21	48	8.3	2.3
III	203	21	12	2.6	11.6
IV	34	21	93	1.6	0.0
V	76	18	3	6.8	10.3

EMB, endomyocardial biopsy; GL, graft lymphocyte.

Phenotypical analysis of GL cultures

After thawing, the GL were placed directly in a well of a 96-well culture plate (NUNC, Roskilde, Denmark) containing 10 μl PerCP-conjugated anti-human CD3 (clone SK7, BD Biosciences, San Jose, CA) and 5 μl FITC-conjugated anti-human CD4 (clone S3.5, Biocarta, San Diego, CA) and left at room temperature for 30 minutes. After washing with cold FACSflow (BD Biosciences), the GL were resuspended in 200 μl Fix/Perm buffer (eBioscience, San Diego, CA) and left at 4°C for 45 minutes. The GL were washed again with cold FACSflow and with 200 μl Permeabil buffer (eBioscience). After blocking with 2% normal rat serum (eBioscience) at 4°C for 15 minutes, 10 μl APC-conjugated anti-human FoxP3 (clone PCH101, eBioscience), or 5 μl APC-conjugated rat anti-human immunoglobulin (Ig)G_2 isotypic antibody (clone eBR2a, eBioscience) as a negative control, was added. After 30 minutes at 4°C, the GL were washed twice with Permeabil buffer, resuspended in 200 μl FACSflow, and left at 4°C for 15 minutes. Finally, the cells were centrifuged and resuspended in 200 μl FACSflow. Flow cytometry was performed on a FACSCalibur with CellQuest Pro software (BD Biosciences). Results are expressed as the frequency (%) of CD4$^+$ or CD4$^-$ T cells expressing FoxP3 in the gated CD3$^+$ T-cell population.

Results and discussion

EMB were studied from 5 heart transplant patients with histological signs of acute cellular rejection; 0.5 to 1.0×10^6 GL were propagated from these EMB after 18 to 21 days of culture (Table 1). Flow cytometric analysis revealed that 1.6% to 17.1% of the CD3$^+$ GL expressed FoxP3. A representative example is shown in Figure 1. In each measurement, we also determined the presence of FoxP3 in the CD4$^+$ and CD4$^-$ subset. The GL cultures contained 2.6% (median, range: 1.6%–8.3%) FoxP3$^+$CD3$^+$CD4$^+$ T cells (Table 1). These finding are in line with Gavin et al.,[9] who demonstrated that FoxP3$^+$ T cells constitute 2.5% to 6.2% of CD4$^+$ T cells in PBMC of healthy humans. Moreover, the data show that culturing GL in IL-2-enriched medium does not result in an excessive proportion of FoxP3 protein-expressing T cells. However, this does not exclude the possibility that in IL-2-enriched culture medium expansion of GL results in selection of cells that express the high affinity IL-2 receptor CD25, and thus also influences the frequency of GL that express FoxP3.

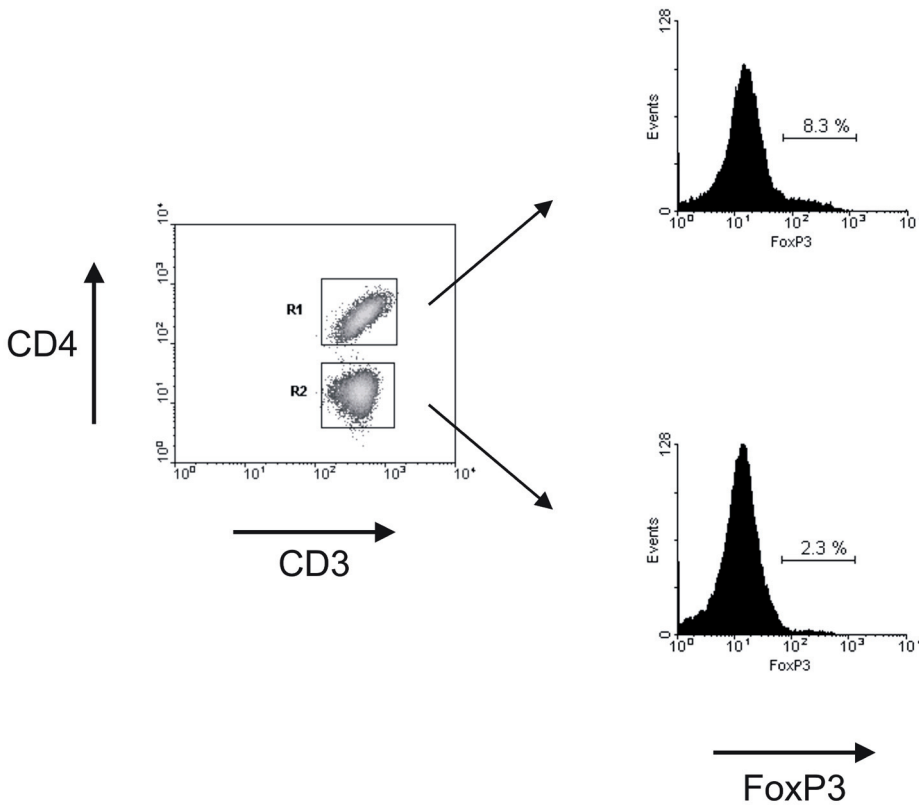

Figure 1. Phenotypical analysis of graft lymphocytes. Graft lymphocytes were cultured from EMB for 18 to 21 days in IL-2-enriched medium. The cells were analyzed by flow cytometry for the extracellular markers CD3 and CD4 as well as the intracellular protein FoxP3. One representative example is shown.

FoxP3$^+$ T cells were also found in the CD3$^+$CD4$^-$ T-cell population in 4 of the GL cultures (range: 1.2%–11.6%; Table 1). This finding suggests that a regulatory T-cell population different from the CD4$^+$ regulatory T-cell population may play a role in the immunological processes in grafts of heart recipients. The group of Suciu-Foca described a CD8$^+$CD28$^-$ T-cell population with immunoregulatory function in heart transplant patients.[10] These cells expressed FOXP3 mRNA at similar levels as the naturally occurring CD4$^+$CD25$^+$ regulatory T cells.[11] Subsequent characterization of the CD3$^+$CD4$^-$FoxP3$^+$ T-cell population of the GL should elucidate whether these cells are similar to the CD8$^+$CD28$^-$ regulatory T-cell subset.

Many speculations have been made about the possible mechanisms involved in graft acceptance and the role of different kinds of cells and cytokines in this process. Among the mechanisms that have been proposed are the FoxP3$^+$ regulatory T cells.[12] For both phenotypical and functional characterization of the graft FoxP3$^+$ T cells *ex vivo* expansion is warranted, as probably only small numbers of FoxP3$^+$ T cells are present within the graft. In this study, we have shown that it is possible to culture FoxP3$^+$ T cells from EMB after heart transplantation. This provides us with unique tools to functionally characterize these cells in depth in forthcoming studies, and will address whether graft acceptance is dependent on FoxP3$^+$ regulatory T cells that as a result of anti-donor immune response are present the allograft.

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Chapter 4

Donor-specific immune regulation by CD8$^+$ lymphocytes expanded from rejecting human cardiac allografts

Dijke IE, Caliskan K, Klepper M, de Kuiper R, Balk AHMM,
Maat APWM, Weimar W, Baan CC.

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Abstract

To assess whether regulatory T cells are present in rejecting human cardiac allografts, we performed functional analysis of graft lymphocytes (GL) expanded from endomyocardial biopsies (EMB; n=5) with histological signs of acute cellular rejection. The GL cultures were tested for their proliferative capacity and regulatory activity on allogeneic-stimulated peripheral blood mononuclear cells (PBMC) of the patient (ratio PBMC:GL = 5:1). Three of these GL cultures were hyporesponsive to donor antigens and suppressed the anti-donor proliferative T-cell response of PBMC, but not the anti-third-party response. Interestingly, it was the $CD8^+$ GL subset of these cultures that inhibited the anti-donor response (65-91% inhibition of the proportion of proliferating cells); the $CD4^+$ GL of the expanded GL cultures were not suppressive. In conclusion, $CD8^+$ GL expanded from rejecting human cardiac allografts can exhibit donor-specific immune regulatory activities *in vitro*. We suggest that during acute cellular rejection, GL may not only consist of graft-destructing effector T cells, but also of cells of the $CD8^+$ type with the potential to specifically inhibit anti-donor immune reactivity.

Introduction

In organ transplantation, regulatory T cells are considered to play a relevant role in the control of immune responses directed to the allograft. To understand this role, several clinical studies analyzed the frequency and/or function of these cells in the peripheral blood in relation to immunological processes, such as immunological quiescence and rejection.[1] Lower frequencies and inadequate immune regulatory function have been observed in acute and chronic rejecting organ transplant patients compared with clinically stable ones.[2-4] Yet, whether immune responses in the allograft are reflected by circulating cells remains a matter of debate. Investigation of graft tissue is therefore inevitable to elucidate the role of regulatory T cells in the control of intragraft immune reactivity.

So far, studies investigated the presence of regulatory T cells in the graft by analyzing the mRNA expression levels of FOXP3, the key regulatory gene for the development and function of regulatory T cells.[5] We demonstrated in heart transplant (HTx) patients that FOXP3 mRNA levels were more highly expressed in endomyocardial biopsies (EMB) collected during an acute cellular rejection than in those without histologically proven myocardial damage.[6] Our findings suggest that intragraft $FOXP3^+$ regulatory T cells are an integral component of alloreactivity. In clinical kidney transplantation, Veronese et al. and Bunnag et al. also found high FOXP3 mRNA levels in biopsies taken during T-cell-mediated rejection.[7,8] In addition, Muthukumar et al. observed higher FOXP3 mRNA levels in urinary samples of kidney transplant patients with an acute rejection than in that of patients with normal biopsy results.[9] Moreover, very high levels of FOXP3 during rejection was associated with successful reversal of this rejection, indicating that intragraft $FOXP3^+$ cells may be involved in "damage control".

In humans, however, FOXP3 mRNA expression is not confined to regulatory T cells, as effector T cells may also transiently express FOXP3 upon activation.[10] It cannot be excluded that high intragraft FOXP3 mRNA levels during rejection are partially caused by activated effector T cells. Thus, in order to determine whether cells with immune regulatory capacities are actually present in the rejecting graft, functional analysis of graft lymphocytes (GL) are necessary. Up till now, only one study has attempted to confirm the presence of regulatory T cells in the graft with functional analysis: Eljaafari et al. expanded skin-infiltrating lymphocytes from a biopsy of a well-accepted human hand transplant and observed that these cells were unresponsive to donor antigens and inhibited donor-directed blood T-cell alloresponses.[11] We were able to expand FoxP3 protein-expressing GL from EMB taken during an acute cellular rejection after clinical heart transplantation.[12] In this study, we investigated whether GL expanded from rejecting human cardiac allografts exhibit immune regulatory activities.

Materials and methods

Study group

We analyzed EMB of 5 HTx patients (median time after transplantation: 36 days, range: 15-144 days). All patients gave informed consent and the study was approved by the local medical ethical committee. Demographics of the patients are summarized in Table 1. Monitoring of acute rejection was performed by histological diagnosis of EMB collected according to the scheduled surveillance heart biopsy program. Only biopsies with ISHLT rejection grade ≥2R were considered as clinically relevant acute cellular rejection necessitating therapy.[13]

Table 1. Demographics of the studied patients

Patient	Age at transplant	Gender	Primary disease	Ischemia time (min)	Maintenance IS therapy	HLA-A/B MM	HLA-DR MM
I	57	M	IHD	not known	CsA/Pred	3	2
II	48	M	IHD	170	CsA/Pred	3	2
III	42	M	DCM	135	CsA/Pred/AZA	3	2
IV	62	M	IHD	185	CsA/Pred/AZA	3	2
V	56	M	IHD	158	CsA/Pred	3	1

IHD, ischemic heart disease; DCM, dilated cardiomyopathy; IS, immunosuppressive; CsA, cyclosporine A; Pred, prednisone; AZA, azathioprine; MM, mismatches.

GL culture method

GL cultures were grown from EMB with ISHLT rejection grade 2R (n=5) as described before.[12] Briefly, GL were expanded in RPMI 1640-DM (Gibco BRL, Paisley, Scotland) supplemented with 2 mM L-Glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (all Gibco BRL) [hereafter termed: supplemented RPMI 1640-DM], and with 10% heat-inactivated pooled human (HIPH) serum, 30 U/ml exogenous Interleukin (IL)-2 (lectin-free lymphocult-T-LF; Biotest AG, Dreieich, Germany) and 10^5 irradiated (40 Gy) autologous peripheral blood mononuclear cells (PBMC). GL cultures were incubated at 37°C/5% CO_2 for 16 days (median, range: 10–27 days), thereafter frozen in supplemented RPMI 1640-DM with 10% HIPH-serum and 10% dimethyl sulfoxide (Merck Schuchardt, Hohenbrunn, Germany) and stored at -140°C. For functional assays, due to insufficient amounts, thawed GL cultures were further expanded in supplemented RPMI 1640-DM with 10% HIPH-serum, 25 U/ml rIL-2 (PeproTech, Inc., Rocky Hill, NJ), 5 ng/ml rIL-15 (PrepoTech, Inc.), 4 μg/ml phytohemagglutinin (PHA; Murex Biotech Ltd, Dartford, UK) and 10^5 irradiated donor spleen cells/10^4 GL. Cells were cultured for 18 days (median, range: 14–20 days), before being tested for their proliferative and immune regulatory capacities.

Isolation of PBMC and spleen cells

PBMC were isolated from heparinized blood samples by density gradient centrifugation using Ficoll-Paque (density 1.077 g/ml; Amersham Pharmacia Biotech, Uppsala, Sweden). PBMC were collected from the interphase, washed twice, frozen in supplemented RPMI 1640-DM with 10% fetal bovine serum (FBS; BioWhittaker Verviers, Belgium) and 10% dimethyl sulfoxide and stored at –140°C. For all patients, donor spleen cells were available. Donor and third-party spleen cells were obtained by homogenizing the spleen in supplemented RPMI 1640-DM containing 10 μg/ml DNAse (Roche Molecular Biochemicals, Mannheim, Germany). The cell suspension was centrifuged over a Ficoll-Paque density gradient, collected, washed, frozen and stored at –140°C.

Isolation of $CD4^+$ and $CD8^+$ GL

To separate the $CD8^+$ GL from the $CD4^+$ GL, expanded GL were washed with cold MACS buffer prepared according to the manufacturer's protocol (Miltenyi, Bergisch Gladbach, Germany) and resuspended in 80 μl MACS buffer supplemented with 20 μl CD8-MicroBeads (Miltenyi). After 15 minutes at 4°C, the GL were washed with MACS buffer and resuspended in 500 μl MACS buffer. Subsequently, the "possel"-separation protocol was used on the autoMACS (Miltenyi).

PKH labelling

For the functional assays, fluorescence-based analysis of cell proliferation was used. The GL populations, thawed patient PBMC and spleen cells were washed in supplemented RPMI 1640-DM. Subsequently, the cells were resuspended in 500 μl Diluent C (Sigma Aldrich, St. Louis, MO) and stained as follows: GL populations were labelled with 2 μM PKH67 (Sigma Aldrich), patient PBMC with 2 $\mu M/10^7$ cells PKH26 (Sigma Aldrich) and spleen cells were double-labelled with 2 $\mu M/10^7$ cells PKH67 and 2 $\mu M/10^7$ cells PKH26. The solutions were prepared by diluting 2 μl of the 10^{-3} M ethanolic dye stock in 500 μl Diluent C. Staining was stopped after 4 minutes by addition of 1 ml FBS and the cells were washed three times with supplemented RPMI 1640-DM with 10% HIPH-serum.

Functional analysis of GL cultures

To investigate the regulatory capacity of GL, $5x10^4$ PKH26-labelled patient PBMC were stimulated with $5x10^4$ irradiated (40 Gy) PKH26/PKH67-labelled spleen cells from donor or third party (fully HLA-A, -B and -DR mismatched with patient and donor) in the absence and presence of $10x10^4$ PKH67-labelled $CD3^+$ GL, $CD8^+$ GL or $CD4^+$ GL. To assess the proliferative capacity of GL, the same mixed

lymphocyte reactions (MLR) were performed, but then with irradiated PKH26-labelled patient PBMC. Experiments were performed at least in triplicate in 96-well round-bottom culture plate (NUNC, Roskilde, Denmark). After 7 days at 37°C/5% CO_2, the cultures were washed with FACSflow (BD Biosciences, San Jose, CA) and incubated for 30 minutes at room temperature with PerCP-conjugated CD3, APC-conjugated CD4 and APC-Cy7-conjugated CD8 (all BD Biosciences). The samples were measured on an eight-color FACSCanto II (BD Biosciences) and analyzed using FACS Diva version 6.1.1 (BD Biosciences) and Flow Jo version 7.2.2. (Tree Star Inc., Ashland, OR) software.

Results

The proliferative capacity of graft lymphocytes

For functional analysis, we used five GL cultures expanded from EMB with histological signs of acute cellular rejection (median time after transplantation: 36 days, range: 15-144 days). We assessed the proliferative response of these GL to alloantigens (Figure 1). Culture II and V had a significant proliferative response to donor antigens: approximately a third of the $CD3^+$ GL in these cultures were proliferating cells. In contrast, Culture I, III and IV poorly proliferated upon donor-antigens stimulation, as only 2–13% of the total population were proliferating cells. After third-party-antigens stimulation, proliferating $CD3^+$ GL were detected in all cultures, ranging from 25 to 89% of the total population.

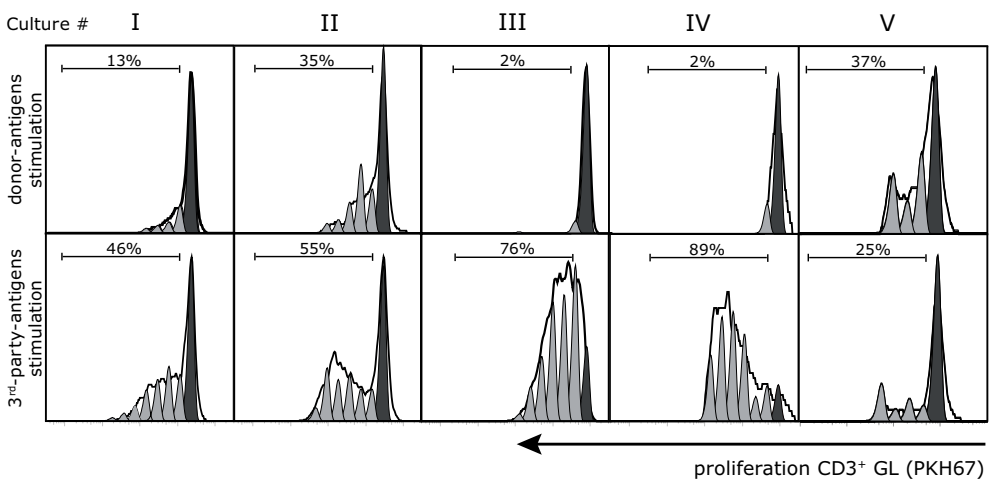


Figure 1. The proliferative capacity of graft lymphocytes. The proliferation of five PKH67-labelled expanded GL cultures stimulated with irradiated PKH26/PKH67-labelled donor or third-party spleen cells was analyzed at day 7 by flow cytometry. To exclude the stimulator cells from analysis, we gated all but PKH26/PKH67-labelled cells. The proliferative response of $CD3^+$ GL of the five cultures is depicted. The percentages represent the proportions of the total $CD3^+$ GL population that are proliferating cells.

The regulatory effect of graft lymphocytes on anti-donor reactivity of peripheral blood lymphocytes

To investigate whether GL exhibit regulatory activities on T-cell proliferation, patient PBMC were stimulated with donor or third-party antigens in the absence and presence of GL (ratio PBMC:GL = 5:1). Figure 2A represents two examples (Culture I and II) of a FACS analysis. We analyzed the regulatory effect of GL on the proliferative T-cell response by calculating the percentage inhibition of the proportion of proliferating $CD3^+$ PBMC. Three GL cultures had a suppressive effect on the anti-donor response, as an inhibition of 60–78% was measured (Figure 2B). Within the $CD3^+$ PBMC population, the proliferation of both $CD4^+$ and $CD8^+$ PBMC was inhibited (data not shown). The three suppressive GL cultures were those that were hyporesponsive to donor antigens (Culture I, III and IV; Figure 1). The two cultures that proliferated upon donor-antigens stimulation (Culture II and V) did not inhibit the anti-donor proliferative response of patient PBMC. The anti-third-party proliferative response of the patient PBMC was not or hardly inhibited by the GL cultures (Figure 2B).

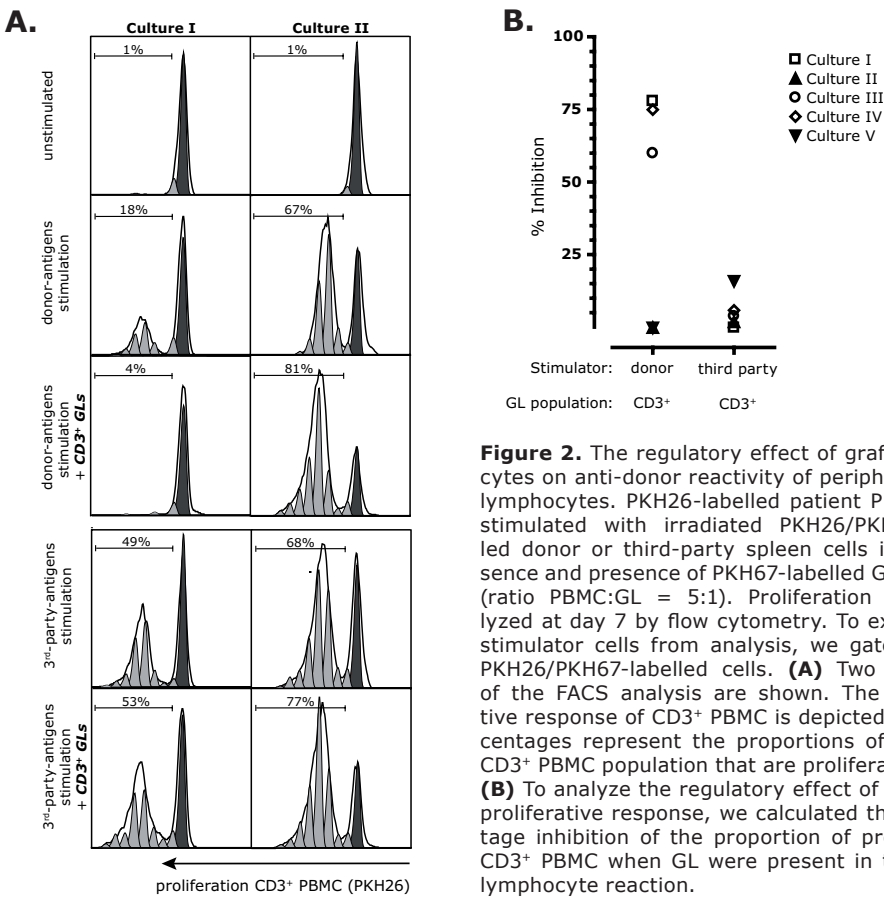


Figure 2. The regulatory effect of graft lymphocytes on anti-donor reactivity of peripheral blood lymphocytes. PKH26-labelled patient PBMC were stimulated with irradiated PKH26/PKH67-labelled donor or third-party spleen cells in the absence and presence of PKH67-labelled GL cultures (ratio PBMC:GL = 5:1). Proliferation was analyzed at day 7 by flow cytometry. To exclude the stimulator cells from analysis, we gated all but PKH26/PKH67-labelled cells. **(A)** Two examples of the FACS analysis are shown. The proliferative response of $CD3^+$ PBMC is depicted. The percentages represent the proportions of the total $CD3^+$ PBMC population that are proliferating cells. **(B)** To analyze the regulatory effect of GL on the proliferative response, we calculated the percentage inhibition of the proportion of proliferating $CD3^+$ PBMC when GL were present in the mixed lymphocyte reaction.

The regulatory effect of either CD4+ or CD8+ graft lymphocytes on anti-donor reactivity

We questioned whether the anti-donor immune regulatory activity was exhibited by either CD4+ or CD8+ GL, or by both populations. We therefore separated the CD4+ GL from the CD8+ GL. Before separation, the CD4+/CD8+ T-cell ratio of Culture I, II and III was 71/29%, 76/24% and 91/9%, respectively. Culture IV consisted mainly of CD8+ GL (>98%) and Culture V of CD4+ GL (>97%). After separation, the purity of the CD8+ GL populations ranged from 78 to 99%, and that of the CD4+ GL populations from 90 to 99%. We examined the proliferative response of these GL subsets to donor antigens (Figure 3A). Three of four CD4+ GL subsets had a significant anti-donor proliferative response, as 33-74% of the total population were proliferating cells. In contrast, three of four CD8+ GL subsets did not proliferate after donor-antigens stimulation (Culture I, III and IV). We analyzed the regulatory effect of each population on the anti-donor proliferative T-cell response of patient PBMC (Figure 3B depicts two examples). Interestingly, the regulatory activity of Culture I, III and IV was found within the CD8+ GL population: the addition of CD8+ GL to the MLR suppressed the proportion of proliferating CD3+ PBMC, ranging from 65 to 91% inhibition (Figure 3C). This inhibition was not observed when only CD4+ GL were added to the MLR.

Discussion

The clinically relevant role of FoxP3+ regulatory T cells in the control of anti-donor immune responses after organ transplantation is still under debate. We and others have demonstrated that FOXP3 mRNA levels were highly expressed during an acute cellular rejection, indicating that FOXP3 mRNA-expressing T cells are an integral component of alloreactivity.[6-9] Yet, as FOXP3 mRNA is not only expressed by regulatory T cells, but also by activated effector T cells,[10] functional analysis of GL are necessary to determine whether these cells actually comprise regulatory T cells. Here, we investigated whether GL populations expanded from rejecting human cardiac allografts contain cells with potential immune regulatory capacities. We demonstrated that, when expanded GL populations were hyporesponsive to donor antigens, they suppressed the anti-donor T-cell proliferation of patient PBMC, but not the anti-third-party proliferation. This donor-specific regulatory function was found within the CD8+ GL subset, suggesting that graft-protective cells may have a CD8+ phenotype. However, not all CD8+ GL were suppressive: in one culture these cells proliferated upon donor-antigens stimulation and did not inhibit the anti-donor T-cell proliferation of PBMC. So, the balance between regulators and effectors in a CD8+ GL population may determine whether this population has a protective or destructive function.

CD8+ T cells with immune regulatory capacities have been described in autoimmune diseases, cancer and transplantation.[14-16] They have been defined by their lack

of CD28 expression or their Qa-1 (HLA-E in humans) restricted cytotoxicity, although it is uncertain whether these characteristics represent the same $CD8^+$ regulatory T-cell population.[14,15] The mechanisms by which Qa-1-restricted $CD8^+$ regulatory T cells mediate their function include direct killing of their target cells, whereas $CD8^+CD28^-$ FoxP3 expressing regulatory T cells inhibit alloreactivity by inducing the upregulation of inhibitory receptors on antigen-presenting cells and endothelial cells. As this latter population has been found in the circulation of HTx patients,[17] we investigated in another cohort of GL populations expanded from rejecting human cardiac allografts (n=9) whether the $CD8^+$ GL populations include cells with a similar phenotype (data not shown). $FoxP3^+$ cells were present within the $CD8^+$ GL subsets and, in some cultures, the majority of these cells indeed had the $CD28^-$ phenotype. In other cultures, however, the $CD8^+FoxP3^+$ GL were predominantly $CD28^+$. This discrepancy may be caused by the culture method: CD28 is upregulated on $CD8^+$ T cells when cocultured with allogeneic cells.[18] Another possibility may be that $CD8^+CD28^-FoxP3^+$ GL are regulators, whereas $CD8^+CD28^+$ GL are activated effector T cells that transiently express FoxP3.[10]

It is conceivable that the observed inhibition of the proliferative response of patient PBMC is due to the lysis of donor APC by the $CD8^+$ GL. Previously, our group demonstrated in heart transplant patients that cytotoxic $CD8^+$ T cells with high avidity for donor antigens home in the graft to induce rejection.[19] As we used the total $CD8^+$ GL population in our present study, we cannot exclude that cytotoxic T cells in this population lyse donor APC and that, consequently, the PBMC do not proliferate. Yet, we demonstrated that only the GL populations that did not proliferate after donor-antigens stimulation inhibited the T-cell proliferation of the donor-antigen-stimulated PBMC. No inhibition of the proliferation of the PBMC was observed when proliferating GL were added to the MLR. So, if the observed *in vitro* regulation is rather due to donor-directed cytotoxic GL than to GL with immune regulatory function, this would mean that nonproliferating GL lyse donor APC and that proliferating GL do not lyse donor APC. This does not seem very plausible. Still, we cannot rule out that the observed *in vitro* regulation may be mediated by expression of effector function.

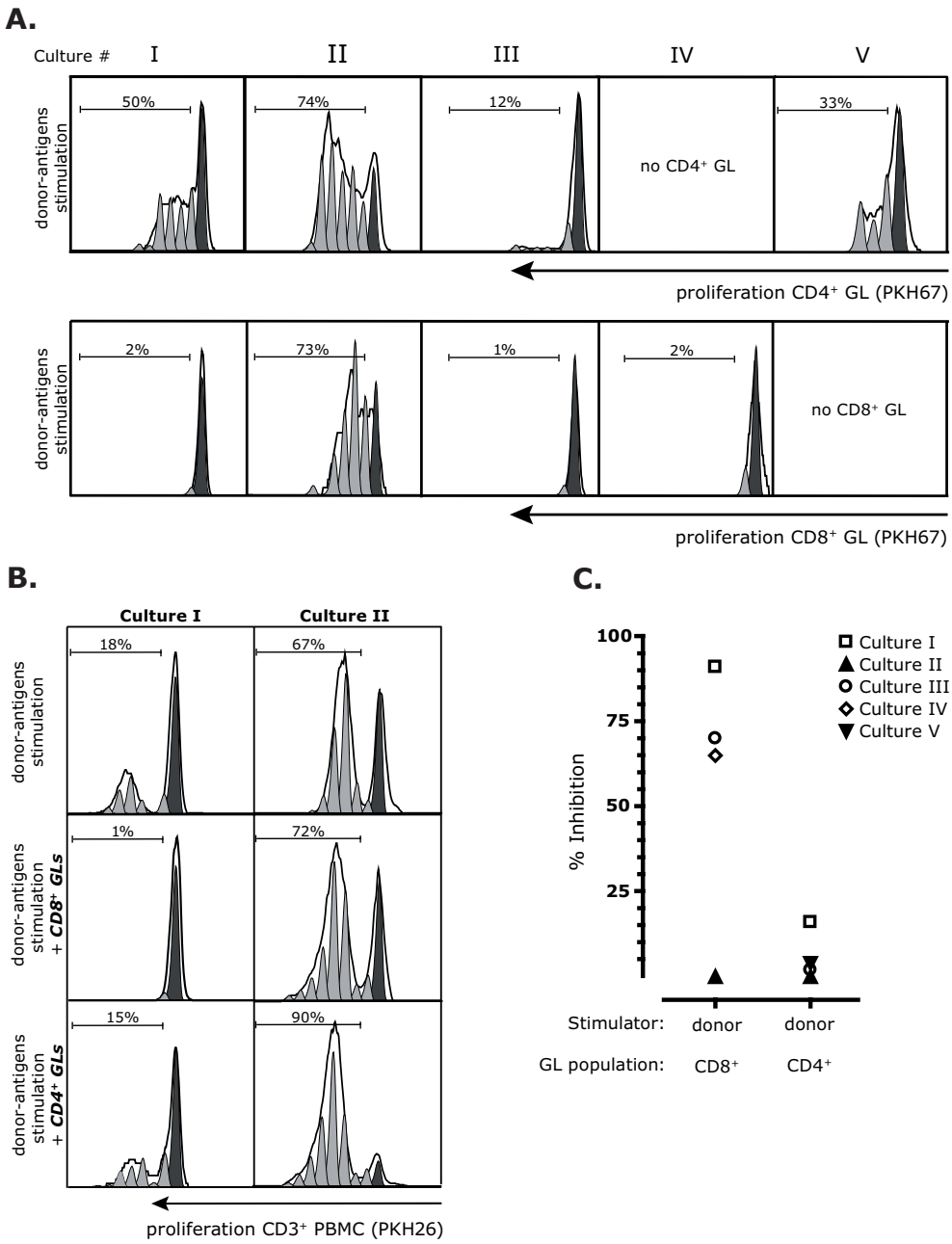
In organ transplantation, attention has mainly focused on $CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells. Studies in experimental transplant models have demonstrated that these regulators prevented acute allograft rejection.[20,21] In this clinical study, however, we were not able to detect $CD4^+$ cells with suppressive capacities in the expanded GL populations. Nonetheless, phenotypic analysis of another cohort of GL populations expanded from rejecting human cardiac allografts (n=9) showed that $FoxP3^+$ cells were present within $CD4^+$ GL subsets (data not shown).[12] One explanation for the lack of inhibition may be that the frequency of regulatory $CD4^+FoxP3^+$ T cells in the expanded GL populations is too low for active suppression of T-cell proliferation. As this might be due to the culture method (other cells may have outgrown the $CD4^+$ regulatory GL), we cannot exclude the contribution of regulatory $CD4^+$ GL *in vivo*. On the other hand, the $CD4^+FoxP3^+$ GL may be

effector T cells that upregulated FoxP3 expression upon activation.[10] Selective isolation of $CD4^+FoxP3^+$ GL to analyze the regulatory function of this population specifically, however, is difficult, as we have not yet found an extracellular marker that distinguishes these cells from conventional expanded GL.

We are aware that we performed our assays with GL expanded in culture. Nevertheless, these cells originate from lymphocytes that are present in rejecting cardiac allografts of immunosuppressed HTx patients. Thus, these intragraft lymphocytes comprise cells that under certain conditions (i.e., in the presence of cytokines and donor antigens) have the potential to expand and exhibit immune regulatory activities. Interestingly, the patients whose expanded $CD8^+$ GL population exhibited immune regulatory activities remained free from acute cellular rejection episodes after this rejection, whereas the patient whose expanded $CD8^+$ GL were not suppressive experienced three more acute rejection episodes. So, in the clinical setting, intragraft allogeneic immune regulation may be established during alloreactivity. Cells with potential immune regulatory activities in the graft during allograft rejection may play a role in "damage control".[9,22] The number of experiments performed, however, is too small to draw any conclusions about the relation between the suppression assays and the clinical events. Further research on GL expanded from EMB with histological signs of acute cellular rejection may help us to address the question whether the control of alloreactivity is dependent on regulatory T cells that as a result of an anti-donor immune response accumulate in the graft.

In conclusion, $CD8^+$ GL expanded from rejecting cardiac allografts can exhibit donor-specific immune regulatory activities *in vitro*. These results suggest that during acute cellular rejection, lymphocyte populations present in the graft may not only consist of graft-destructive effector T cells, but also of cells of the $CD8^+$ phenotype with the potential to specifically inhibit anti-donor immune responses.

Figure 3. The regulatory effect of either $CD4^+$ or $CD8^+$ graft lymphocytes on anti-donor reactivity. The $CD4^+$ GL were separated from the $CD8^+$ GL. Both populations were labelled with PKH67 and stimulated with irradiated PKH26/PKH67-labelled donor spleen cells. In addition, PKH26-labelled patient PBMC were stimulated with irradiated PKH26/PKH67-labelled donor spleen cells in the absence and presence of either PKH67-labelled $CD4^+$ GL or PKH67-labelled $CD8^+$ GL (ratio PBMC: GL = 5:1). Proliferation was analyzed at day 7 by flow cytometry. To exclude the stimulator cells from analysis, we gated all but PKH26/PKH67-labelled cells. **(A)** The proliferative responses of $CD4^+$ GL and $CD8^+$ GL are depicted. The percentages indicate the proportions of the total GL subset that are proliferating cells. **(B)** Two examples of the FACS analysis are shown. The proliferative response of $CD3^+$ PBMC is depicted. The percentages indicate the proportions of the total $CD3^+$ PBMC population that are proliferating cells. **(C)** To analyze the effect of the GL subsets on the proliferative response we calculated the percentage inhibition of the proportion of proliferating $CD3^+$ PBMC when GL were present in the mixed lymphocyte reaction. As Culture IV consisted mainly of $CD8^+$ GL (>98%) and Culture V of $CD4^+$ GL (>97%), no experiments could be performed with $CD4^+$ GL of Culture IV and $CD8^+$ GL of Culture V due to insufficient amounts of these cells.


A.
Culture #
I
II
III
IV
V
donor-antigens stimulation
50%
74%
12%
no CD4+ GL
33%
proliferation CD4+ GL (PKH67)
2%
73%
1%
2%
no CD8+ GL
proliferation CD8+ GL (PKH67)
B.
Culture I
Culture II
18%
67%
1%
72%
+ CD8+ GLs
15%
90%
+ CD4+ GLs
proliferation CD3+ PBMC (PKH26)
C.
Culture I
Culture II
Culture III
Culture IV
Culture V
% Inhibition
100
75
50
25
Stimulator:
donor
donor
GL population:
CD8+
CD4+

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Regulatory T cells in the peripheral blood

Part III

Chapter 5

FOXP3 mRNA expression analysis in the peripheral blood of heart transplant patients

Dijke IE, Caliskan K, Korevaar SS, Maat APWM, Zondervan PE,
Balk AHMM, Weimar W, Baan CC.

Transplant Immunology 2008; 18:250-254

Abstract

Previously, we demonstrated in heart transplant patients that FOXP3, a gene required for the development and function of regulatory T cells, was highly expressed in the graft during an acute cellular rejection. In this study, we analyzed whether the FOXP3 gene expression in the peripheral blood also reflects anti-donor immune responses, and therefore may provide clues for noninvasive detection of nonresponsiveness or acute rejection. We examined the FOXP3 expression patterns of peripheral blood mononuclear cells (PBMC; n=69) of 19 heart transplant patients during quiescence and rejection in comparison with those of endomyocardial biopsies (EMB; n=75) of 24 heart transplant patients. While the FOXP3 mRNA levels were abundantly expressed in rejection EMB (ISHLT rejection grade 2R) compared with EMB without histological evidence of myocardial damage (ISHLT rejection grade 0R–1R; p=0.003), no association with rejection or nonresponsiveness was found for the FOXP3 mRNA levels in the peripheral blood. Thus, in contrast to intragraft FOXP3 gene expression, the peripheral FOXP3 mRNA levels lack correlation with anti-donor immune responses in the graft. Consequently, FOXP3 does not appear to be a potential candidate gene for noninvasive diagnosis of nonresponsiveness or rejection.

Introduction

The transcription factor FOXP3 serves as the key regulatory gene for the development and function of cells with immunoregulatory activities.[1] This gene inhibits the activity of nuclear factor of activated T cells (NFAT) and NF-κB, two transcription factors that are essential for cytokine gene expression and T-cell functions.[2] *Ex vivo* gene transfer of FOXP3 converted naive T cells into a regulatory T-cell phenotype with suppressive capacities, indicating that FOXP3 is a specific marker for regulatory T cells.[3] In transplantation, it is speculated that FOXP3$^+$ T cells may play an important role in the suppression of donor-activated effector T cells. Experimental transplant models have demonstrated that the mRNA expression of FOXP3 is upregulated in skin and cardiac allografts of tolerant recipients, suggesting that FOXP3$^+$ regulatory T cells accumulate and act locally in the tolerated tissue.[4,5]

In clinical heart transplantation, we recently demonstrated that the FOXP3 mRNA expression was high in endomyocardial biopsies (EMB) taken during an acute cellular rejection compared to those without histologically proven myocardial damage.[6,7] In addition, Muthukumar et al. observed higher FOXP3 mRNA levels in the urine of renal transplant patients with an acute rejection than in that of patients with normal biopsy results.[8] Thus, in human organ transplantation, intragraft FOXP3 mRNA expression is associated with anti-donor immune reactivity. Yet, it is unknown whether the FOXP3 mRNA levels in the peripheral blood of transplant patients also reflect anti-donor immune responses and, consequently, could provide clues for noninvasive detection of nonresponsiveness or rejection. Therefore, we analyzed whether FOXP3 mRNA levels in peripheral blood mononuclear cells (PBMC) correlate with the immunological processes in the graft of patients after heart transplantation.

Materials and methods

Patients and materials

EMB and PBMC samples of 41 heart transplant patients were studied. All patients gave informed consent and the study was approved by the local medical ethical committee. EMB were collected according to the scheduled surveillance heart biopsy program to monitor histological signs of acute rejection.[9] Only patients with ISHLT rejection grade ≥2R were considered to have a clinically relevant acute cellular rejection necessitating therapy. Twenty-six patients underwent one or more acute rejection episodes within the first year posttransplantation (rejectors); 15 patients had no histological signs of acute rejection (nonrejectors). The demographics of the rejectors and nonrejectors are summarized in Table 1.

Peripheral blood samples were collected at the time of an EMB procedure. PBMC were isolated by density gradient centrifugation using Ficoll-Paque (density 1.077 g/ml; Amersham Pharmacia Biotech, Uppsala, Sweden). PBMC were collected from the interphase, washed twice with RPMI 1640 (BioWhittaker, Verviers, Belgium) and frozen in RPMI 1640-DM (Gibco BRL, Paisley, Scotland) supplemented with 2 mM L-Glutamine (Gibco BRL), 100 IU/ml penicillin (Gibco BRL), 100 μg/ml streptomycin (Gibco BRL), 10% fetal bovine serum (BioWhittaker), and 10% dimethyl sulfoxide (Merck, Schuchardt, Germany) and stored at –140°C until analysis. In total, we analyzed 75 EMB from 22 patients and 69 PBMC samples from 17 patients; from two patients both PBMC and EMB samples were available for analysis. No significant differences were observed in clinical characteristics between the patient group whose EMB were investigated and the group whose PBMC samples were analyzed (data not shown).

Table 1. Demographics of the studied patients

	No AR+ #	AR+ #	p value
No. of subjects (n)	15	26	
Age at time of HTx (y)	52 ± 11*	49 ± 19*	p=0.58 ‡
Gender (M/F)	12/3	15/11	p=0.27 §
Primary disease (n):			
Ischemic heart disease	7	8	
Dilated cardiomyopathy	7	12	p=0.34 §
Other	1	6	
Cold ischemia time (min)	177 ± 46*	175 ± 40*	p=0.88 ‡
Induction therapy (n):			
ATG/OKT3	15/0	25/1	
Immunosuppressive therapy (n):			
CsA-MMF-Pred	12	14	
Tacro-MMF-Pred	2	8	p=0.39 §
Tacro-Evero-Pred	1	3	
Tacro-Pred	0	1	
HLA-A/B mismatches (n)	3.1 ± 0.8*	3.2 ± 0.7*	p=0.68 ‡
HLA-DR mismatches (n)	1.4 ± 0.6*	1.8 ± 0.4*	p=0.01 ‡

#Acute rejection was defined as ISHLT rejection grade ≥2R.[9]
HTx, heart transplantation; CsA, cyclosporine A; MMF, mycophenolate mofetil; Pred, prednisone; Tacro, tacrolimus; Evero, everolimus; AR+, acute rejection.
*Mean±SD; P values were obtained by the Student's *t* test ‡ or the χ^2-test §.

Quantitative real-time polymerase chain reaction

Messenger RNA extraction from the EMB, cDNA transcription and DNA amplification was performed as described before.[6] From PBMC samples, total RNA was isolated using the High Pure RNA Isolation Kit (Roche Applied Science, Penzberg, Germany). RNA concentrations were measured by the RiboGreen RNA quantification reagent and kit (Molecular Probes, Eugene, OR), and complimentary DNA was synthesized with random primers. We used quantitative real-time polymerase chain reaction (Q-PCR) to quantify the amount of FOXP3, the T-cell marker CD3ε and the housekeeping gene 18S mRNA expression. FOXP3 (Hs00203958_m1) and CD3ε (Hs00167894_m1) analyses were performed using Assay-on-Demand products (Applied Biosystems, Foster City, CA). A 5 μl sample of cDNA was added to 20 μl PCR mixture containing 12.5 μl Universal PCR Master Mix (Applied Biosytems), 0.625 μl of each specific Primer & Probe-on-Demand mix, and 6.875 μl H_2O. The pre-developed TaqMan Assay (PDAR) was used to measure 18S (Hs99999901_s1) concentrations (Applied Biosystems). A 5 μl sample of cDNA was added to 20 μl PCR mixture containing 12.5 μl Universal PCR Master Mix (Applied Biosytems), 1.25 μl primers and probe (Vic labelled), and 6.25 μl H_2O.

The PCR reaction was performed after a first step of 2 minutes 50°C and 10 minutes 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C using the ABI Prism 7700 sequence detector (Applied Biosystems). Each run contained several negative controls and two positive reference samples to check intra- and inter-assay variations. There were no significant differences in cycle threshold (Ct) values within and between the experiments (all <0.5 Ct). The amount of each target molecule was quantified by measuring the Ct, which was transformed to the number of cDNA copies [$2^{(40-Ct)}$] on a TaqMan Real-Time PCR system. The relative concentration of FOXP3 was normalized to the relative concentration of 18S or to that of CD3ε and multiplied by 10^6 or 10^3 respectively due to the lower concentration of the target gene compared with the concentration of 18S and CD3ε.

Statistical analysis

A log transformation was performed on the relative mRNA levels of FOXP3 to reduce the positive skew of the distribution. We used the Kruskal-Wallis test to identify differences among >2 groups and the Mann-Whitney test to compare mRNA levels between two groups. The Student's *t* test and the χ^2-test were used to analyze differences of clinical data. P values ≤0.05 were considered to be significant. GraphPad Version 4.0 statistical program (San Diego, CA) was applied for analyses and graphics.

Results

Clinical characteristics

EMB and PBMC samples were studied of patients who remained free from rejection in the first year posttransplantation (nonrejectors; n=15) and of patients who experienced at least one histologically proven acute rejection episode (rejectors; n=26). No significant differences were found in age, gender, primary disease, cold ischemia time, induction therapy, type of immunosuppression and HLA-A/B mismatches with the donor between the nonrejectors and the rejectors (Table 1). The rejectors, however, had more HLA-DR mismatches with their donor than the nonrejectors (p=0.01; Table 1).

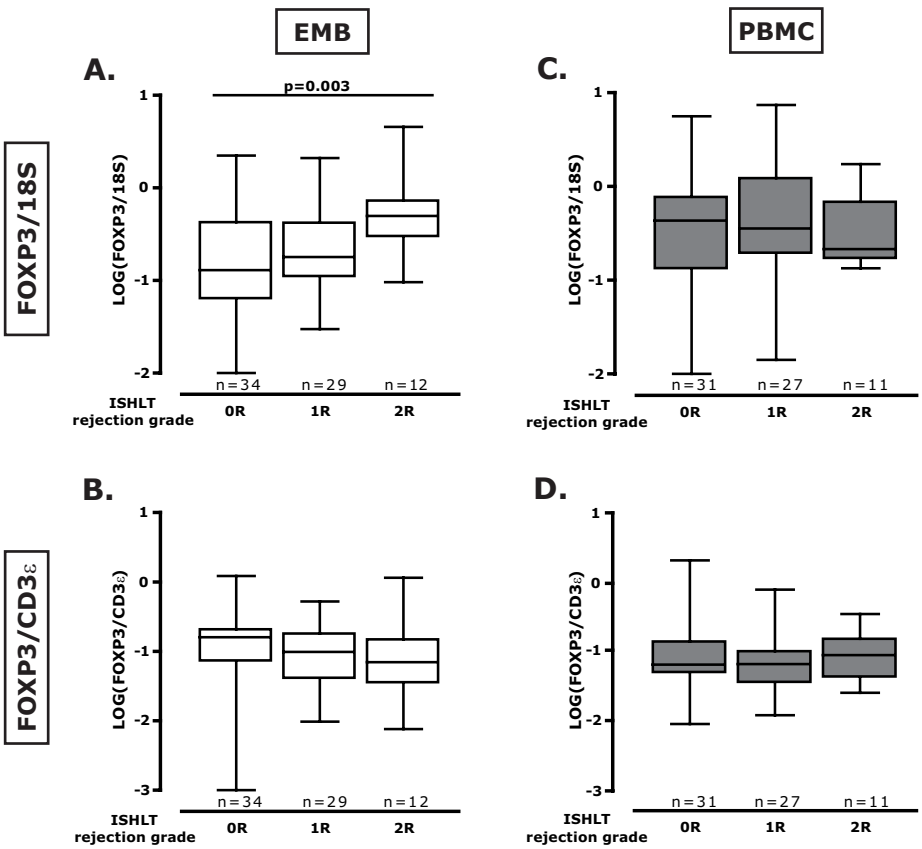

Figure 1. The FOXP3 gene expression in EMB and PBMC samples stratified to ISHLT rejection grades. **(A, C)** The 18S-normalized, log-transformed FOXP3 mRNA levels measured in EMB (white) and PBMC samples (gray) stratified to the ISHLT rejection grades.[9] High FOXP3 mRNA expression levels in the graft, but not in the peripheral blood, were associated with acute rejection. **(B, D)** The CD3ε-normalized, log-transformed FOXP3 mRNA levels measured in EMB (white) and PBMC samples (gray) stratified to ISHLT rejection grades.[9] P-values are based on the Kruskal-Wallis test.

FOXP3 gene expression

First, we analyzed the FOXP3 mRNA expression levels in 75 EMB and 69 PBMC samples in relation to the ISHLT rejection grades.[9] The 18S-normalized, log-transformed mRNA levels of FOXP3 were highly expressed in EMB with histological signs of acute rejection (ISHLT rejection grade 2R) compared with nonrejection EMB (rejection grades 0R–1R; p=0.003; Figure 1A). The CD3ε-normalized, log-transformed intragraft FOXP3 mRNA levels, however, were comparable between the different ISHLT rejection grades, suggesting that the increase of intragraft FOXP3 gene expression during acute rejection is proportionally correlated to the increase of CD3ε expressing infiltrate (Figure 1B). In PBMC samples, the 18S-normalized FOXP3 mRNA expression was comparable between ISHLT rejection grades 0R, 1R and 2R (Figure 1C). In addition, no differences in CD3ε-normalized FOXP3 mRNA levels were measured between the ISHLT rejection grades (Figure 1D). Next, we questioned whether the FOXP3 mRNA levels in nonrejection EMB and PBMC samples (ISHLT rejection grades 0R–1R) were differently expressed between nonrejectors and rejectors. For both EMB and PBMC samples, no significant differences in FOXP3 gene expression were found (data not shown). Thus, the association between high intragraft FOXP3 mRNA levels and anti-donor immune reactivity was only found during the acute rejection episode.

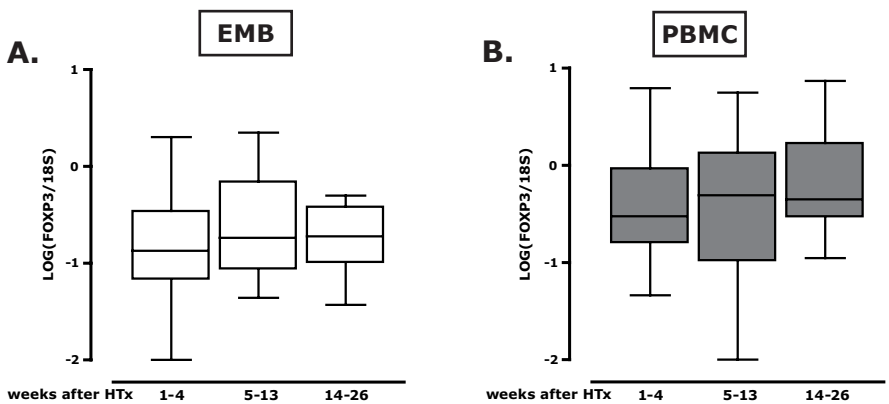

Figure 2. The relationship of FOXP3 gene expression with time after transplantation. **(A, B)** The FOXP3 mRNA levels in the first month (1–4 weeks), the second/third month (5–13 weeks) and fourth/sixth month (14–26 weeks) posttransplantation in EMB (white) and PBMC samples (gray). No relation between FOXP3 mRNA levels and the time after heart transplantation was observed for both EMB and PBMC samples.

To investigate whether the FOXP3 gene expression in the peripheral blood predicts anti-donor immune responses in the cardiac allograft, we first determined the relationship between FOXP3 mRNA levels and time after transplantation. To rule out the influence of acute rejection on the mRNA expression, we excluded the EMB

and PBMC samples taken during an acute rejection episode. No relation between FOXP3 mRNA levels and the time after heart transplantation was observed for both EMB and PBMC samples (Figure 2A-B). Subsequently, we analyzed PBMC samples taken at consecutive time points after transplantation of 12 rejectors and 4 nonrejectors and linked the measured FOXP3 mRNA levels with the ISHLT rejection grade diagnosed at that time point in the cardiac allograft. In rejectors as well as nonrejectors, the FOXP3 mRNA levels in the peripheral blood showed no correlation with the ISHLT rejection grades diagnosed in the cardiac allograft (data not shown).

Discussion

At present, the state of anti-donor immune responses in heart transplant patients is monitored by histological diagnosis of surveillance heart biopsies. Yet, as this is an invasive procedure, the development of a reliable less invasive assay that identifies nonresponsiveness or acute rejection is an important goal in clinical heart transplantation. We and others have explored different *in vitro* assays to monitor intragraft anti-donor immune responses in the peripheral blood of heart transplant patients, such as limiting dilution assays with different readouts (cytokine production or cytotoxicity) and the ELIspot assay.[10-13] However, findings have not been unambiguous.[14,15] Besides, these assays are labor intensive and require a long incubation period and, therefore, not preferred for routinely use to monitor anti-donor responses. A sensitive approach that is less time-consuming is measuring gene expression levels with the use of Q-PCR. Previously, we described that FOXP3, a gene required for the development and function of regulatory T cells, was highly expressed in the graft of heart transplant patients during an acute cellular rejection.[6,7] In this study, we analyzed whether the FOXP3 gene expression in the peripheral blood also reflects anti-donor immune responses and, consequently, may provide clues for noninvasive detection of nonresponsiveness or acute rejection. In contrast to intragraft FOXP3 gene expression, however, the FOXP3 mRNA expression levels in the peripheral blood of heart transplant patients lack correlation with anti-donor immune responses.

Previous studies have investigated whether analyzing gene expression profiles in the peripheral blood can prove cardiac allograft rejection. Several genes involved in immunological processes, such as transcription factors (NFAT, GATA3), costimulatory molecules (PDCD1), cytotoxic molecules (perforin, granzyme B) and chemokines (RANTES and CXCR3), were found to be elevated in the peripheral blood during an acute cellular cardiac allograft rejection.[16-18] Many of these genes were also upregulated in the cardiac allograft itself during an allograft rejection.[19-21] The results from these studies together suggest that although the effector phase of allograft rejection represents a local immune process, activated cells can be detected in the circulation. In our present study, however, cardiac allograft rejection was only associated with high FOXP3 mRNA levels in the graft, and not

with those in the peripheral blood. This may suggest that the increase of FOXP3 gene expression during anti-donor immune reactivity is solely a local immune process. Yet, we should bear in mind that 'random noise' in transcript studies may masquerade as molecular signatures, and replication of the data is necessary.[22] For instance, our results of FOXP3 mRNA levels in the peripheral blood are not in line with the results of Vlad et al., who showed a negative correlation between FOXP3 gene expression levels in peripheral T-cell populations and the grade of rejection.[23] Nevertheless, quantitative analysis of the expression of genes may provide a valuable tool for noninvasive diagnosis of allograft rejection. The lack of correlation between peripheral FOXP3 mRNA levels and the immunological processes in the graft, however, indicates that this gene may not be a potential candidate for noninvasive detection of nonresponsiveness or rejection.

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Chapter 6

Inadequate immune regulatory function of $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cells in heart transplant patients who experience acute cellular rejection

Dijke IE, Korevaar SS, Caliskan K, Balk AHMM, Maat APWM, Weimar W, Baan CC.

Transplantation; in press

Abstract

$CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells are suppressors of antigen-activated immune reactivity. Here, we assessed the clinically relevant role of these cells in the control of immune responses directed to a transplanted heart. We investigated the phenotype and function of peripheral $CD4^+CD25^{bright+}FoxP3^+$ T cells in heart transplant patients free from acute rejection (n=9) and in rejectors (n=12) before and during acute cellular rejection. Between rejectors and nonrejectors, the proportion of $CD4^+CD25^{bright+}$ T cells and of $FoxP3^+$ cells within this population was comparable. Yet, $CD4^+CD25^{bright+}FoxP3^+$ T cells of rejectors had a higher CD127 expression than those of nonrejectors ($p<0.0001$). Depletion of $CD4^+CD25^{bright+}$ T cells from PBMC increased the anti-donor proliferative response of both nonrejectors ($p=0.0005$) and rejectors ($p=0.03$). In rejectors, however, only a 2-fold increase was measured, whereas the nonrejectors' response became 14 times higher ($p=0.002$). Reconstitution of $CD4^+CD25^{bright+}$ T cells only suppressed the overall anti-donor proliferative response of $CD25^{neg/dim}$ responder cells of nonrejectors significantly ($p=0.001$). Moreover, the percentage inhibition of the response was higher in nonrejectors than in rejectors ($p=0.02$). Analyses of pretransplant samples revealed that $CD4^+CD25^{bright+}$ T cells of rejectors already had a lower suppressive capacity than those of nonrejectors before transplantation ($p=0.04$). In conclusion, $CD4^+CD25^{bright+}FoxP3^+$ T cells of heart transplant patients who experience acute rejection had an upregulated CD127 expression and an inadequate regulatory function compared with those of nonrejecting patients. Our observations suggest that the function of circulating $CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells may be pivotal for the prevention of acute cellular rejection after clinical heart transplantation.

Introduction

After heart transplantation, patients are treated with immunosuppressive medication to prevent graft rejection. At present, a combination of immunosuppressive drugs consisting of calcineurin inhibitors, anti-proliferative agents or mammalian target of rapamycin (mTOR) inhibitors, and steroids, seems to be the best choice to prevent acute rejection effectively.[1] Yet, although the majority of heart transplant (HTx) patients treated with this immunosuppressive regimen remain free from acute rejections, a considerable number still experience one or more acute rejection episodes in the first year after transplantation.[2] This discrepancy suggests that other factors may play a role. So far, studies in clinical heart transplantation have mainly focused on immunostimulatory factors, such as effector T cells: we and others found that high numbers and avidity of these cells were associated with acute cellular rejection episodes.[3-5] Currently, immunoregulatory factors are suggested to play an important role in anti-donor immune responses as well. Several mechanisms have been described that inhibit or eliminate alloreactive T-cell activation including clonal deletion, anergy and active suppression by regulatory T cells.[6]

Regulatory T cells are suppressors of antigen-activated immune responses to self and nonself antigens.[7] Several regulatory T-cell populations have been identified, but attention has focused on a specific subset of $CD4^+$ T cells that constitutively expresses the interleukin (IL)-2 receptor α chain (CD25).[8] Besides the bright expression of CD25, these $CD4^+CD25^{bright+}$ regulatory T cells express other markers such as cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), glucocorticoid-induced tumor necrosis factor (TNF) receptor family-related gene (GITR) and CD62L.[9-11] The most generally used marker to identify $CD4^+CD25^{bright+}$ regulatory T cells, however, is the intracellular expression of the transcription factor forkhead box P3 (FoxP3), which is the key regulatory gene for the development and function of cells with immunoregulatory activities.[12,13] Recently, Liu et al. suggested that the expression of the IL-7 receptor α chain (CD127) may be an additional marker for the identification of $CD4^+CD25^{bright+}$ regulatory T cells, as CD127 inversely correlated with FoxP3 expression and regulatory T-cell activity.[14] $CD4^+CD25^{bright+}$ regulatory T cells suppress the proliferation and cytokine production of effector T cells;[15] the underlying mechanisms include the secretion of cytokines such as transforming growth factor (TGF)-β and direct cell contact through binding of cell-surface molecules such as CTLA-4.[16]

In organ transplantation, $CD4^+CD25^{bright+}$ regulatory T cells are considered to play a relevant role in the control of immune reactivity directed to the donor allograft.[6,17] Studies in experimental mouse models showed that these regulators prevented acute allograft rejection.[18,19] More recently, their counterparts in clinical transplantation have been investigated. We and others demonstrated in clinically stable organ transplant patients that circulating $CD4^+CD25^{bright+}$ T cells have the potential to inhibit anti-donor immune responses *in vitro*: depletion of these cells

from the peripheral blood mononuclear cells (PBMC) increased the proliferative response directed to donor antigens and the production of IL-2 and interferon (IFN)-γ significantly.[20-22] Yet, whether these cells indeed play a clinically relevant role in the control of acute cellular rejection after heart transplantation remains to be elucidated. Hence, we analyzed the phenotype and the immune regulatory function of peripheral $CD4^+CD25^{bright+}$ T cells of HTx patients free from acute rejection and those who experience an acute rejection episode.

Materials and methods

Study group

Twenty-one HTx patients transplanted between July 2002 and September 2006 were included in this study. Monitoring of acute rejection was performed by histological diagnosis of endomyocardial biopsies collected according to the scheduled surveillance heart biopsy program. Only biopsies with ISHLT rejection grade ≥2R were considered as clinically relevant acute cellular rejection (AR+) necessitating therapy.[23] Twelve HTx patients had one or more AR+ in the first year after transplantation with the first AR+ within 3 months (rejectors): six patients had one AR+, four patients had two AR+, and two patients had three AR+ (Table 1). We focused on the first AR+, as at that time point all patients had similar immunosuppressive medication and no one had yet been treated with anti-rejection therapy. Of each rejector, we examined peripheral blood samples taken before the first AR+ (preAR+; median time before AR+: 7 days, range: 7–34 days) and during this first AR+ (median time after transplantation: 33 days, range: 7–71 days). Nine patients without histological signs of AR+ were included in the nonrejector group. Of each nonrejector, we analyzed two peripheral blood samples matched in time after transplantation with the samples of rejectors.

All 21 patients received horse anti-thymocyte globuline induction therapy for 5-7 days and maintenance immunosuppressive therapy consisting of cyclosporine A or tacrolimus, mycophenolate mofetil or everolimus, and prednisone. The demographics of the patients are summarized in Table 1.

Isolation of peripheral blood lymphocytes

Blood sampling was approved by the local medical ethical committee on human research. All patients gave informed consent. PBMC were isolated by density gradient centrifugation using Ficoll-Paque (density 1.077 g/ml; Amersham Pharmacia Biotech, Uppsala, Sweden). The PBMC were collected from the interphase, washed twice in RPMI 1640 (BioWhittaker, Verviers, Belgium) and frozen in RPMI 1640-DM (Gibco BRL, Paisley, Scotland) supplemented with 2 mM L-Glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin (all Gibco BRL), 10% fetal bovine

Table 1. Demographics of the studied patients

Patient	Age at transplant	Gender	Primary disease	Ischemia time (min)	Maintenance immunosuppressive therapy	AR+ at 1 year posttransplant
Nonrejectors						
1	38	F	HCM	238	CsA-MMF-Pred	0
2	45	M	DCM	165	CsA-MMF-Pred	0
3	58	M	IHD	188	CsA-MMF→X-Pred*	0
4	60	M	DCM	150	CsA-MMF-Pred	0
5	56	M	DCM	220	CsA-MMF-Pred	0
6	60	M	DCM	210	CsA-MMF-Pred	0
7	45	M	IHD	159	Tacro-MMF-Pred	0
8	38	M	DCM	190	CsA-MMF-Pred	0
9	50	F	IHD	179	Tacro-MMF-Pred	0
Rejectors						
10	49	M	IHD	224	CsA-MMF-Pred	3
11	26	M	RCM	208	Tacro-MMF-Pred	1
12	41	M	IHD	205	Tacro-Evero→MMF-Pred#	1
13	35	F	HCM	197	Tacro-MMF-Pred	2
14	65	M	DCM	174	CsA-MMF-Pred	2
15	42	M	DCM	144	CsA-MMF-Pred	2
16	46	M	DCM	149	Tacro-Evero-Pred	1
17	38	M	HCM	163	Tacro-MMF-Pred	1
18	25	M	DICM	174	Tacro-MMF-Pred	1
19	50	M	IHD	219	Tacro-Evero-Pred	1
20	54	M	DCM	269	CsA-MMF→Evero-Pred§	3
21	45	M	IHD	205	Tacro-Evero-Pred	2

Acute rejection was defined as ISHLT rejection grade ≥2R.[23] HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; IHD, ischemic heart disease; RCM, restrictive cardiomyopathy; DICM, drug-induced cardiomyopathy; CsA, cyclosporine A; MMF, mycophenolate mofetil; Pred, prednisone; Tacro, tacrolimus; Evero, everolimus.
In the course of analysis: *MMF was withdrawn; #Evero was converted to MMF; §MMF was converted to Evero.

serum (BioWhittaker), and 10% dimethyl sulfoxide (Merck, Schuchardt, Germany) and stored at –140°C until analysis. In total, 41 posttransplant PBMC samples of the 21 HTx patients were examined. In addition, 14 pretransplant PBMC samples were available for analysis.

Stimulator cells

For all patients, donor spleen cells were available. Donor and third-party spleen cells were obtained by mechanical dissociation of small pieces of spleen through a stainless steel strainer in RPMI 1640 containing 10 μg/ml DNAse (Roche Molecular Biochemicals, Mannheim, Germany), 100 IU/ml penicillin and 100 μg/ml streptomycin. Subsequently, the cell suspension was filtrated through a 40 μm cell strainer (Falcon, Franklin Lakes, NJ) and washed. Thereafter, the cells were centrifuged over a Ficoll-Paque density gradient, collected, washed, frozen and stored at –140°C until analysis.

Flow cytometry

To analyze the frequency of $CD4^{+}CD25^{bright+}$ cells, thawed PBMC were incubated with PE-conjugated CD25 (clone M-A251; directed against epitope B), FITC-conjugated CD3, PerCP-conjugated CD4 and APC-conjugated CD8 (all from BD Biosciences, San Jose, CA) for 30 minutes at room temperature. To determine the FoxP3 and CD127 expression, PBMC were incubated with PerCP-conjugated CD4, PE-conjugated CD25 and FITC-conjugated CD127 (eBioscience, San Diego, CA) or IgG1 (BD Biosciences) and left at room temperature for 30 minutes. After washing with cold FACSflow (BD Biosciences), the PBMC were resuspended in Fix/Perm buffer (eBioscience) and left at 4°C for 30 minutes. Subsequently, the PBMC were washed with cold FACSflow and with Permeabil buffer (eBioscience). After blocking with 2% rat serum in Permeabil buffer at 4°C for 15 minutes, APC-conjugated FoxP3 (eBioscience) or IgG2a (eBioscience) was added. After 30 minutes at 4°C, the PBMC were washed twice with Permeabil buffer and resuspended in FACSflow. The samples were measured on a four-color FACSCalibur and analyzed using CellQuest Pro (BD biosciences) and Flow Jo (Tree Star Inc., Ashland, OR) software. The median fluorescence value (MFV) is defined as the fluorescence value below which 50% of the events are found.

Isolation of $CD4^{+}CD25^{bright+}$ T cells and $CD25^{neg/dim}$ responder cells

To isolate $CD4^{+}CD25^{bright+}$ cells, thawed PBMC were washed once with MACS buffer and resuspended in 90 μl MACS buffer/$10x10^{6}$ PBMC prepared according to the manufacturer's protocol (Miltenyi, Bergisch Gladbach, Germany) and supplemented with 10 μl CD25-microbeads/$10x10^{6}$ PBMC (directed against

epitope A; Miltenyi). After 15 minutes at 4°C, the PBMC were washed once again with MACS buffer and resuspended in 500 μl MACS buffer. Subsequently, the "posseld"-separation protocol was used on the autoMACS (Miltenyi).[21] The purity of the isolated $CD4^+CD25^{bright+}$ T cell was 72% (median). Cells not selected by this separation protocol are referred to as $CD25^{neg/dim}$ responder cells; this population contained approximately 1.3% (median) contaminating $CD4^+CD25^{bright+}$ T cells. As a control for the effect of the autoMACS procedure on the cells, PBMC were handled by the same protocol in the absence of CD25-microbeads.

Viability assay

The cells were tested for their viability by trypan-blue exclusion and for their proliferation capacity using the phytohemagglutinin (PHA) proliferation assay. After $5x10^4$ cells were exposed to 1 μg/ml PHA (Murex Biotech LTd, Kent, UK) for 3 days, cell proliferation was measured by ^{3}H-thymidine incorporation (0.5 μCi/well) (Amersham Pharmacia Biotech) during the last 16 hours of culture. A 1450 MicroBeta liquid scintillation spectrophotometer (Wallac, Turku, Finland) was used to analyze the ^{3}H-thymidine incorporation. In 2/41 posttransplant PBMC samples, we observed few viable cells (<50% viable) and no proliferative response to PHA, and these samples were therefore excluded for analysis.

Mixed lymphocyte reaction

$5x10^4$ PBMC and $5x10^4$ $CD25^{neg/dim}$ responder cells were cultured with $5x10^4$ irradiated (40 Gy) stimulator cells from donor or third party (fully HLA-A, -B and -DR mismatched with patient and donor) in RPMI 1640-DM supplemented with 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated pooled human serum. $5x10^4$ PBMC and $5x10^4$ $CD25^{neg/dim}$ responder cells cultured in absence of irradiated stimulator cells served as background control. The experiments were performed in triplicate in 96-well round-bottom culture plates (NUNC, Roskilde, Denmark). After 7 days of incubation at 37°C/5% CO_2, cell proliferation was measured as described above. Subsequently, the Stimulation Indexes (SI) were calculated:

$$SI = \frac{\text{counts per minute (cpm) obtained in presence of irradiated stimulator cells}}{\text{cpm obtained in absence of irradiated stimulator cells}}$$

SI lower than 10 were considered as hyporesponsiveness. The regulatory function of $CD4^+CD25^{bright+}$ T cells was examined by comparing the proliferative response to donor and third-party antigens before and after depletion of these cells. Additionally, $CD25^{neg/dim}$ responder cells were cocultured with isolated $CD4^+CD25^{bright+}$ T cells in a ratio of 10:1.

Statistical analysis

The Mann-Whitney test (unpaired analyses) or the Wilcoxon Signed-Rank test (paired analyses) was performed to identify differences between two groups. The χ^2-test was performed for discrete variables. The clinical characteristics of rejectors and nonrejectors were compared using the Mann-Whitney test for continuous variables, and the χ^2-test or the Fisher's Exact test for discrete variables. P values ≤0.05 were considered to be significant. GraphPad Version 4.0 statistical program (San Diego, CA) and SPSS Version 11.5 (Chicago, IL) were applied for analyses and graphics.

Results

Clinical characteristics

Between nonrejectors and rejectors, no significant differences were observed for the age, gender, primary disease, cold ischemia time, and type of immunosuppression (Table 1). In addition, the HLA-A/B and HLA-DR mismatches were comparable (mean±SD for nonrejectors vs. rejectors: 2.7±0.9 vs. 3.2±0.6 and 1.4±0.7 vs. 1.8±0.5, respectively). Neither the number of cytomegalovirus or of toxoplasmosis seronegative recipient/seropositive donor combinations was significantly different between nonrejectors and rejectors (4/9 vs. 3/12 and 3/9 vs. 3/12, respectively). Cytomegalovirus disease was diagnosed in two rejectors and three nonrejectors.

The proliferative response of PBMC of HTx patients

Significant differences in the anti-donor proliferative response were observed between the PBMC of nonrejectors and those of rejectors: almost none (1/17) of the nonrejectors' PBMC were responsive to donor antigens, whereas the vast majority (16/22) of the rejectors' PBMC proliferated upon donor-antigens stimulation ($p<0.0001$; Figure 1A). Additionally, significantly more PBMC of rejectors (12/21) had a proliferative response to third-party antigens compared with those of nonrejectors (2/15, $p=0.01$; Figure 1B).

The frequency and FoxP3 expression of $CD4^+CD25^{bright+}$ T cells

We defined the $CD4^+CD25^{bright+}$ T cells as the population that highly expressed CD25 in combination with slightly less CD4 expression (Figure 2A).[21] In the PBMC of nonrejectors, the proportion of $CD25^{bright+}$ cells within the $CD4^+$ T-cell population was 4.1% (median, range: 2.2–11.5%); this was comparable to the proportion in the PBMC of rejectors (5.1% (1.1–13.0%); Figure 2B). Within the rejector group,

no differences were found in the proportion of CD25$^{bright+}$ cells between samples taken preAR+ (4.4% (1.1–12.2%)) and those collected during this AR+ (5.4% (3.8–13.0%)).

Next, we analyzed the proportion of FoxP3^{+} cells within the CD4^{+}CD25$^{bright+}$ T-cell population (Figure 2C). Again, no significant differences were found between nonrejectors (59% (45–78%)) and rejectors (66% (39–91%); Figure 2D), neither between preAR+ PBMC (66% (49–91%)) and AR+ PBMC (62% (39–81%)).

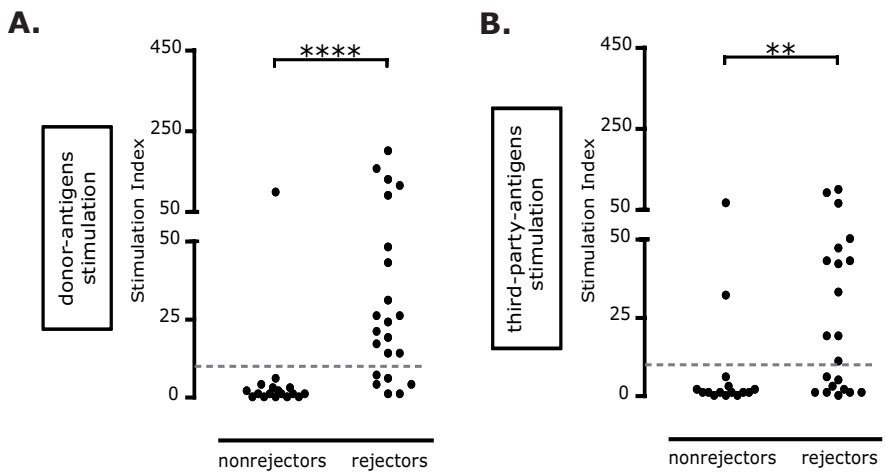


Figure 1. The proliferative response of PBMC of HTx patients. PBMC were stimulated with irradiated donor **(A)** or third-party **(B)** spleen cells. Proliferation was measured by incorporation of ^{3}H-thymidine at day 7. Subsequently, the stimulation indexes (SI) were calculated and plotted. SI <10 were regarded as hyporesponsive. ** p≤0.01 and **** p≤0.0001 represent the outcome of the Mann-Whitney test. Due to insufficient material, three PBMC samples could not be stimulated with third-party antigens.

Effect of depleting CD4^{+}CD25$^{bright+}$ T cells on the proliferative response

To examine the regulatory function of CD4^{+}CD25$^{bright+}$ T cells, we first studied the effect of depleting these cells from the PBMC on the proliferative response towards donor antigens. An example of the obtained CD25$^{neg/dim}$ responder T-cell population after isolation of the CD25$^{bright+}$ T cells is depicted in Figure 3A. In both nonrejectors and rejectors, the anti-donor reactivity of CD25$^{neg/dim}$ responder cells was significantly higher than that of PBMC (p=0.0005 and p=0.03, respectively; Figure 3B). The suppressive capacity of CD4^{+}CD25$^{bright+}$ T cells of rejectors, however, showed significant differences with that of nonrejectors' CD4^{+}CD25$^{bright+}$ T cells: in rejectors only a 2-fold increase (median, range: 0.3–16) in response was measured when these cells were depleted, while the response in nonrejectors was 14 times (median, range: 1–108) higher (p=0.002; Figure 3C).

Depletion of $CD4^+CD25^{bright+}$ T cells increased the overall proliferative response to third-party antigens in both nonrejectors (p=0.0002) and rejectors (p=0.01; Figure 3D) as well, indicating that the suppressive function of these regulators was not shifted to donor specificity within the first 3 months after transplantation. No significant differences were measured in the extent of increase between rejectors (median fold increase (range): 4-fold (0.1–107)) and nonrejectors (8-fold (1–70); Figure 3E).

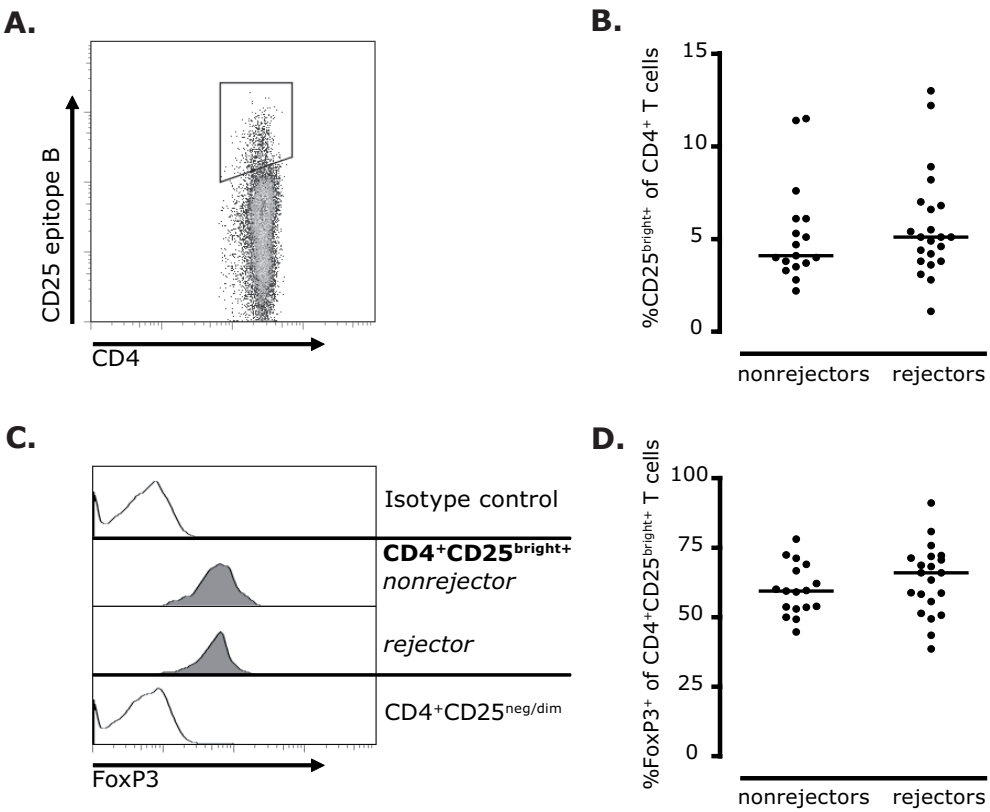


Figure 2. The frequency and FoxP3 expression of $CD4^+CD25^{bright+}$ T cells in HTx patients. **(A)** We defined $CD25^{bright+}$ T cells as the population that highly expressed CD25 in combination with slightly less CD4 expression.[21] **(B)** The percentage of $CD25^{bright+}$ T cells within the $CD4^+$ T-cell population was plotted. **(C)** FoxP3 expression was determined in the $CD4^+CD25^{bright+}$ T cells. **(D)** Each symbol represents the percentage of $FoxP3^+$ cells within the $CD4^+CD25^{bright+}$ T-cell population in one PBMC sample. Due to insufficient material, FoxP3 staining could not be performed in one rejector sample.

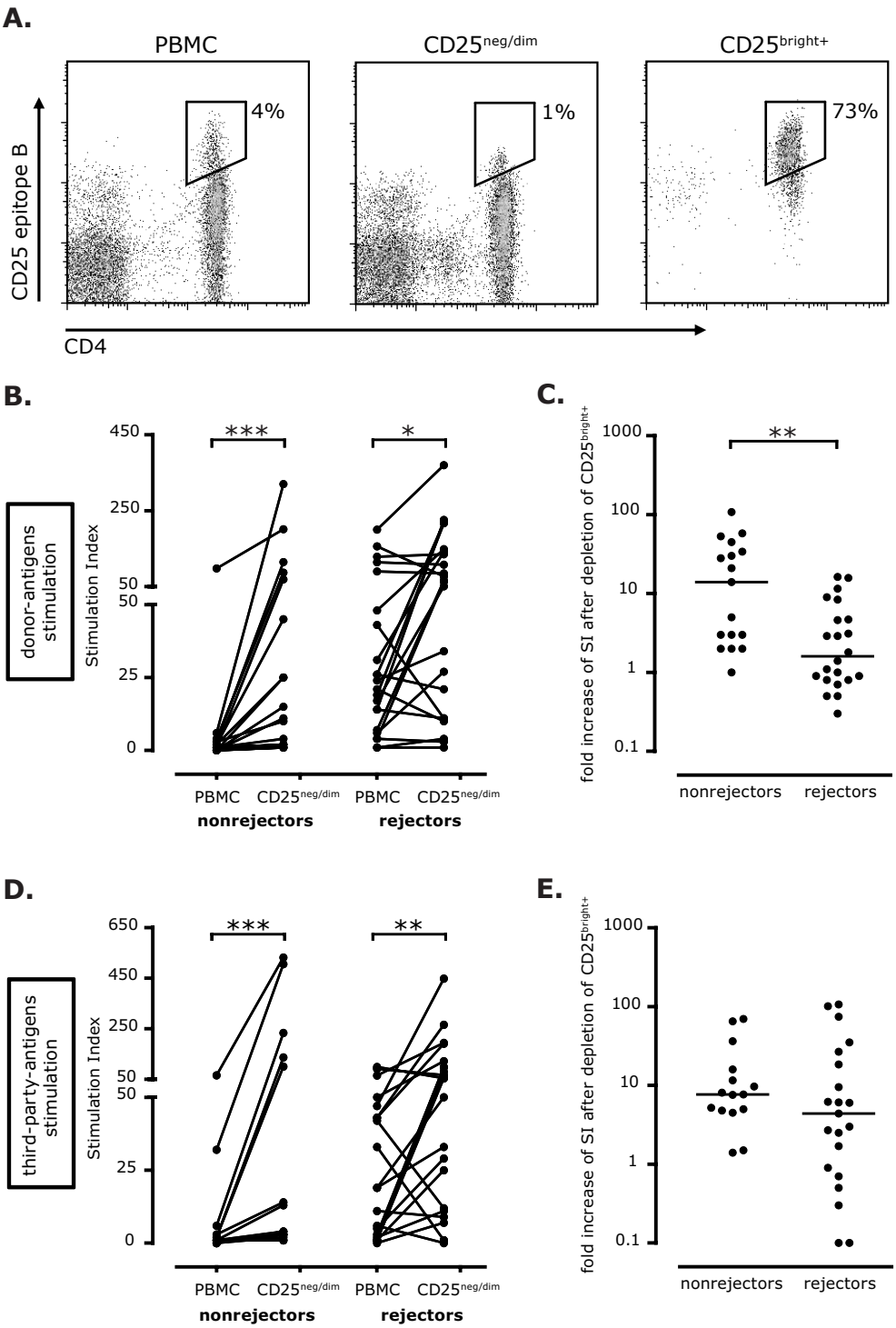
Coculture of CD25$^{neg/dim}$ responder cells with CD4^{+}CD25$^{bright+}$ T cells

The isolated CD4^{+}CD25$^{bright+}$ T-cell population itself (Figure 3A) was anergic to both donor and third-party antigens (data not shown). Coculturing of CD25$^{neg/dim}$ responder cells with CD4^{+}CD25$^{bright+}$ T cells in a fixed ratio of 10:1 resulted in a strong suppression of the proliferative response to donor antigens in nonrejectors (p=0.001; Figure 3F). In contrast, the overall anti-donor response was not significantly inhibited in rejectors (p=0.14; Figure 3F), which was due to 4 samples in which the proliferative response increased instead of decreased after reconstitution. Moreover, the percentage inhibition of the proliferation of the responsive CD25$^{neg/dim}$ responder cells (i.e., those with SI≥10) after reconstitution of CD4^{+}CD25$^{bright+}$ T cells was significantly higher in the nonrejector group (median: 97%) than in the rejector group (66%; p=0.02; Figure 3G).

After third-party-antigens stimulation, reconstituted CD4^{+}CD25$^{bright+}$ T cells inhibited the proliferative response of CD25$^{neg/dim}$ responder cells of both nonrejectors and rejectors (p=0.03 vs. p=0.02, respectively; Figure 3H). The median % inhibition of the proliferation of the responsive CD25$^{neg/dim}$ responder cells was 79% in the nonrejector group and 58% in the rejector group, which was not significantly different (Figure 3I).

Function of CD4^{+}CD25$^{bright+}$ T cells before and during acute rejection

Within the rejector group, we observed a wide variation in the suppressive capacity of CD4^{+}CD25$^{bright+}$ T cells: in some cases these cells were good regulators, whereas in other cases these cells suppressed the anti-donor response poorly or not at all. One explanation for this may be that the results of the preAR+ samples were combined with that of AR+ samples: the suppressive capacity of the CD4^{+}CD25$^{bright+}$ T cells may be altered during the acute rejection episode compared with that before this rejection. Hence, we divided the preAR+ samples from the AR+ samples. In the preAR+ samples, a 3-fold increase (median, range: 0.3–16) of the anti-donor proliferative response was measured after depleting CD4^{+}CD25$^{bright+}$ T cells from the PBMC; this was not significantly different to the extent of increase in the AR+ samples (1-fold (0.5–16)). Furthermore, coculturing of CD25$^{neg/dim}$ responder T cells with CD4^{+}CD25$^{bright+}$ T cells in a fixed ratio of 10:1 resulted in a comparable inhibition of the anti-donor proliferative response between preAR+ and AR+ CD25$^{neg/dim}$ responder cells (median % inhibition: 63% vs. 71%, respectively).


A.
PBMC
CD25neg/dim
CD25bright+
4%
1%
73%
CD25 epitope B
CD4
B.
donor-antigens stimulation
Stimulation Index
450
250
50
50
25
0
***
*
PBMC
CD25neg/dim
PBMC
CD25neg/dim
nonrejectors
rejectors
C.
fold increase of SI after depletion of CD25bright+
1000
100
10
1
0.1
**
nonrejectors
rejectors
D.
third-party-antigens stimulation
Stimulation Index
650
450
250
50
50
25
0
***
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PBMC
CD25neg/dim
PBMC
CD25neg/dim
nonrejectors
rejectors
E.
fold increase of SI after depletion of CD25bright+
1000
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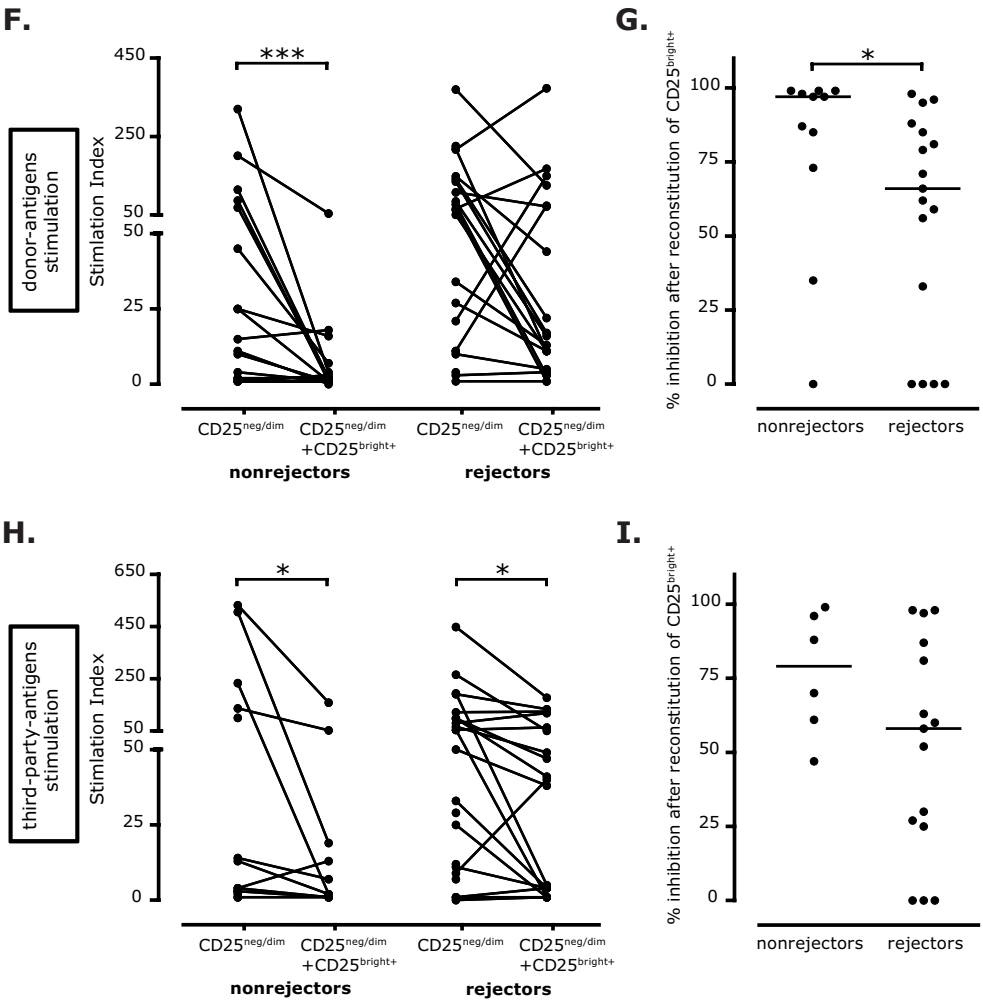

Figure 3. The effect of depletion of and coculturing with $CD4^{+}CD25^{bright+}$ T cells on the proliferative response. **(A)** $CD4^{+}CD25^{bright+}$ T cells were isolated from the PBMC by using the autoMACS. Cells not selected by the separation protocol are referred to as $CD25^{neg/dim}$ responder cells. An example of the obtained populations after isolation is shown. The PBMC and $CD25^{neg/dim}$ responder cells were stimulated with irradiated donor **(B, C)** or third-party spleen cells **(D, E)**. Proliferation was measured by incorporation of ^{3}H-thymidine at day 7. Subsequently, the SI were calculated and plotted. The suppressive capacity of $CD4^{+}CD25^{bright+}$ T cells was determined by calculating the fold increase of the SI after depletion of these cells **(C, E)**. In addition, $CD25^{neg/dim}$ responder cells in the absence and presence of $CD4^{+}CD25^{bright+}$ T cells (ratio 10:1) were stimulated with irradiated donor **(F, G)** or third-party **(H, I)** spleen cells. The suppressive capacity of $CD4^{+}CD25^{bright+}$ T cells was analyzed by calculating the percentage inhibition of the proliferation of the responsive $CD25^{neg/dim}$ cells (i.e., those with SI ≥10) after reconstitution **(G, I)**. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ represent the outcome of the Wilcoxon Signed-Rank test or the Mann-Whitney test, as appropriate. Due to insufficient amount of $CD4^{+}CD25^{bright+}$ T cells, coculture experiments could not be performed for four donor-antigens-stimulated and ten third-party-antigens-stimulated $CD25^{neg/dim}$ responder-cell populations.

Function of pretransplant CD4$^+$CD25$^{bright+}$ T cells

As we observed no differences in regulatory function of CD4$^+$CD25$^{bright+}$ T cells between preAR+ PBMC and AR+ PBMC, we wondered whether the lower suppressive capacity of these cells from rejectors was caused by the transplantation and/or immunosuppressive therapy or was already present before transplantation. We therefore investigated the function of pretransplant CD4$^+$CD25$^{bright+}$ T cells of both rejectors (n=7) and nonrejectors (n=7). Depletion of CD4$^+$CD25$^{bright+}$ T cells from pretransplant PBMC significantly increased the overall anti-donor proliferative response of nonrejectors (p=0.02), but not that of rejectors (Figure 4A). Moreover, the response of nonrejectors was 20 times (median, range: 3 – 312) higher after depletion, whereas only a 2-fold (median, range 0.7 – 123) increase was detected in the rejector group (p=0.04; Figure 4B). Coculturing of CD25$^{neg/dim}$ responder T cells with CD4$^+$CD25$^{bright+}$ T cells in a fixed ratio of 10:1 only significantly inhibited the overall anti-donor proliferative response of nonrejectors (p=0.03; Figure 4C). The % inhibition was higher in the nonrejector group (median: 96%) than in the rejector group (median: 53%), albeit not significantly (p=0.09; Figure 4D).

CD127 expression of CD4$^+$CD25$^{bright+}$FoxP3$^+$ T cells of HTx patients

We analyzed the expression level of CD127 on the CD4$^+$CD25$^{bright+}$FoxP3$^+$ T cells of nonrejectors and rejectors (Figure 5A). An isotype control served as a CD127^{neg} control and CD4$^+$CD25$^{neg/dim}$ T cells as a CD127^{high} control, as these cells have a high expression of the IL-7 receptor α chain (median MFV (range): 48 (34-64)).[24] Interestingly, CD4$^+$CD25$^{bright+}$FoxP3$^+$ T cells of rejectors had a significantly higher expression of CD127 than those of nonrejectors (28 (16–31) vs. 19 (15–26), respectively; p<0.0001; Figure 5B).

This higher expression of CD127 on the CD4$^+$CD25$^{bright+}$FoxP3$^+$ T cells of rejectors may be due to more activated CD25$^+$ responder T cells that transiently express FoxP3 present in the lower percentages of the CD25$^{bright+}$ gate. Hence, we analyzed the CD127 expression of the CD4$^+$ T cells that had the brightest expression of CD25 (top 2% of the gate). Yet, even the CD4$^+$CD25$^{brightest+}$FoxP3$^+$ T cells of rejectors had a higher expression of CD127 compared with those of nonrejectors (27 (16–30) vs. 18 (13–24), respectively; p<0.0001; Figure 5C). Thus, CD127 expression was elevated on all CD4$^+$CD25$^{bright+}$FoxP3$^+$ T cells of rejectors.

Next, we wondered whether CD127 was upregulated on CD4$^+$CD25$^{bright+}$FoxP3$^+$ T cells during allogeneic immune reactivity. No differences in expression level, however, were observed between these cells of preAR+ PBMC and those of AR+ PBMC (28 (22–31) vs. 28 (16–31)), indicating that the CD127 expression was elevated before the histological diagnosis of an allogeneic immune response. We therefore analyzed the expression of CD127 on CD4$^+$CD25$^{bright+}$FoxP3$^+$ T cells of pretransplant PBMC samples. Indeed, CD4$^+$CD25$^{bright+}$FoxP3$^+$ T cells of rejectors

had already a significantly higher expression of CD127 than these cells of nonrejectors before transplantation (22 (18 – 27) vs. 17 (10 – 20), respectively; p=0.004; Figure 5D).

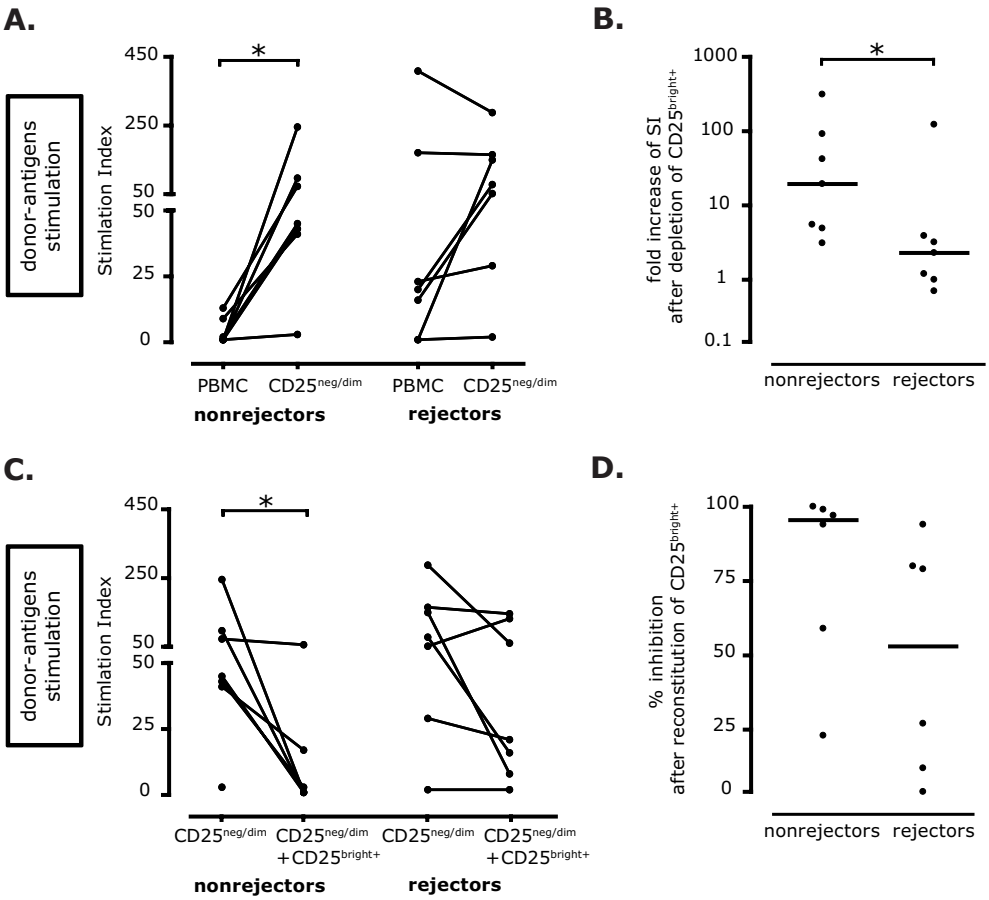

Figure 4. The regulatory function of pretransplant $CD4^{+}CD25^{bright+}$ T cells. Pretransplant PBMC samples of seven nonrejectors and seven rejectors were available for analysis. **(A)** Pretransplant PBMC and $CD25^{neg/dim}$ responder cells were stimulated with irradiated donor spleen cells. Proliferation was measured by incorporation of ^{3}H-thymidine at day 7. The SI were calculated and plotted. **(B)** The suppressive capacity of pretransplant $CD4^{+}CD25^{bright+}$ T cells was determined by calculating the fold increase of the SI after depletion of these cells. **(C)** Subsequently, donor-antigens-stimulated pretransplant $CD25^{neg/dim}$ responder cells were cocultured with $CD4^{+}CD25^{bright+}$ T cells in a fixed ratio of 10 to 1. **(D)** The percentage inhibition of the proliferation of the responsive $CD25^{neg/dim}$ cells (i.e., those with SI ≥10) after reconstitution was calculated. * p≤0.05 represent the outcome of the Wilcoxon Signed-Rank test or Mann-Whitney test, as appropriate.

Discussion

To investigate whether $CD4^+CD25^{bright+}$ regulatory T cells play a clinically relevant role in acute cellular rejection after heart transplantation, we analyzed the function of these cells in HTx patients who remained free from acute rejection and in rejectors before and during an acute rejection episode. We observed that $CD4^+CD25^{bright+}$ T cells of nonrejectors had the capacity to inhibit the allogeneic proliferative response of responder T cells. Our findings that functional $CD4^+CD25^{bright+}$ regulatory T cells are present in the peripheral blood of clinically stable HTx patients are in line with our and other studies in clinically stable kidney transplant patients, in whom functional $CD4^+CD25^{bright+}$ regulatory T cells were readily detectable.[20-22] Functional $CD4^+CD25^{bright+}$ regulatory T cells were also present in the majority of PBMC samples of rejectors, as depletion of these cells increased the proliferation. The extent of increase in these patients, however, was lower than that in nonrejectors. This discrepancy may not be explained by differences in the potency of the effector T-cell population, as the proliferation of the $CD25^{neg/dim}$ responder cells was comparable between rejectors and nonrejectors (Figure 3). Nonetheless, there may be a higher number of activated T cells in rejectors, and such activated T cells may be more resistant to the suppression induced by $CD4^+CD25^{bright+}$ T cells. To test this possibility, we added posttransplant $CD4^+CD25^{bright+}$ T cells of rejectors (n=7) to pretransplant $CD25^{neg/dim}$ responder cells of the same patient, as these cells are not activated by donor antigens. The suppression of the anti-donor proliferative responses of pretransplant $CD25^{neg/dim}$ responder cells, however, was comparable with that measured in posttransplant responder populations (data not shown). So, the differences in the extent of increase between rejectors and nonrejectors must be due to an inadequate immune regulatory function of $CD4^+CD25^{bright+}$ T cells of rejectors.

One hypothesis is that the most vigorous $CD4^+CD25^{bright+}$ regulatory T cells in rejecting HTx patients are not circulating through the peripheral blood, but have migrated to other compartments, such as the secondary lymphoid organs or the transplanted graft. We and others recently demonstrated that FOXP3 mRNA expression levels in the graft were most highly expressed during an acute cellular rejection,[25-27] indicating that intragraft $FOXP3^+$ T cells are an integral component of acute rejection. Our intragraft analyses, however, revealed that the FOXP3 mRNA levels were only highly expressed during the rejection episode and not before this rejection, suggesting that intragraft $FOXP3^+$ T cells are a response to anti-donor effector T-cell activity.[25,27] In this study we observed that the suppressive capacity was already lower in preAR+ PBMC.

An alternative hypothesis is that some patients just have more vigorous $CD4^+CD25^{bright+}$ regulatory T cells in the periphery than others. In these patients, these circulating regulators may control activation of donor-reactive T cells, thereby preventing an immune response directed to the transplanted heart. In the patients with less vigorous circulating $CD4^+CD25^{bright+}$ regulatory T cells, these

cells may not be able to suppress the activation of effector cells effectively and, consequently, acute cellular rejection may occur. A second line of regulation may then be initiated: regulatory T cells home into the graft and adaptive regulators may develop in this compartment to control the damage of graft-destructive effector cells.[28]

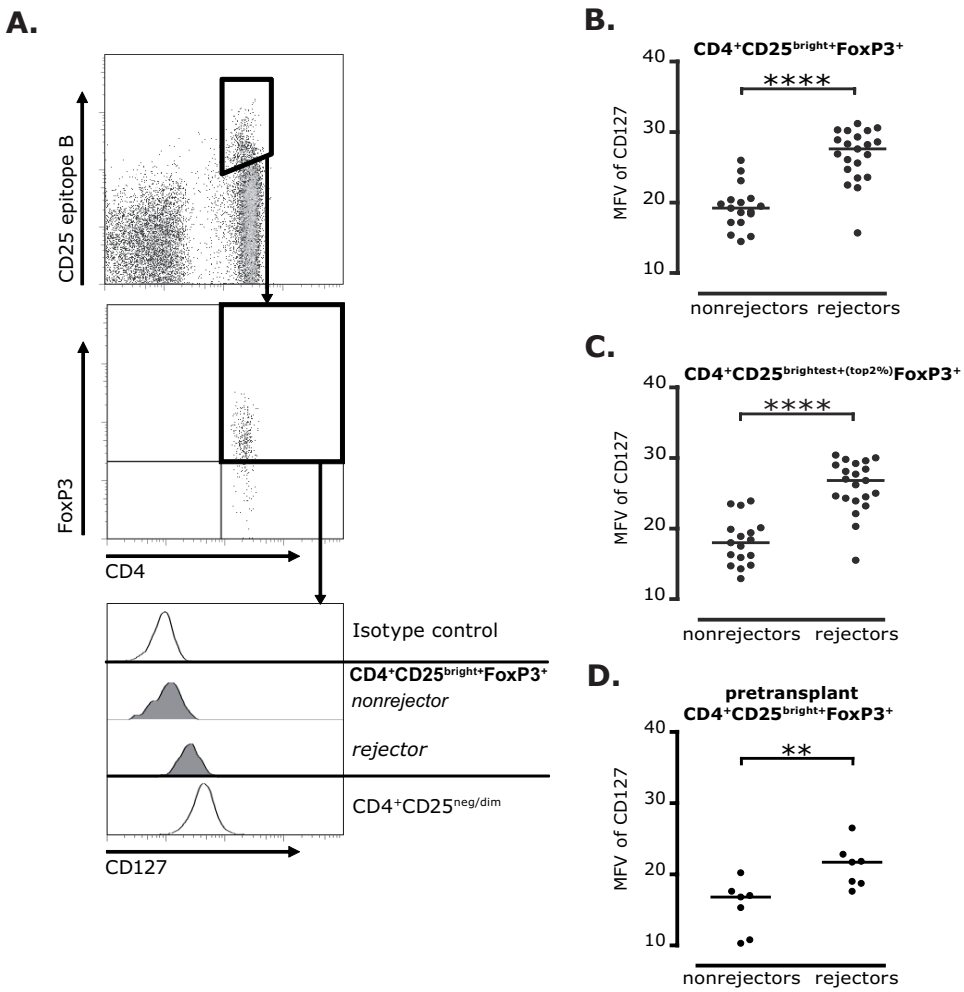

Figure 5. The expression of CD127 of $CD4^+CD25^{bright+}FoxP3^+$ T cells in HTx patients. **(A)** The $CD4^+CD25^{bright+}FoxP3^+$ T cells were analyzed for their expression of CD127. An isotype control served as a $CD127^{neg}$ control, and $CD4^+CD25^{neg/dim}$ cells were used as a $CD127^{high}$ control.[24] The median fluorescence values (MFV; the fluorescence value below which 50% of the events are found) of CD127 of $CD4^+CD25^{bright+}FoxP3^+$ T cells **(B)** and of the $CD4^+CD25^{brightest+}FoxP3^+$ T cells (top 2% of CD25 expressing cells) **(C)** were plotted. Additionally, the CD127 expression of pretransplant $CD4^+CD25^{bright+}FoxP3^+$ T cells **(D)** was analyzed. ** $p \leq 0.01$ and **** $p \leq 0.0001$ represent the outcome of the Mann-Whitney test. Due to insufficient material, flow cytometric analysis could not be performed on one rejector sample.

Recently, Liu et al. suggested that expression of CD127 may be an additional marker for the identification of regulatory T cells, as CD127 inversely correlates with FoxP3 expression and regulatory T-cell activity in healthy human individuals.[14] Interestingly, in HTx patients, the expression of CD127 was higher on $CD4^+CD25^{bright+}FoxP3^+$ T cells of rejectors than on those of nonrejectors. This may be explained by the presence of activated effector T cells in the $CD25^{bright+}$ population. Effector T cells downregulate CD127 early in the course of T-cell activation.[24,29] Along with an upregulated expression of CD25 and transient expression of FoxP3,[29] these cells may be confused with regulatory T cells. Baecher-Allan et al. stated that only the highest CD25 expressors exert significant suppressive effects.[30] We therefore analyzed the CD127 expression in the 2% $CD4^+$ T cells with the brightest CD25 expression. Yet, even the $CD25^{brightest+}FoxP3^+$ T cells of rejectors had a higher expression of CD127 than those of nonrejectors, indicating that the total $CD4^+CD25^{bright+}FoxP3^+$ population of rejectors had this phenotype. A second possibility is that $CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells upregulate CD127 expression in an activated milieu. Allan et al., however, activated $CD4^+CD25bright^+$ regulatory T cells and demonstrated that CD127 expression remained low over the course of activation.[29]

Liu et al. showed that the CD127 promotor is a target for FoxP3 binding and upregulated FoxP3 expression may result in CD127 gene repression.[14] So, a third explanation may be a variation in FoxP3 function or a lower capacity of this transcription factor to interact with or repress the CD127 promotor in rejectors. As FoxP3 is a critical regulator of regulatory T-cell function,[12] this variation in FoxP3 activity may also account for the inadequate function of $CD4^+CD25^{bright+}$ regulatory T cells. Of course, this explanation is purely speculative and needs experimental confirmation. Molecular and functional analyses of sorted $CD4^+CD25^{bright+}$ T cells with different expression levels of CD127 may further clarify the relation of an elevated expression of this receptor, an inadequate immune regulatory function and the occurrence of acute rejections.

In conclusion, $CD4^+CD25^{bright+}FoxP3^+$ T cells of HTx patients who experience an acute cellular rejection episode had an inadequate immune regulatory function compared with those of nonrejecting patients. Additionally, these cells had a higher expression of CD127. These observations suggest that the function of circulating $CD4^+CD25^{bright+}$ regulatory T cells may be pivotal for the prevention of acute cellular rejection after clinical heart transplantation.

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Chapter 7

Donor-specific cytotoxic hyporesponsiveness associated with high IL-10 mRNA expression in heart transplant patients

Dijke IE, Velthuis JHL, Balk AHMM, Korevaar SS, Maat APWM, Weimar W, Baan CC.

Journal of Heart and Lung Transplantation 2006; 25:955-964

Abstract

After transplantation, $CD4^+CD25^+FOXP3^+$ and interleukin (IL)-10 producing regulatory cells may regulate immune responses toward donor antigens. In this study, we determined whether heart transplant patients show donor-specific hyporesponsiveness and studied the underlying mechanisms. We analyzed the donor-specific T-cell responses by mixed lymphocyte reactions and limiting dilution assays to define whether heart transplant patients (n=21) show proliferative and cytotoxic hyporesponsiveness to donor antigens long after transplantation (range: 1.5-7 years). The mechanisms controlling immune responses, i.e., $FOXP3^+/GITR^+$ T cells and IL-10 producing cells, were studied by quantitative real-time PCR. In the presence of a proliferative response to donor antigens, no cytotoxic responsiveness could be measured in a number of patients in the absence (73%) and presence of exogenous IL-2 (29%), IL-15 (31%) and IL-15 plus IL-2 receptor (R)α chain blockade (88%). Overall, the cytotoxic response to donor cells was significantly lower than the reactivity to third-party cells after the addition of IL-2 ($p=0.004$) and IL-15 plus IL-2Rα blockade ($p<0.001$). After donor stimulation, the peripheral blood mononuclear cells expressed higher mRNA levels of IL-10, but not of FOXP3 or GITR, than after third-party stimulation ($p=0.003$). Moreover, the IL-10 mRNA expression was inversely correlated with the donor-specific cytotoxic responsiveness ($p=0.01$). In conclusion, a significant proportion of patients showed donor-specific cytotoxic hyporesponsiveness long after heart transplantation, which was associated with high mRNA levels of IL-10, but not of FOXP3 or GITR. This observation suggests that IL-10 producing cells participate in the donor-specific cytotoxic hyporeactivity.

Introduction

Transplantation has become a well-accepted treatment for end-stage diseases of organs. Immunosuppressive medication induces a state of pseudo-tolerance for the transplanted organ. However, true transplant tolerance (i.e., long-lasting nonreactivity of the immune system to donor antigens in the absence of immunosuppression) has been described in selected liver and kidney transplant patients.[1-3] Decreased alloreactivity to the donor graft has also been found in heart transplant patients. This is reflected by the observation that the frequency of acute rejection episodes decreases with time after transplantation, and by the fact that the immunosuppressive dosage can be gradually lowered.[4] The mechanism of this "transplant acceptance", however, remains unclear. Studying the donor-specific responsiveness and mechanisms controlling these responses *in vitro* several years after transplantation may provide insights into transplant tolerance in heart transplant patients.

Previously, we reported that the cytotoxic T-lymphocyte precursor (CTLp) assay is an informative functional assay to measure and quantify donor-specific responsiveness. For example, kidney transplant patients who experienced acute rejection after withdrawal of immunosuppression had a significantly higher CTLp frequency (CTLpf) than recipients who remained free from rejection.[5] Tapering of immunosuppression in patients with low CTLpf was associated with stable donor-specific hyporeactivity during follow-up.[6,7] In addition, when analyzed by stimulating patient cells with donor antigens via the direct antigen-presenting pathway, several studies have shown that donor-specific cytotoxic reactivity gradually declines over time. Baker et al. found significantly reduced frequencies of donor-specific cytotoxic T cells several years (median: 13 y) after kidney transplantation.[8] Likewise, Hornick et al. showed that donor-specific hyporesponsiveness was demonstrated in 50% of heart transplant patients longer after transplantation (median: 4 y).[9] Thus, cytotoxic hyporesponsiveness to donor antigens can be found after allograft transplantation.

Several immunological mechanisms may participate in the hyporesponsiveness of alloreactive T cells to a donor allograft. T cells with receptors that enable them to recognize donor antigens might be deleted *in vivo*.[10] Alternatively, these cells may become anergic or even completely ignore donor antigen-bearing cells.[11,12] Another mechanism that could be responsible for acceptance of the allograft is active regulation. Several regulatory T-cell populations have been identified, but it seems that 2 types are particularly important in transplantation: the naturally occurring $CD4^+CD25^+FOXP3^+$ regulatory T cells that regulates immune responses in a cell-contact-dependent manner, and the antigen-specific type 1 regulatory T cells (Tr1) that inhibits the immune reactivity through the secretion of interleukin (IL)-10.[13-16]

In all of the above-mentioned immunological mechanisms, cytokines of the IL-2 family play important roles. IL-2 has a dual role, as it is involved in both immunostimulatory and immunosuppressive actions. It is a growth factor for antigen-stimulated T lymphocytes, induces cytotoxicity by upregulating perforin and granzymes and it restores the reactivity of anergic T cells.[17,18] However, IL-2 also controls overall clonal expansion of $CD4^+$ T cells through induction of activation induced cell death and is involved in the development and function of $CD4^+CD25^+$ regulatory T cells.[19,20] Another cytokine of the IL-2 family that functions in both an immunostimulatory and immunoinhibitory manner is IL-15. This cytokine stimulates T-cell survival and proliferation, is an important growth factor for memory phenotype $CD8^+$ T cells and induces the expansion of Tr1 cells.[21,22]

In the present study, we analyzed the potential direct donor-specific proliferative and cytotoxic T-cell responses of peripheral blood mononuclear cells (PBMC) derived from patients long after heart transplantation. To clarify the role of IL-2 and IL-15 in possible regulatory mechanisms, tests were performed in various *in vitro* conditions, including in the absence and presence of exogenous IL-2, IL-15 and IL-15 plus an IL-2 receptor (R)α chain blockade. We also examined the mRNA expression levels of the $CD4^+CD25^+$ regulatory T-cell markers FOXP3 and GITR, and the immunoregulatory cytokine IL-10. Altogether, we observed a donor-specific cytotoxic hyporesponsiveness that was associated with high expression of IL-10 mRNA, suggesting the involvement of IL-10 producing regulatory cells.

Materials and methods

Patients

Twenty-one heart transplant patients were studied at a median of 4 years (range: 1.5-7 years) after heart transplantation. The characteristics are described in Table 1. Maintenance immunosuppressive therapy consisted of calcineurin inhibitors and low-dose steroids for all patients, and 10 patients were also treated with anti-proliferative agents. Monitoring of rejection was performed by endomyocardial biopsies, and specimens were graded according to the guidelines of the 1990 International Society for Heart and Lung Transplantation.[23] Coronary artery disease was defined as all vascular wall changes, including minor irregularities, as assessed by visual analysis of coronary angiograms.[24]

Isolation of peripheral blood lymphocytes

PBMC were isolated by density gradient centrifugation using Ficoll-Paque (density 1.077 g/ml; Amersham Pharmacia Biotech, Uppsala, Sweden). The PBMC were washed twice with RPMI 1640 (BioWhittaker, Verviers, Belgium) and frozen in

RPMI 1640-DM (Gibco BRL, Paisley, Schotland) supplemented with 2 mM L-Glutamine (Gibco BRL), 100 IU/ml penicillin (Gibco BRL), 100 μg/ml streptomycin (Gibco BRL), 10% fetal bovine serum (FBS) (Biowhittaker) and 10% dimethyl sulfoxide (Merck, Schuchardt, Germany) and stored at -140ºC until analysis.

Stimulator cells

For all patients, spleen cells from the donor were available. Donor and third-party spleen cells were obtained by homogenizing spleen in RPMI 1640-DM containing 10 μg/ml DNAse (Roche Molecular Biochemicals, Mannheim, Germany), 100 IU/ml penicillin and 100 μg/ml streptomycin. The cell suspension was centrifuged over a Ficoll-Paque gradient, collected, washed and frozen and stored at -140°C until analysis.

Table 1. Characteristics of the heart transplant patients

No.	21
Recipient age (at HTx) (y)	46,6 ± 12,4*
Recipient gender (M/F)	20/1
Primary heart disease (n):	
Cardiomyopathy/ischemic heart disease	11/10
Cold ischemia time (min)	160,6 ± 34,6*
HLA-A/B mismatches (n)	3,0 ± 1,1*
HLA-DR mismatches (n)	1,6 ± 0,5*
Induction therapy (n):	
None/ATG/anti-CD25	10/8/3
Immunosuppressive therapy (n):	
CNI/Pred	11
CNI/Pred/AZA	4
CNI/Pred/MMF	6
No. of acute rejections 1 year	1,4 ± 1,4*
coronary artery disease 1 year (no/yes)	15/6

HTx, heart transplantation; ATG, anti-thymocyte globulin; CNI, calcineurin inhibitors; Pred, prednisone; MMF, mycophenolate mofetil; AZA, azathioprine.
*Mean±SD

Culture medium

Culture medium consisted of RPMI 1640-DM supplemented with 2 mM L-Glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated pooled human serum.

Cytokines and antibodies

Recombinant human cytokines were added to the cultures in final concentrations of 200 U/ml IL-2 (PeproTech, Inc., Rocky Hill, NJ) and 10 ng/ml IL-15 (PeproTech, Inc.) and 5 mg/ml αCD25 (Daclizumab; Hoffman-La Roche, Basel, Switzerland) was used to block the IL-2Rα chain. After addition of αCD25, the plates were pre-incubated for 1 hour at 37°C/ 5% CO_2 before to the addition of the stimulator cells.

Viability assay

The viability of the PBMC and spleen cells was tested by using the phytohemagglutinin (PHA) proliferation assay. After $5x10^4$ cells were exposed to 1 μg/ml PHA (Murex Biotech LTd, Kent, United Kingdom) for 72 hours, cell proliferation was measured by ^{3}H-thymidine incorporation (0.5 μCi/well; Amersham Pharmacia Biotech) during the last 8 hours of culture. A 1450 MicroBeta liquid scintillation spectrophotometer (Wallac, Turku, Finland) was used to analyze ^{3}H-thymidine incorporation. All patient PBMC, donor and third-party spleen cells showed a proliferative response to PHA.

Mixed lymphocyte reaction

After thawing, $5x10^4$ patient PBMC with $5x10^4$ irradiated (40 Gy) donor or third-party spleen cells (fully HLA-A, -B and -DR mismatched with patient and donor), were cultured in round-bottomed wells (triplicate) of a 96-well culture plate (NUNC, Roskilde, Denmark) without exogenous cytokines (standard MLR), with exogenous IL-2, IL-15 and IL-15 plus αCD25. The plates were incubated at 37ºC/5% CO_2. After 7 days, cell proliferation was measured by ^{3}H-thymidine incorporation.

Limiting dilution assay

Limiting dilution assays (LDA) were performed in the absence and presence of exogenous cytokines, e.g., IL-2 (standard LDA), IL-15 and IL-15αCD25, respectively. Patient PBMC were titrated in 24 replicates in 7-fold double dilution, starting from 50,000 cells down to 781 cells per well in 96-well round-bottomed plates. To each well were added $5x10^4$ irradiated (40 Gy) donor or third-party spleen cells. Wells containing irradiated stimulator cells alone served as background control. The cultures were incubated for 7 days at 37ºC/5% CO_2. After 7 days the cytotoxicity was determined by the europium release assay.

PHA blasts

$7x10^6$ Donor spleen cells and third-party spleen cells were cultured for 7 days in RPMI 1640-DM supplemented with 2 mM L-Glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10% FBS, 1 μg/ml PHA, and 200 U/ml IL-2.

Europium release assay

The europium release assay, as described by Bouma et al., was used.[25] Briefly, the PHA blasts were washed twice with PBS at 4°C and labeled with europium chloride (Fluka, Buchs, Switzerland) and diethylene triamine pentaacetic acid (DTPA; Sigma, St. Louis, MO) in the presence of dextran sulfate (10 mg/ml; Fluka). After 20 minutes, the blasts were sealed by adding calcium chloride (Merck) and left on ice for another 10 minutes. After washing, 5,000 blasts were added per well to the cells cultured in the LDA. The plates were incubated at 37°C for 4 hours. After centrifugation, 20 μl supernatant was harvested from each well and transferred to flat-bottom Fluoronunc microtiter plates (NUNC), 100 μl enhancement solution (LKB-Wallac) was added and the fluorescence was measured in a time-resolved fluorometer (Victor 1420 Multilabel Counter, LKB-Wallac). As a control for every blast, spontaneous lysis (target cell plus culture medium) and maximum lysis (target cells plus 1% Triton X-100 (Sigma)) was determined.

mRNA expression analysis by quantitative real-time PCR

Quantitative real-time PCR (Q-PCR) technique was used to quantify mRNA expression levels of FOXP3, GITR, IL-10 and the housekeeping gene 18S in patient PBMC after allogeneic stimulation.[26] Total RNA was isolated using the High Pure RNA Isolation kit (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. RNA concentrations were measured by the RiboGreen RNA quantification reagent and kit (Molecular Probes, Eugene, OR), and cDNA was synthesized from 500 ng RNA with random primers. FOXP3, GITR and IL-10 analysis was performed using Assay on Demand; pre-developed TaqMan assays (PDAR) were used to measure 18S concentrations (Applied Biosystems, Foster City, CA). For FOXP3, GITR and IL-10, 5 μl of cDNA was added to 20 μl PCR mixture containing 12.5 μl Universal PCR Master Mix (Applied Biosytems), 0.625 μl primers and FAM-labeled probe, and 6.875 μl H_2O. For 18S, 5 μl of cDNA was added to 20 μl PCR mixture containing 12.5 μl Universal PCR Master Mix (Applied Biosytems), 1.25 μl primers and probe (Vic labeled), and 6.25 μl H_2O.

The PCR cycling program consisted of 40 2-step cycles of 15 seconds at 95°C and 60 seconds at 60°C, and was performed with the ABI 7700 sequence detector system (Applied Biosystems). Real-time measurements were taken, and Ct values were calculated by determining the background-exceeding point

of >0.04. The relative concentration of FOXP3, GITR and IL-10 was normalized to the relative concentration of 18S. To investigate whether culturing in the presence of IL-2, IL-15 and IL-15 plus αCD25 affected mRNA expression levels, we set the mRNA expression levels measured in donor-antigens-stimulated PBMC cultured in de absence of exogenous cytokines as 100% and analyzed the relative mRNA expression levels. Also, the IL-10 mRNA expression levels in third-party-stimulated PBMC was compared with the IL-10 mRNA expression measured after donor-antigens stimulation, which was set as 100%.

Statistical analysis

After the europium release assay, the mean fluorescence+3xSD of the wells in which only stimulator cells were present, was considered as background. The experimental wells were considered negative if the fluorescence did not exceed the background. The number of negative wells for each LDA concentration was determined and the CTLp frequency was calculated with a computer program designed by Strijbosch et al.[27] The calculated frequencies were accepted when the goodness-of-fit did not exceed 12. The frequencies are expressed as the number of CTLp per 10^6 cells. The Wilcoxon Signed-Rank test and the Spearman's coefficient were used for statistical analysis. Statistical significance was set at $p \leq 0.05$.

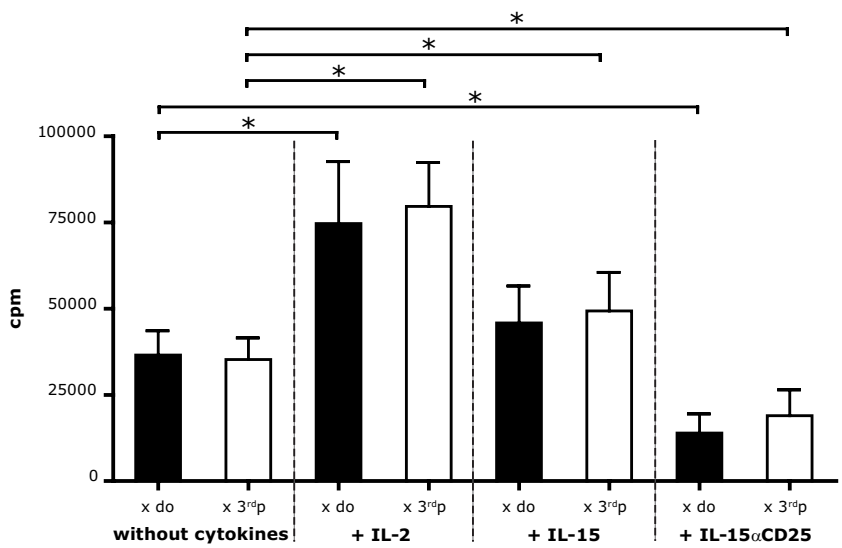

Figure 1. The proliferative response of PBMC of heart transplant patients. The proliferative response in counts per minute (cpm) of heart recipient PBMC to donor antigens (black bars) and third-party antigens (white bars) was measured in the absence of exogenous cytokines (n=21) (standard MLR) or in the presence of IL-2 (n=10), IL-15 (n=13) or IL-15 plus αCD25 (n=15). Due to a shortage of material, the proliferative response of some patient PBMC could not be tested under all the different growth conditions. The mean±SEM is depicted in this figure. * p≤0.05.

Results

The proliferative response

In all the MLR, irrespective of the growth conditions, PBMC had comparable proliferative responses to donor and third-party antigens (Figure 1). In the presence of IL-2, all patient PBMC had a proliferative response to the donor and third-party antigens that was higher than in the MLR without exogenous cytokines ($p=0.02$ and $p=0.03$, respectively). IL-15 also had a stimulatory effect on the proliferative response to both donor and third-party antigens ($p=0.07$ and $p=0.01$, respectively), albeit less outspoken than that of IL-2. However, when IL-15 was added to the cultures in combination with αCD25, a lower response was measured to donor and third-party antigens compared with the MLR without exogenous cytokines ($p=0.02$ and $p=0.01$, respectively).

The cytotoxic response

To investigate the cytotoxic response of patient PBMC to donor and third-party antigens, the CTLp frequencies (CTLpf) were determined. The results are depicted in Figure 2. In the absence of exogenous cytokines, no difference in CTLpf to the donor (median: $3/10^6$ PBMC) and third-party antigens (median: $13/10^6$ PBMC) was measured ($p=0.19$). The addition of exogenous IL-2 resulted in a significantly higher CTLpf to both donor and third-party antigens compared with the LDA without exogenous cytokines ($p<0.001$). Moreover, the donor-specific CTLpf (median: $20/10^6$ PBMC) was significantly lower than the CTLpf to third-party antigens (median: $108/10^6$ PBMC; $p=0.004$). Thus, IL-2 facilitated the outgrowth of cytotoxic T cells directed against third-party antigens and, to a lesser extent, against donor antigens.

In the presence of exogenous IL-15, the cytotoxic response of donor-antigens-stimulated PBMC (median: $11/10^6$ PBMC) was comparable with the response of these PBMC cultured in the absence of exogenous cytokines ($p=0.38$), although the addition of IL-15 in combination with αCD25 led to a lower CTLpf (median: $1/10^6$ PBMC; $p=0.08$). In contrast, the CTLpf to third-party antigens was significantly higher when the PBMC were cultured in the presence of exogenous IL-15 (median: $41/10^6$ PBMC) compared with the CTLpf without exogenous cytokines ($p=0.005$), whereas addition of IL-15 plus αCD25 resulted in a similar CTLpf (median: $10/10^6$ PBMC; $p=0.38$). Furthermore, also in the presence of exogenous IL-15 and certainly when this cytokine was added in combination with αCD25, the CTLpf of patient PBMC to donor antigens was lower than to third-party antigens ($p=0.09$ and $p<0.001$, respectively). Clearly, there is a cytotoxic hyporesponsiveness to donor antigens compared with third-party antigens. We therefore set out to determine the possible involvement of $CD4^+CD25^+FOXP3^+$ or IL-10 producing regulatory cells.

mRNA expression levels

From donor and third-party-antigens-stimulated patient PBMC, mRNA was extracted and quantitative real-time PCR was performed to determine mRNA expression levels of FOXP3, GITR and IL-10. Overall, FOXP3 and GITR mRNA expression levels did not differ between donor antigens and third-party-antigens-stimulated PBMC (p=0.49 and p=0.62, respectively; data not shown). In contrast, donor-antigens-stimulated PBMC had significantly higher IL-10 mRNA expression levels than PBMC stimulated with third-party antigens (p=0.003). Furthermore, the IL-10 mRNA expression levels in the allogeneic-stimulated PBMC were clearly influenced by the addition of cytokines to the culture. As shown in Figure 3A, the expression levels measured in PBMC stimulated with donor cells in the absence of exogenous cytokines were set as 100%. In the presence of IL-2, 75% less IL-10 mRNA expression was found. Exogenous IL-15 did not lead to different mRNA expression levels, but the addition of IL-15 in combination with αCD25 resulted in 150% more IL-10 mRNA expression. Additionally, a lower percentage IL-10 mRNA expression was found in all third-party-antigens-stimulated PBMC compared with donor-antigens-stimulated PBMC cultured under the same growth conditions (Figure 3B).

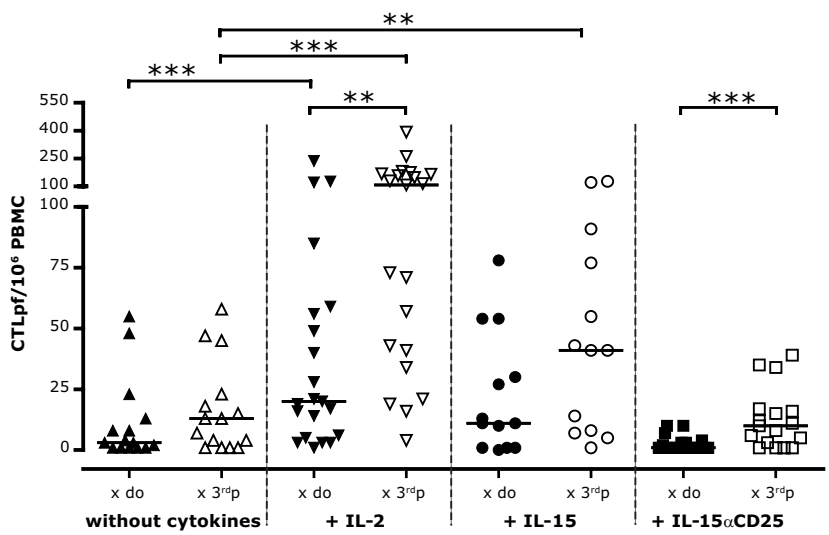

Figure 2. The cytotoxic response of PBMC of heart transplant patients. The CTLp frequencies of heart recipient PBMC to donor antigens (black) and third-party antigens (white) were measured in the absence of exogenous cytokines (n=15) or in the presence of IL-2 (n=21) (standard LDA), IL-15 (n=13) or IL-15 plus αCD25 (n=17). Due to a shortage of material, the cytotoxic response of some patient PBMC could not be tested under all the different growth conditions. ** p≤ 0.01; *** p≤ 0.001.

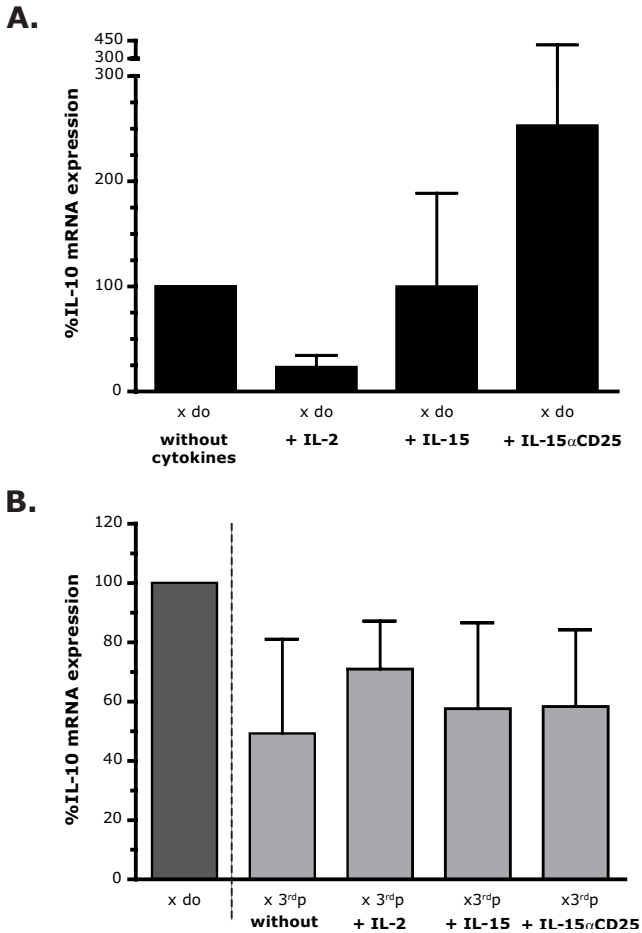

Figure 3. mRNA expression levels of the immunoregulatory cytokine IL-10. **(A)** To investigate whether culturing PBMC under different growth conditions, i.e., in the presence of IL-2, IL-15 or IL-15 plus αCD25, affected the IL-10 mRNA expression levels, we set the mRNA expression levels measured in donor-antigens-stimulated PBMC cultured in de absence of exogenous cytokines as 100% and analyzed whether the different growth conditions led to a higher or lower percentage of mRNA expression. **(B)** In addition, to examine whether the IL-10 mRNA expression levels in third-party-antigens-stimulated PBMC differ from the expression in donor-antigens-stimulated PBMC cultured under the same growth conditions, we set the IL-10 mRNA expression measured after donor-antigens stimulation as 100% and analyzed whether third-party stimulation resulted in a higher or lower percentage of mRNA expression. Due to a shortage of material, the IL-10 mRNA expression levels of several patient PBMC could not be tested under all the different growth conditions. The mean±SEM of 10 patients are depicted in this figure.

Correlation between the CTLp frequency and IL-10 mRNA expression

We hypothesized that IL-10 producing cells may participate in the regulation of donor-specific immune responses. Therefore we analyzed whether the CTLp frequency was related to the IL-10 mRNA expression levels. We found a close inverse correlation between the CTLpf and IL-10 mRNA expression in donor-antigens-stimulated PBMC cultured in the standard LDA conditions, i.e., in the presence of exogenous IL-2 ($R=-0.76$; $p=0.01$; Figure 4A). In addition, the impact of additive exogenous cytokines on the CTLpf to donor antigens proportionally correlated with the effect on IL-10 mRNA expression levels ($R=-0.63$; $p=0.01$). In the absence of exogenous cytokines, the CTLpf and the IL-10 mRNA expression levels were set as 100% (Figure 4B). Addition of IL-2 resulted in a 6-fold higher CTLpf, whereas the IL-10 mRNA expression was decreased with a factor 4 ($p<0.001$). The addition of IL-15 to the culture did not influence the CTLpf toward donor antigens and the IL-10 mRNA expression levels also remained unaffected ($p=0.38$ and $p=0.99$, respectively). In contrast, IL-15 in combination with αCD25 resulted in a 2-fold lower CTLpf, whereas the IL-10 mRNA expression increased with a factor 2.5 ($p=0.08$).

In contrast, no inverse correlation was found between the CTLpf and IL-10 mRNA expression in third-party-antigens-stimulated PBMC ($R=-0.35$; $p=0.33$; Figure 4C). The impact of the additive exogenous cytokines on the CTLpf to third-party antigens and IL-10 mRNA expression levels is depicted in Figure 4D. The CTLpf and the IL-10 mRNA expression levels of third-party-antigens-stimulated PBMC cultured without exogenous cytokines were set as 100%. Addition of IL-2 led to a 9-fold higher CTLpf, whereas the IL-10 mRNA expression was 1.7-fold lower ($p<0.001$ and $p=0.14$, respectively). The addition of IL-15 resulted in an increase of the CTLpf with a factor 3 ($p=0.005$). Nevertheless, the IL-10 mRNA expression in the PBMC cultured in the presence of IL-15 was not different from those cultured with exogenous IL-2 ($p=0.64$). Finally, the CTLpf of the PBMC cultured in the absence of cytokines and those cultured in IL-15αCD25 were comparable, whereas the IL-10 mRNA expression was almost 3 times higher cultured in the presence of the exogenous cytokine and the blocking antibody, albeit not significantly ($p=0.38$ and $p=0.22$).

Discussion

To define whether immunosuppressed heart transplant patients exhibit antigen-specific hyporesponsiveness, we measured both the proliferative and cytotoxic reactivity against donor and third-party antigens. Although most of the PBMC had a proliferative response to both donor and third-party antigens, a significant proportion showed a lower cytotoxic responsiveness to donor antigens than to third-party antigens. No differences were detectable in the number of donor-recipient HLA mismatches, donor ischemia time, the number of acute rejections

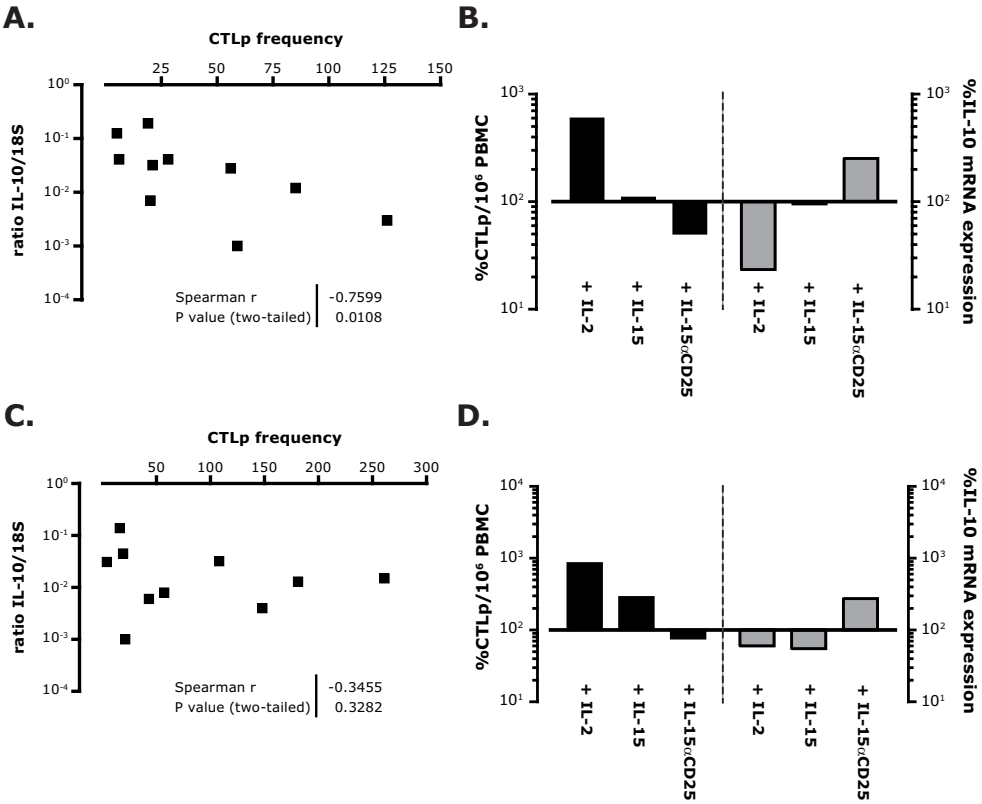

Figure 4. Correlation between the CTLp frequency and IL-10 mRNA expression. Correlation between the CTLp frequency and the IL-10 mRNA expression levels of patient PBMC after stimulation with donor antigens **(A)** and third-party antigens **(C)** cultured in the standard LDA conditions (i.e., in the presence of exogenous IL-2). In addition, the impact of exogenous cytokines on the CTLpf (black bars) was compared with the effect on the IL-10 mRNA expression levels measured in the PBMC (grey bars) after stimulation with donor antigens **(B)** and third-party antigens **(D)**. The CTLpf and the IL-10 mRNA expression measured after allogeneic stimulation without exogenous cytokines were set as 100%. Due to a shortage of material, the IL-10 mRNA expression levels of several patient PBMC could not be tested under all the different growth conditions. The results of 10 patients are depicted in this figure.

in the first year, or the development of coronary artery disease between the patients with a cytotoxic response and those hyporesponsive to donor antigens. The type of immunosuppressive medication and the presence or absence of anti-proliferative agents did not make a difference in proliferation and cytotoxic responsiveness (data not shown).

The data of the MLR without exogenous cytokines is consistent with an earlier study, in which no donor-specific proliferative hyporesponsiveness was found in well-functioning heart transplant patients in a long-term follow-up

(range: 3.5-6 years).[28] The addition of growth factors had a stimulatory effect on the proliferative response, but again, no difference in proliferation to donor and third-party antigens was observed. However, the MLR examines the overall proliferation of the alloreactive T cells and no information is obtained on the number of responding cells, so differences between donor and third-party responses could be overlooked.

A more sensitive assay to examine the reactivity of specific alloreactive T cells is the limiting dilution assay. This assay can measure different effector functions, such as proliferation, cytokine secretion, and cytotoxicity, and quantifies the number of responsive cells, such as helper T-lymphocyte precursors (HTLp) or cytotoxic T-lymphocyte precursors (CTLp). Several studies have found lower HTLp and CTLp frequencies to donor antigens than to third-party antigens in kidney, heart and lung transplant patients several years after transplantation.[6,8,9,29,30] Because of limited patient and donor material we focused on the cytotoxic cell. We found no differences in cytotoxic response to donor and third-party antigens in the absence of exogenous cytokines. However, this assay requires addition of the T-cell growth factor IL-2 because of the low frequency of responder T cells in the culture.[5] Indeed, we found much higher frequencies when IL-2 was added. Interestingly, a significantly lower CTLpf was found to donor antigens than to third-party antigens ($p=0.004$) and no donor-specific cytotoxic response could be measured at all in 6 (29%) of 21 patients.

Apart from IL-2, we also investigated the effect of IL-15 and IL-15 plus αCD25 on the cytotoxic responsiveness of patient PBMC to donor antigens. Grabstein et al. showed that IL-15 can substitute IL-2 in the induction of alloantigen-specific cytotoxic T lymphocytes.[31] Yet again, a lower cytotoxic reactivity was found to donor antigens than to third-party antigens, and a considerable number of patients showed no donor-specific reactivity. These data suggests that donor-specific cytotoxic hyporesponsiveness is present in a number of heart transplant patients long after heart transplantation that cannot be annihilated by IL-2 and IL-15.

Several immunological mechanisms may contribute to the observed donor-specific cytotoxic hyporesponsiveness: anergy, ignorance, deletion and regulation.[10-12,16] Anergic as well as ignorant T cells do not proliferative upon antigen-recognition.[12,32] The state of anergy can subsequently be overcome by addition of exogenous IL-2.[18] In our experiments PBMC proliferated well upon stimulation with irradiated donor cells. Thus, the PBMC are neither anergic nor ignorant. Addition of IL-2 led to an increase in proliferation, but this is due to growth enhancing effect of IL-2 on activated T cells and not to the reversal of an anergic state.

Deletion of T cells with a T-cell receptor capable of recognizing donor antigens seems unlikely, since the PBMC proliferated upon donor antigens. However, it cannot completely be excluded that $CD4^+$ T cells are still present in the peripheral

blood of heart transplant patients that can respond to donor antigens, but that the $CD8^+$ T cells that could recognize these antigens were deleted. Since the MLR depends mostly on $CD4^+$ T cells and the CTLpf on $CD8^+$ T cells, this would explain the discrepancy. Nevertheless, it seems more plausible that if T cells with a specific T-cell receptor were deleted *in vivo*, the $CD4^+$ T cells were also deleted. An alternative explanation therefore seems more likely.

Another mechanism that might contribute to graft acceptance is active regulation. Two well-characterized regulatory T cells are the naturally occurring $CD4^+CD25^+FOXP3^+$ regulatory T cells and the antigen-specific IL-10 producing Tr1 cells. Since no differences in the specific regulatory T-cell marker FOXP3[33] expression levels could be detected after stimulation, it seems unlikely that these regulatory T cells are involved in the donor-specific hyporesponsiveness. This is in line with Game et al.[34] who suggested that regulation by $CD4^+CD25^+$ T cells does not account for the direct pathway hyporesponsiveness that occurs in most kidney transplant patients. Nevertheless, the addition of IL-2 to the culture may influence the suppressive capacity of the $CD4^+CD25^+$ regulatory T cells.[20,35]

In contrast to FOXP3, large quantities of IL-10 mRNA were detected in PBMC stimulated with donor antigens. Moreover, this IL-10 mRNA showed an inverse correlation with the CTLp frequency (Figure 4; $p=0.01$) to donor antigens. Our experiments support the findings by VanBuskirk et al., who showed that donor-antigen-triggered regulation relied on IL-10 in humans.[1] In experimental transplant models, Hara et al. demonstrated that antigen-specific regulatory T cells were responsible for the maintenance of donor-specific tolerance and required IL-10.[36] Moreover, Alberu et al. observed a higher IL-10 production by the PBMC in long-term kidney transplant patients with excellent graft function.[37] Yet, we should bear in mind that these studies measured IL-10 protein levels in the culture supernatants, whereas we measured IL-10 mRNA expression levels in our limited patient material. Nonetheless, Wang et al. found little evidence for posttranscriptional regulation of IL-10 mRNA, so the IL-10 mRNA levels are expected to reflect the protein levels.[38]

Our findings suggest a role for IL-10 producing regulatory cells in the donor-specific cytotoxic hyporesponsiveness. From these experiments, however, it is unclear whether the IL-10 mRNA was produced by regulatory Tr1 cells or by Th2 cells, B lymphocytes or dendritic cells, all capable of producing high amounts of IL-10.[39-41] It is suggested that the Th2 cell cytokine production mediates induction of tolerance toward the transplanted organ by suppressing Th1 responses.[42] As for B cells, Fillatreau et al. described a role in regulating immune responses by controlling the T-cell differentiation through the production of IL-10.[40] An important role for patient and donor dendritic cells (DC) in the induction of T-cell hyporesponsiveness has also been demonstrated. The group of Thomson showed that donor-derived DC had an inhibitory effect on allogeneic T-cell proliferation and cytokine production.[43,44] In addition, several other studies have reported

that the IL-10 producing recipient DC induced the generation of IL-10 secreting, antigen-specific regulatory T cells.[14,45,46] Phenotyping the IL-10 producing cells should elucidate whether regulatory T cells or other potent IL-10 producers are responsible for the high IL-10 mRNA expression levels.

One possible explanation for the involvement of IL-10 producing regulatory cells but not the $CD4^+CD25^+FOXP3^+$ regulatory T cells may be the immunosuppressive treatment. Previously, we have shown that in de presence of the calcineurin inhibitors cyclosporine A and tacrolimus, no significant induction of FOXP3 mRNA in allogeneic MLR could be detected.[26] Therefore, the presence of calcineurin inhibitors may impair the outgrowth and function of $CD4^+CD25^+FOXP3^+$ regulatory T cells. Moreover, calcineurin inhibitors prevents the activation of nuclear factor of activated T cells (NFAT), an important transcription factor for a variety of cytokine genes like IL-2, but not IL-10.[47]

In conclusion, we found that a significant proportion of patients showed donor-specific cytotoxic hyporesponsiveness long after heart transplantation that could not be broken by IL-2 and IL-15. Furthermore, the donor-specific hyporesponsiveness was associated with IL-10 mRNA levels, but not with FOXP3 or GITR mRNA levels. These observations suggest that within the recipient's PBMC IL-10 producing regulatory cells are present, although from these experiments it can not be excluded that the IL-10 mRNA was produced by B lymphocytes, dendritic cells or Th2 cells. Obviously, this aspect needs further clarification.

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Summary and discussion

Part IV

Chapter 8

Summary and discussion

Discussion is partially based on:

Regulatory T cells after organ transplantation: where does their action take place? Dijke IE, Weimar W, Baan CC.
Human Immunology 2008; 69:389-398

Summary

T-cell-mediated regulation of donor-reactive cells is one of the mechanisms that may be involved in the induction and maintenance of graft acceptance after organ transplantation. The identification and characterization of regulatory T cells that can control the anti-donor immune reactivity has therefore become the focus of many studies. Research in experimental transplant models demonstrated that these regulators are important for the prevention of allograft rejection and the induction of transplant tolerance.[1-4] Yet, it remains to be elucidated whether regulatory T cells control anti-donor immune reactivity in immunosuppressed organ transplant patients, thereby inducing and maintaining donor-specific nonresponsiveness. The central aim of this thesis was to investigate the role of regulatory T cells in the control of immune responses directed to the graft of heart transplant recipients.

Part I of this thesis comprises a general introduction and the outline of the thesis. In **Chapter one**, we described the regulatory T-cell populations that have been identified in the past three decades. Furthermore, we reviewed the current knowledge about the presence of functional donor-directed regulatory T cells in the secondary lymphoid organs, peripheral blood and the transplanted organ itself. A description of the objectives of our studies is also included in this chapter.

In **Part II**, we defined our studies on the presence of regulatory T cells in the transplanted heart. **Chapter two** addressed the question whether the presence of these cells is associated with immunological processes, such as immunological quiescence and acute cellular rejection. We analyzed the gene expression profiles of regulatory T-cell (associated) and effector T-cell markers in endomyocardial biopsies (EMB) taken from heart transplant recipients who remained free from rejection and those who experienced at least one acute cellular rejection episode. We found that the mRNA levels of FOXP3, the key regulatory transcription factor for the development and function of cells with immune regulatory activities,[5] were higher in EMB with histological signs of acute rejection than in EMB without evidence of myocardial damage ($p<0.003$). In addition, the mRNA levels of other regulatory T-cell-associated markers (CD25, GITR, CTLA-4) as well as effector T-cell markers (IL-2 and Granzyme B) were also significantly higher in rejection EMB compared with nonrejection EMB. The relation of high FOXP3 mRNA levels with acute cellular rejection, but not with immunological quiescence, suggest that intragraft $FOXP3^+$ T cells do not prevent acute rejection, but rather are a response to anti-donor effector T-cell activity.

Next, we aimed to elucidate whether lymphocytes present in rejecting human cardiac allografts indeed exhibit immune regulatory activities on donor-directed immune responses. For studying the regulatory capacities of graft lymphocytes (GL), *ex vivo* expansion of these cells is warranted to extract the lymphocytes

from the biopsies. In **Chapter three**, a preliminary study is presented in which we investigated whether FoxP3 protein-expressing GL could be cultured from EMB with histological signs of acute cellular rejection. We observed that 2-17% of expanded $CD3^+$ GL expressed FoxP3. Thus $FoxP3^+$ T cells can be grown from EMB, providing a tool to functionally characterize these cells. In **Chapter four**, we described our study in which expanded GL cultures were tested for their regulatory capacities on allogeneic immune reactivity. We observed that, when GL cultures were hyporesponsive to donor antigens, these cells suppressed the anti-donor proliferative T-cell response of patient peripheral blood mononuclear cells (PBMC), but not the anti-third party T-cell response. Further analysis of GL subsets to determine which population is responsible for the observed suppression revealed that the $CD8^+$ GL vigorously inhibited the anti-donor response (65-91% inhibition of proliferating T cells), whereas the tested $CD4^+$ GL of the expanded GL cultures were not suppressive. Our intragraft studies showed that lymphocyte populations present in the transplanted heart during acute cellular rejection not only consist of graft-destructive effector T cells, but also of cells with the potential to specifically inhibit anti-donor immune reactivity.

In **Part III** of this thesis, we outlined the frequency and function of regulatory T cells in the peripheral blood of heart transplant recipients. In consideration of the high of FOXP3 mRNA expression levels in the graft during acute cellular rejection, we addressed in **Chapter five** the question whether FOXP3 mRNA levels in the peripheral blood also reflect anti-donor immune responses. In that case, these levels could provide clues for noninvasive detection of immunological processes in the graft. FOXP3 mRNA expression patterns were examined in PBMC of heart transplant patients collected during immunological quiescence and acute cellular rejection. Yet, in contrast to the intragraft expression of FOXP3, the peripheral FOXP3 mRNA levels lack correlation with immunological processes in the graft. Consequently, this gene does not appear to be a potential candidate for noninvasive diagnosis of quiescence or rejection.

In **Chapter six**, we described our study on the clinically relevant role of peripheral regulatory T cells in the control of acute cellular rejection. For this research, we focused on a specific subset of regulatory T cells: the $CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells (Chapter one). The phenotype and the immune regulatory function of these regulators of patients free from acute rejection were compared with that of $CD4^+CD25^{bright+}FoxP3^+$ T cells of patients who experienced an acute rejection episode. No significant differences in the proportion of $CD25^{bright+}$ cells within the $CD4^+$ T-cell population were found between the two patient groups. In addition, the proportion of $FoxP3^+$ cells within the $CD4^+CD25^{bright+}$ T cells was comparable. Yet, phenotypic differences were observed for the CD127 expression: $CD4^+CD25^{bright+}FoxP3^+$ T cells of rejectors had a higher expression of this marker than those of nonrejectors ($p<0.0001$). Functional analysis revealed that the $CD4^+CD25^{bright+}$ T cells of rejectors had an inadequate immune regulatory function compared with those of nonrejecting patients. Depletion of these cells

from PBMC of rejectors resulted in only a two-fold increase of the anti-donor proliferative response, whereas the response of nonrejectors became 14 times higher (p=0.002). Coculture experiments with $CD4^{+}CD25^{bright+}$ cells confirmed these findings: the percentage inhibition of the proliferative response of $CD25^{neg/dim}$ responder cells was much higher in the nonrejector group (median: 97%) than in the rejector group (66%; p=0.02). Analysis of pretransplant samples showed that $CD4^{+}CD25^{bright+}$ T cells of rejectors already had a lower suppressive capacity than those of nonrejectors before transplantation. The findings of this study indicate that the function of peripheral $CD4^{+}CD25^{bright+}FoxP3^{+}$ regulatory T cells may be pivotal for the prevention of acute cellular rejection after clinical heart transplantation.

Finally we aimed to define whether heart transplant patients exhibit antigen-specific hyporesponsiveness some years after transplantation. We reported our results of the potential donor-specific proliferative and cytotoxic T-cell responses of PBMC collected long after transplantation (median: 4 years) in **Chapter seven**. To clarify the role of cytokines of the IL-2 family in possible regulatory mechanisms, the tests were performed in various *in vitro* conditions, including in the absence and presence of exogenous IL-2, IL-15, and IL-15 plus an IL-2 receptor α chain blockade. In addition, the mRNA expression levels of FOXP3 and GITR, and the immunoregulatory cytokine IL-10 were examined. Although most PBMC had a proliferative response to both donor and third-party antigens, a significant proportion of patients (73%) showed donor-specific cytotoxic hyporesponsiveness that could not be broken by IL-2 and IL-15. In addition, the mRNA expression levels of IL-10, but not of FOXP3 or GITR, were higher after donor-antigens stimulation than after third-party-antigens stimulation (p=0.003). Moreover, the IL-10 mRNA expression was inversely correlated with the donor-specific cytotoxic responsiveness (p=0.01), suggesting a role for IL-10 producing cells in the donor-specific cytotoxic hyporeactivity some years after transplantation.

Discussion

Experimental transplant studies have demonstrated that regulatory T cells are important for the prevention of acute rejection and the prolongation of graft survival.[1,3,6] Moreover, regulatory T cells with the potential to suppress an anti-donor immune response have been found in both secondary lymphoid organs and the transplanted organ itself, indicating that immune regulation may occur at several sites.[4,7] Likewise, in human transplant recipients, evidence of the presence of potent regulatory T cells in different compartments is rising.[8-12] Our studies described in this thesis demonstrated that cells with immune regulatory activities were present in both the graft and the peripheral blood of heart transplant recipients.

Differences in FOXP3 gene expression in the transplanted heart found between immunological quiescence and anti-donor immune reactivity, however, suggest that the localization of regulatory T cells may depend on the immunological state in the patient. We observed that the intragraft FOXP3 mRNA levels were most highly expressed during acute cellular rejection. Functional analysis of lymphocytes present in the graft during anti-donor immune reactivity demonstrated that this population indeed comprises cells with the potential to specifically inhibit anti-donor immune responses (Table 1). Our results are supported by studies in clinical kidney transplantation. Muthukumar et al. observed higher FOXP3 mRNA levels in urinary samples of kidney transplant recipients with an acute rejection than in that of patients with normal biopsy results.[10] In addition, Veronese et al. and Bunnag et al. also found high FOXP3 mRNA levels during cellular T-cell-mediated rejection.[13,14] The findings in clinical organ transplantation are in contrast to findings in experimental transplant models in which high intragraft FOXP3 mRNA levels were associated with graft acceptance.[15,16] So, in the clinical setting, intragraft immune regulation may be established during alloreactivity. Cells with potential immune regulatory activities in the graft may play a role in "damage control" rather than prevent the allograft rejection.

On the other hand, the results of our studies in the peripheral blood of heart transplant recipients seem to suggest a preventive role for regulatory T cells. We showed that patients free from acute rejection had more vigorous suppressing peripheral $CD4^{+}CD25^{bright+}FoxP3^{+}$ regulatory T cells than patients who experienced an acute rejection episode. In addition, the presence of IL-10 producing cells in the peripheral blood was associated with donor-specific cytotoxic hyporesponsiveness some years after transplantation (Table 1). So far, few studies have compared the function of peripheral regulatory T cells between immunological quiescence and alloreactivity. Demirkiran et al. examined the function of $CD4^{+}CD25^{bright+}$ regulatory T cells in rejecting and nonrejecting liver transplant recipients.[17] They, however, found no differences in the function of these cells between the two patient groups. Yet, the function was analyzed at one year after transplantation, whereas the acute rejection episodes occurred in the first three months after transplantation. So the function of the $CD4^{+}CD25^{bright+}$ T cells before or during the acute rejection is not known. Akl et al. analyzed the function of $CD4^{+}CD25^{bright+}$ regulatory T cells in kidney transplant recipients with stable graft function and in those with biopsy-proven chronic allograft rejection.[18] While these cells in the stable recipients exhibited strong donor-antigens-directed suppressive capacities, the $CD4^{+}CD25^{bright+}$ T cells in chronically rejecting patients showed impaired regulatory activities. Thus, in contrast to the intragraft regulatory T cells, peripheral regulatory T cells may play a role in preventing anti-donor immune reactivity.

Based on the findings in this thesis, and supported by findings reported by others, we propose the following hypothesis on the role of regulatory T cells in the control of anti-donor immune reactivity. During immunological quiescence, regulatory

Table 1. Overview of the results described in this thesis

Time after Tx	In the transplanted heart	In the peripheral blood	Regulatory T-cell population
< 1 year	High FOXP3 mRNA expression levels are associated with anti-donor immune reactivity *(Chapter two)*	Lack of correlation between FOXP3 mRNA expression levels and immunological processes in the graft *(Chapter five)*	FOXP3 mRNA expressing cells
	During acute cellular rejection, cells with the potential to specifically inhibit anti-donor immune reactivity can be present *(Chapter three and four)*		$CD8^+$ T cells
		Inadequate immune regulatory function and upregulated expression of CD127 in patients who experience an acute cellular rejection *(Chapter six)*	$CD4^+CD25^{bright+}FoxP3^+$ T cells
1 – 7 years		Donor-specific cytotoxic hyporesponsiveness is associated with high mRNA expression levels of IL-10 *(Chapter seven)*	IL-10 mRNA expressing cells

T cells circulate among secondary lymphoid organs, blood and the transplanted organ. In these compartments, regulators control both activation and expansion of donor-reactive T cells, thereby preventing the induction of an immune response directed to the donor antigens (Figure 1A). However, when circulating regulatory T cells fail to suppress the activation of effector T cells and the anti-donor immune reactivity is escalating out of control, a second line of regulation is initiated: naturally occurring regulatory T cells home into the graft and antigen-specific adaptive regulators develop in this compartment to control the damage of graft-destructive effector cells (Figure 1B). In this way, regulatory T cells play a role in the control of the anti-donor reactivity at various stages of the immune response.

In the past three decades, several regulatory T-cell populations have been identified (Chapter one). In organ transplantation, attention has nowadays mainly focused on the role of $CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells in the induction of graft acceptance.[19] Nonetheless, clinical studies have shown that other regulatory T-cell populations, such as $CD8^+CD28^-FoxP3^+$ suppressor T cells and Type 1 regulatory (Tr1) T cells, may be involved in the control of anti-donor immune responses as well.[20,21] In our experiments, we detected in the peripheral blood a possible regulatory role for the $CD4^+CD25^{bright+}FoxP3^+$ T cells shortly after heart transplantation. Longer after transplantation, we observed that IL-10 producing cells may be involved in anti-donor immune responses. In the transplanted graft, evidence of $CD8^+$ cells with potential immune regulatory activities was found (Table 1). These findings suggest that several regulatory T-cell populations can be involved in the control of alloreactivity. Thus, by focusing on just one regulatory T-cell population we may overlook significant information.

Whether all these regulatory T-cell populations play a role in the control of the anti-donor reactivity at the various stages of the immune response remains to be elucidated. It is possible that certain regulatory T cells are involved in preventing the induction of an immune response, while others are more important for 'damage control'. Studies in experimental inflammation models have demonstrated that naive $CD4^+CD25^+$ regulatory T cells controlled the induction phase of an immune response, while effector/memory-like $CD4^+CD25^+$ regulators were present at the site of inflammation to control ongoing responses.[22,23] So, within the $CD4^+CD25^+$ regulatory T-cell population, regulators with a specific role, i.e., prevention of anti-donor immune reactivity or 'damage control', are present. $CD8^+$ regulatory T cells and IL-10 producing regulatory T cells can be induced after repeated stimulation with antigens in the presence of the IL-2 and IL-10, respectively.[24,25] These cells can thus be generated after transplantation. The conditions for the generation of $CD8^+$ regulatory T cells are optimal in the transplanted heart (presence of antigens) during alloreactivity (presence of IL-2), and these cells may thus play a role in the control of alloreactivity in the graft. In contrast, we observed no increase of intragraft IL-10 gene expression during acute cellular rejection, so this may not be the compartment were the action of IL-10 producing regulators takes place. Further research on the different regulatory T-cell populations

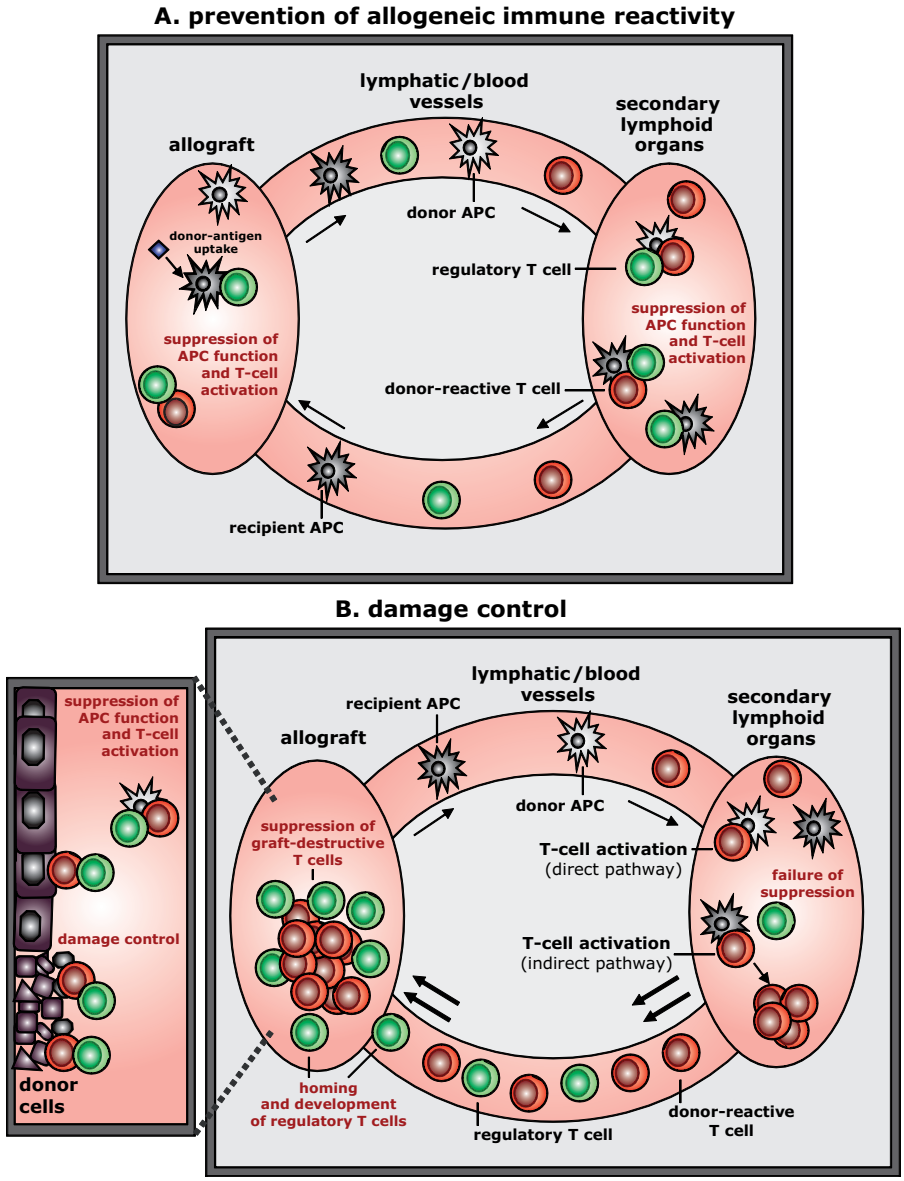

Figure 1. Schematic overview of the different localization and migration patterns of regulatory T cells during immunological quiescence and anti-donor immune reactivity. **(A)** During immunological quiescence, regulatory T cells circulate among secondary lymphoid organs, blood and the transplanted organ. In these compartments, regulators control both activation and expansion of donor-reactive T cells, thereby preventing the induction of an immune response directed to donor antigens. **(B)** When circulating regulatory T cells fail to suppress the activation of effector T cells and the anti-donor immune reactivity is escalating out of control, a second line of regulation is initiated: naturally occurring regulatory T cells home into the graft and antigen-specific adaptive regulators develop in this compartment to control the damage of graft-destructive effector cells.

will clarify where and when the action of a certain population takes place.

This thesis provides new insights on the role of regulatory T cells in immune regulation after heart transplantation. Obviously, the next question is: can we use these new insights for clinical applications? We presume that phenotypical and functional analyses of regulators may be of great value for the immunological monitoring of heart transplant recipients. For example, we observed a relation between an elevated expression of CD127 on inadequate regulating $CD4^{+}CD25^{bright+}$ T cells and the occurrence of acute cellular rejection. Flow cytometric analyses of the expression of this marker may thus identify patients who are more susceptible to acute cellular rejection episodes. The advantage of flow cytometry is that it's a rapid, quantitative and validated technique, which makes it useful for monitoring heart transplant patients. If we can discriminate patients who will reject their graft from those who will not, we may stratify patients towards more individualized immunosuppressive regimens thereby improving long term survival and quality of life. To confirm that CD127 is a useful marker to predict acute cellular rejection, a prospective study should be conducted.

A clinical application for regulatory T cells that is of great interest is the usage of these cells as cellular therapeutic agents. These cells may be generated *in vitro* by manipulating T cells isolated from the recipient. Several studies have reported the *in vitro* development of regulatory T cells using either mouse or human T cells.[26-29] Ideally, these cells should only suppress immune responses directed to donor antigens. Thus, before regulators can be used as cellular therapeutic agents, their donor-antigens specificity should be characterized. In this thesis, we demonstrated that the source for donor-antigens-specific regulatory T cells is the transplanted heart: cells with donor-specific immune regulatory activities were expanded *in vitro* from rejecting human cardiac allografts. These cells may thus theoretically be potential candidates for cellular therapy. Yet, if these cells can only be expanded from rejecting allografts, it would mean that these cells may just be used as therapeutic agents for anti-rejection treatment or for the prevention of another acute cellular rejection. The danger of *ex vivo* expansion of regulatory T cells, however, is that the behavior of these cells after infusion in the transplant recipient is unknown. Do these cells maintain their regulatory activity? Is their behavior affected by the environment in which they reside? Do they migrate to the compartments where they should exhibit their regulatory function? And what is the effect of immunosuppressive drugs on the function of expanded regulatory T cells? All these factors will require careful evaluation in relevant experimental transplant models before graft-derived regulatory T cells may be considered as cellular therapy for the treatment of transplant patients.

We conclude that various regulatory T-cell populations present in the peripheral blood and the transplanted heart play a role in immunological processes, such as immunological quiescence and acute cellular rejection, in heart transplant patients.

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Chapter 9

Nederlandse samenvatting (Dutch summary)

Samenvatting

Orgaantransplantatie is een laatste behandelingsmogelijkheid voor een patiënt wiens orgaan door ziekte of aangeboren afwijking zijn functie verloren heeft. Tijdens de transplantatie wordt het orgaan van de patiënt vervangen door een gezond orgaan van een donor. Het immuunsysteem van de patiënt ziet het nieuwe orgaan echter als lichaamsvreemd en stoot het transplantaat af. Om afstoting te voorkomen moet een patiënt langdurig behandeld worden met medicijnen die het immuunsysteem onderdrukken. Deze immunosuppressieve medicijnen verminderen het risico van afstoting na transplantatie. Zij onderdrukken het immuunsysteem echter op een niet-specifieke manier, waardoor de patiënten een verhoogd risico hebben op infecties en kanker. Daarnaast kent het gebruik van deze medicijnen ook andere ernstige bijwerkingen, zoals nierfalen, botontkalking en hart- en vaatziekten.

Een alternatieve benadering om afstoting te voorkomen, en zo ook het gebruik van immunosuppressiva terug te brengen, is het induceren van tolerantie ten opzichte van het getransplanteerde orgaan. Tolerantie kan mogelijk tot stand gebracht worden door een speciale subpopulatie van de witte bloedcellen, de regulatoire T cellen (of suppressor T cellen). Deze cellen kunnen immuunresponsen tegen eigen en lichaamsvreemde (allogene) antigenen onderdrukken. Onderzoek in de transplantatie immunologie richt zich dan ook op de identificatie en karakterisatie van regulatoire T cellen die de immuunreactiviteit tegen donorantigenen onderdrukken. In proefdiermodellen is al aangetoond dat deze cellen een rol spelen in de preventie van afstoting en het induceren van tolerantie. Kennis van de rol van regulatoire T cellen in immunologische processen in orgaantransplantatiepatiënten die behandeld worden met immunosuppressieve medicijnen is echter nog beperkt. Verder is het onduidelijk waar in het lichaam deze cellen een interactie aangaan met de cellen die de afstotingsreactie in gang zetten. Het onderzoek beschreven in dit proefschrift had als doel de rol van regulatoire T cellen in de controle van immuunresponsen tegen het transplantaat van hartontvangers te onderzoeken. We hebben ons gericht op twee plaatsen waar functionele regulatoire T cellen mogelijk aanwezig zijn, namelijk het getransplanteerde hart en het perifere bloed.

Het eerste gedeelte (**Part I**) van dit proefschrift omvat een algemene introductie en de opzet van het proefschrift. In **Hoofdstuk 1** beschrijven we de verschillende regulatoire T celpopulaties die in de afgelopen jaren geïdentificeerd zijn. Verder bespreken we de huidige literatuur over de aanwezigheid van functionele donorgerichte regulatoire T cellen in de secundaire lymfoïde organen, het perifere bloed en het getransplanteerde orgaan. Ook wordt het doel van ons onderzoek behandeld.

In het tweede gedeelte (**Part II**) van dit proefschrift behandelen we ons onderzoek naar de aanwezigheid van regulatoire T cellen in het getransplanteerde

hart. In **Hoofdstuk 2** vroegen we ons af of de aanwezigheid van deze cellen geassocieerd is met immunologische processen, zoals immunologische rust en acute afstoting. We analyseerden de mRNA expressieprofielen van regulatoire T cel (geassocieerde) en effector T cel markers in biopten genomen van harttransplantatiepatiënten die vrij bleven van acute afstoting en van patiënten die minstens één acute afstotingsepisode doormaakten in het eerste jaar na transplantatie. We vonden dat de mRNA expressieniveaus van FOXP3, een eiwit dat betrokken is bij de ontwikkeling en functie van regulatoire T cellen,[1] hoger waren in biopten met histologische tekenen van acute afstoting dan in biopten waarin geen beschadiging aan het hartspierweefsel was waargenomen. Ook de mRNA expressieniveaus van andere regulatoire T cel geassocieerde markers (CD25, GITR, CTLA-4) en van effector T cel markers (IL-2 en Granzyme B) waren hoger in afstotingsbiopten dan in de biopten zonder tekenen van afstoting. De relatie van hoge FOXP3 mRNA expressie met acute afstoting, maar niet met immunologische rust, suggereert dat $FOXP3^+$ T cellen in het transplantaat acute afstoting niet voorkomen, maar een reactie zijn op antidonor effector T cel activiteit.

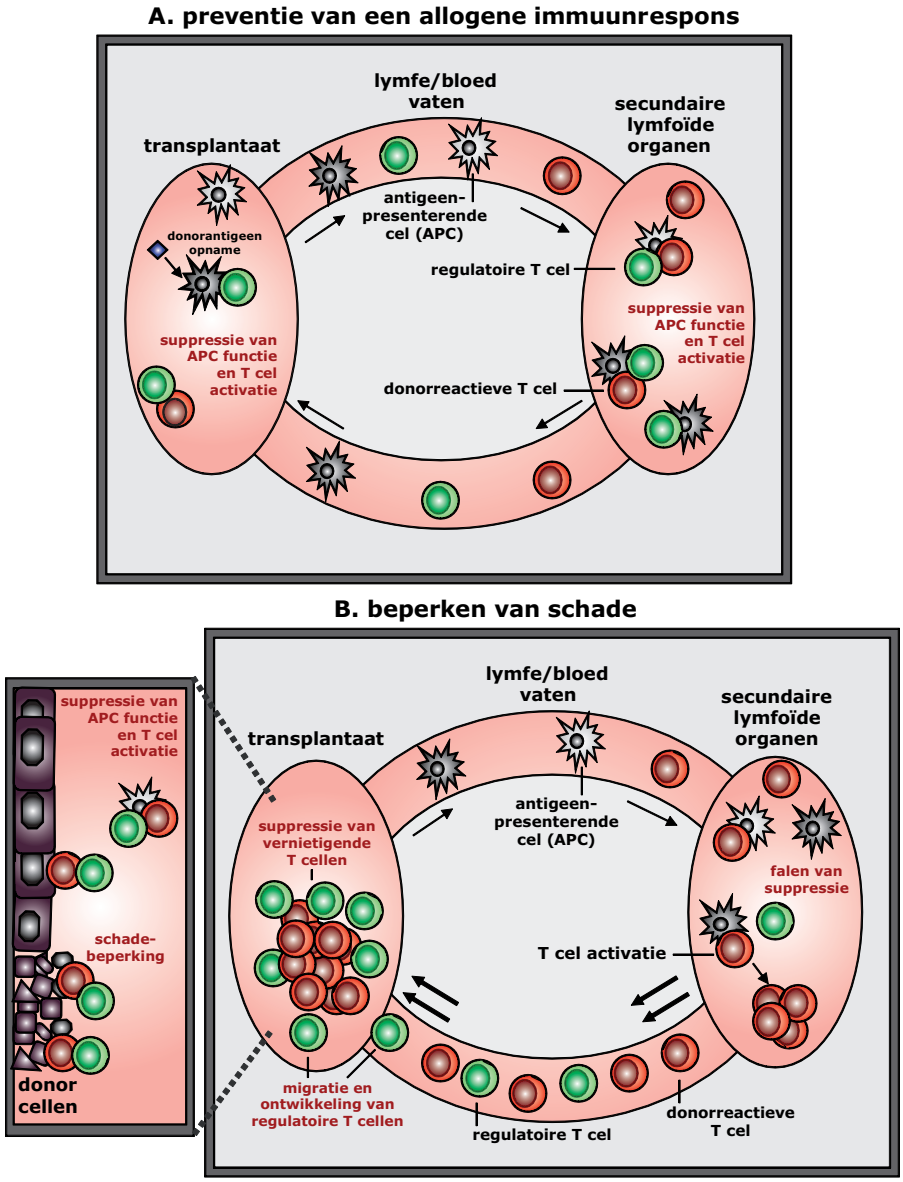
Hierop wilden we onderzoeken of lymfocyten die aanwezig zijn in het transplantaat tijdens acute afstoting daadwerkelijk suppressieve activiteiten op de afweerreactie vertonen. Om de suppressieve capaciteiten van transplantaat (graft) lymfocyten (GL) te kunnen bestuderen moeten deze cellen eerst uit een biopt gekweekt worden. In **Hoofdstuk 3** wordt een preliminaire studie beschreven waarin we bekeken hebben of $FoxP3^+$ GL gekweekt konden worden uit biopten met histologische tekenen van acute afstoting. We vonden dat 2-17% van de GL FoxP3-eiwit tot expressie bracht. Dus $FoxP3^+$ GL kunnen gekweekt worden uit een afstotingsbiopt, wat ons de mogelijkheid geeft deze cellen functioneel te karakteriseren. In **Hoofdstuk 4** bespreken we de studie waarin de gekweekte GL getest werden op hun suppressieve activiteit op allogene immuunresponsen. We constateerden dat, wanneer GL zelf niet prolifereren (=vermenigvuldigen van het aantal cellen) na stimulatie met donorantigenen, deze cellen de antidonor T cel proliferatie van lymfocyten uit het perifere bloed onderdrukten, maar niet de T cel proliferatie geïnduceerd door andere 'vreemde' antigenen (derde-partij antigenen). Verdere analyses van GL subsets om te onderzoeken welke subpopulatie verantwoordelijk is voor de suppressie lieten zien dat de $CD8^+$ GL de antidonor respons sterk onderdrukten. De geteste $CD4^+$ GL van de kweken waren niet suppressief. Samengevat, de studies gericht op het getransplanteerde hart lieten zien dat, tijdens een acute afstoting, lymfocytenpopulaties in het transplantaat niet alleen bestaan uit transplantaatvernietigende effector T cellen, maar ook uit cellen die de potentie hebben om de afweerreactie te onderdrukken en zo de schade mogelijk kunnen beperken.

In deel drie (**Part III**) van het proefschrift beschrijven we het onderzoek dat gericht is op de frequentie en functie van regulatoire T cellen in het perifere bloed van harttransplantatiepatiënten. Met het oog op de hoge FOXP3 mRNA

expressieniveaus in het transplantaat tijdens een acute afstoting, vroegen we ons in **Hoofdstuk 5** af of de FOXP3 mRNA expressieniveaus in het bloed ook geassocieerd zijn met antidonor immuunresponsen. Deze niveaus kunnen we dan mogelijk gebruiken voor non-invasieve detectie van immunologische processen die in het transplantaat plaatsvinden. FOXP3 mRNA expressiepatronen werden geanalyseerd in perifere bloed mononucleaire cellen (PBMC) van harttransplantatiepatiënten afgenomen tijdens immunologische rust en acute afstotingen. We vonden echter, in tegenstelling tot de FOXP3 mRNA expressie in het transplantaat, geen relatie tussen de perifere FOXP3 mRNA expressieniveaus en de immunologische processen in het transplantaat. FOXP3 lijkt daardoor geen potentiële kandidaat voor non-invasieve diagnose van rust of afstoting.

In **Hoofdstuk 6** behandelen we de klinisch relevante rol van perifere regulatoire T cellen in de controle van acute afstoting. Voor dit onderzoek hebben we ons gericht op een specifieke subset van regulatoire T cellen: de $CD4^{+}CD25^{bright+}FoxP3^{+}$ regulatoire T cellen (Hoofdstuk 1). Het fenotype en de suppressieve functie van deze cellen van patiënten die vrij bleven van acute afstoting werden vergeleken met dat van $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cellen van patiënten die wel een acute afstotingsreactie doormaakten. Er werden tussen de twee patiëntengroepen geen significante verschillen gevonden in het percentage cellen dat CD25 sterk tot expressie bracht binnen de $CD4^{+}$ T celpopulatie. Ook het percentage $FoxP3^{+}$ cellen binnen deze $CD4^{+}CD25^{bright+}$ T celsubset was vergelijkbaar. Fenotypische verschillen werden wel gevonden in expressie van CD127: de $CD4^{+}CD25^{bright+}FoxP3^{+}$ T cellen van afstoters hadden een hogere expressie van deze marker dan deze cellen van niet-afstoters. Functionele analyses lieten zien dat de $CD4^{+}CD25^{bright+}$ T cellen van afstoters een slechte suppressieve functie hadden vergeleken met de $CD4^{+}CD25^{bright+}$ cellen van niet-afstoters. Het verwijderen van deze cellen uit de PBMC van niet-afstoters resulteerde in een 14 keer zo hoge respons tegen donorantigenen, terwijl de respons van afstoters maar 2 keer hoger werd. Het weer toevoegen van de $CD4^{+}CD25^{bright+}$ T cellen bevestigde de gevonden resultaten: het percentage remming van de proliferatie van $CD25^{neg/dim}$ responder cellen was veel hoger in de niet-afstotersgroep (mediaan: 97%) dan in de afstotersgroep (mediaan: 66%). Analyses van pretransplantatie PBMC toonden dat de $CD4^{+}CD25^{bright+}$ T cellen van afstoters al een lagere suppressieve capaciteit hadden voor transplantatie dan deze cellen van niet-afstoters. De bevindingen van deze studie doen vermoeden dat de functie van circulerende $CD4^{+}CD25^{bright+}FoxP3^{+}$ regulatoire T cellen belangrijk is voor de preventie van acute afstoting na harttransplantatie.

Ten slotte vroegen we ons af of harttransplantatiepatiënten enkele jaren na transplantatie een verlaagde immuunrespons tegen donorantigenen hebben. Onze resultaten over de donorspecifieke proliferatieve en cytotoxische (=het vernietigen van cellen) T cel responsen van PBMC die lang na transplantatie verzameld zijn (mediaan: 4 jaar) worden beschreven in **Hoofdstuk 7**. Om de rol van cytokines (eiwitten die de interacties tussen cellen reguleren) van de IL-2 familie in mogelijke



Figuur 1. Schematische weergave van verschillende plaatsen waar regulatoire T cellen aanwezig zijn en naartoe verplaatsen tijdens immunologische rust en een antidonor immuunrespons. **(A)** Tijdens immunologische rust circuleren regulatoire T cellen tussen de secundaire lymfoïde organen, het bloed en het getransplanteerde orgaan. Op deze plaatsen onderdrukken de regulatoire T cellen zowel de activatie als de proliferatie van donorreactieve T cellen, waardoor een immuunrespons gericht tegen donorantigenen voorkomen wordt. **(B)** Wanneer circulerende regulatoire T cellen niet in staat zijn om de activatie van effector T cellen te onderdrukken en de antidonor immuunrespons uit de hand gaat lopen, wordt een tweede lijn van regulatie geïnitieerd: 'natuurlijk voorkomende' regulatoire T cellen migreren naar het transplantaat en antigeenspecifieke adaptieve regulatoire T cellen ontstaan mogelijk op deze plaats om zo de schade van transplantaatvernietigende effector T cellen te beperken.

regulerende mechanismen te verklaren werden de experimenten uitgevoerd in verschillende *in vitro* condities, waaronder in afwezigheid en aanwezigheid van IL-2, IL-15 en IL-15 in combinatie met een antilichaam dat een bepaald gedeelte van de IL-2 receptor, de α keten, afdekt. Daarnaast zijn mRNA expressieniveaus van FOXP3, GITR en het immunoregulatoire cytokine IL-10 bestudeerd. Ofschoon de meeste PBMC een proliferatieve respons gericht tegen zowel donor- als derde-partij antigenen hadden, vertoonde een groot gedeelte van de PBMC een lage donorspecifieke cytotoxische responsiviteit dat niet opgeheven kon worden door het toevoegen van IL-2 of IL-15. De mRNA expressieniveaus van IL-10, maar niet van FOXP3 of GITR, waren hoger na stimulatie met donorantigenen, dan na stimulatie met derde-partij antigenen. Deze hoge IL-10 mRNA expressie was gerelateerd aan de lage cytotoxische responsiviteit gericht tegen donorantigenen, wat een rol suggereert voor IL-10 producerende regulatoire cellen enkele jaren na harttransplantatie.

Discussie

Studies in proefdiermodellen hebben laten zien dat regulatoire T cellen belangrijk zijn voor de preventie van acute afstotingen en voor transplantaat-overleving.[2-4] Verder is beschreven dat regulatoire T cellen met de potentie om antidonor immuunresponsen te onderdrukken aanwezig zijn in zowel secundaire lymfoïde organen als het getransplanteerde orgaan zelf, wat suggereert dat immuunregulatie op meerdere locaties kan plaatsvinden.[5,6] Ook in orgaantransplantatiepatiënten neemt het bewijs toe dat potente regulatoire T cellen aanwezig zijn op verschillende plaatsen in het lichaam.[7-11] Onze studies beschreven in dit proefschrift lieten zien dat cellen met suppressieve capaciteiten aanwezig zijn in zowel het perifere bloed als het transplantaat van hartontvangers.

Verschil in FOXP3 mRNA expressie in het getransplanteerde hart tussen immunologische rust en een allogene immuunrespons doet vermoeden dat de aanwezigheid van regulatoire T cellen in het transplantaat afhankelijk is van de immunologische staat van de ontvanger. We vonden dat de FOXP3 mRNA expressieniveaus in het transplantaat hoog waren tijdens een acute afstoting. Functionele analyses van lymfocyten die in het hart aanwezig waren tijdens een acute afstoting lieten zien dat deze populatie inderdaad cellen bevat die de potentie hebben de afweerreactie te onderdrukken (Tabel 1). Onze resultaten komen overeen met bevindingen in klinische niertransplantatie studies.[9,12,13] Daarin werden ook hoge FOXP3 mRNA expressieniveaus juist tijdens een acute afstoting gemeten. De bevindingen in klinische orgaantransplantatie staan in contrast met bevindingen in proefdiermodellen waarin hoge FOXP3 mRNA expressie in het transplantaat geassocieerd is met tolerantie.[14,15] Dus in patiënten lijkt immuunregulatie in het getransplanteerde orgaan tot uiting te komen tijdens allogene reactiviteit. Cellen met potentiële suppressieve activiteiten spelen in het

transplantaat dus waarschijnlijk eerder een rol in het beperken van de schade aan hartspiercellen, dan dat ze acute afstotingen voorkomen.

Daartegenover suggereren de resultaten van onze studies in het perifere bloed dat de functie van perifere regulatoire T cellen mogelijk belangrijk is voor preventie van een afweerreactie. Patiënten die vrij bleven van acute afstoting hadden krachtigere onderdrukkende $CD4^+CD25^{bright+}FoxP3^+$ regulatoire T cellen dan patiënten die een acute afstoting doormaakten. Verder was de aanwezigheid van IL-10 producerende cellen in het perifere bloed geassocieerd met een lage cytotoxische responsiviteit (Tabel 1). Tot nu toe hebben maar weinig studies de functie van perifere regulatoire T cellen geanalyseerd in relatie tot immunologische rust en alloreactiviteit. Eén studie heeft de suppressieve functie van $CD4^+CD25^{bright+}$ regulatoire T cellen onderzocht in levertransplantatiepatiënten die vrij bleven van acute afstoting en in patiënten die minstens één acute afstotingsepisode doormaakten.[16] Hierin werd geen verschil in functie gevonden. Echter, de functie werd 1 jaar na transplantatie geanalyseerd, terwijl de acute afstotingen binnen de eerste 3 maanden na transplantatie plaatsvonden. Dus de suppressieve functie van $CD4^+CD25^{bright+}$ regulatoire T cellen voor en tijdens de afstoting is onbekend. Een andere studie heeft de suppressieve functie van $CD4^+CD25^{bright+}$ regulatoire T cellen bestudeerd in niertransplantatiepatiënten met een stabiele nierfunctie en in patiënten met een chronische afstoting.[17] De $CD4^+CD25^{bright+}$ regulatoire T cellen van stabiele niertransplantatiepatiënten vertoonden krachtig onderdrukkende capaciteiten. Deze cellen van chronisch afstotende patiënten, daarentegen, hadden een slechte suppressieve functie. Dus perifere regulatoire T cellen lijken, in tegenstelling tot de regulatoire T cellen in het transplantaat, een rol te hebben in de preventie van antidonor immuunreactiviteit.

Gebaseerd op de resultaten van dit proefschrift, en ondersteund door bevindingen beschreven door anderen, stellen we de volgende hypothese over de rol van regulatoire T cellen in de controle van immuunresponsen tegen het transplantaat. Tijdens immunologische rust circuleren regulatoire T cellen tussen de secundaire lymfoïde organen, het bloed en het getransplanteerde orgaan. Op deze plaatsen onderdrukken de regulatoire T cellen zowel de activatie als de proliferatie van donorreactieve T cellen, waardoor een immuunrespons tegen donorantigenen voorkomen wordt (Figuur 1A). Wanneer circulerende regulatoire T cellen echter niet in staat zijn om de activatie van effector T cellen te onderdrukken en de antidonor immuunrespons uit de hand gaat lopen, wordt een tweede lijn van regulatie geïnitieerd: 'natuurlijk voorkomende' regulatoire T cellen migreren naar het transplantaat en antigeenspecifieke adaptieve regulatoire T cellen ontstaan op deze plaats om zo de schade van transplantaatvernietigende effector T cellen te beperken (Figuur 1B). Op deze wijze spelen regulatoire T cellen een rol in de controle van antidonor reactiviteit tijdens verschillende stadia van een immuunrespons.

Table 1. Overzicht van de resultaten beschreven in dit proefschrift

Tijd na transplantatie	In het getransplanteerde hart	In het perifere bloed	Regulatoire T cel populatie
< 1 jaar	Hoge FOXP3 mRNA expressieniveaus zijn geassocieerd met antidonor immuunreactiviteit *(Hoofdstuk 2)*	Geen relatie tussen FOXP3 mRNA expressieniveaus en immunologische processen in het transplantaat *(Hoofdstuk 5)*	FOXP3 mRNA producerende cellen
	Tijdens een acute afstoting kunnen cellen aanwezig zijn die de antidonor immuunreactiviteit specifiek onderdrukken *(Hoofdstuk 3 en 4)*		$CD8^+$ T cellen
		Slechte suppressieve functie en een verhoogde expressie van CD127 in patiënten die een acute afstoting doormaken *(Hoofdstuk 6)*	$CD4^+CD25^{bright+}FoxP3^+$ T cellen
1 – 7 jaar		Een lage donorspecifieke cytotoxische responsiviteit is geassocieerd met hoge IL-10 mRNA expressieniveaus *(Hoofdstuk 7)*	IL-10 mRNA producerende cellen

In de afgelopen 30 jaar zijn verschillende regulatoire T celpopulaties geïdentificeerd (Hoofdstuk 1). In orgaantransplantatie heeft de aandacht zich tegenwoordig vooral gevestigd op de rol van $CD4^+CD25^{bright+}FoxP3^+$ regulatoire T cellen in het ontstaan van tolerantie ten opzichte van het transplantaat.[18] Toch blijkt uit klinische studies dat ook andere regulatoire T celpopulaties, zoals $CD8^+CD28^-FoxP3^+$ suppressor T cellen en Type-1 regulatoire T cellen, betrokken kunnen zijn bij de controle van antidonor immuunresponsen.[19,20] In onze experimenten vonden we in het perifere bloed dat $CD4^+CD25^{bright+}FoxP3^+$ regulatoire T cellen waarschijnlijk een rol spelen in het onderdrukken van antidonor reactiviteit kort na transplantatie. Enkele jaren na transplantatie zagen we dat IL-10 producerende cellen ook mogelijk betrokken zijn in antidonor immuunregulatie. In het getransplanteerde hart was bewijs gevonden voor $CD8^+$ cellen met potentiële suppressieve activiteiten (Tabel 1). Deze bevindingen suggereren dat meerdere regulatoire T celpopulaties een rol kunnen spelen in de controle van een afweerreactie. Dus wanneer we de aandacht maar op één regulatoire T celpopulatie richten, kunnen we belangrijke informatie mislopen.

Of al deze verschillende populaties een rol spelen in de controle van antidonor reactiviteit tijdens de verschillende stadia van een immuunrespons moet nog verder uitgezocht worden. Het is mogelijk dat bepaalde regulatoire T cellen betrokken zijn bij de preventie van de inductie van een immuunrespons, terwijl anderen belangrijker zijn voor het beperken van schade aan hartspiercellen. Studies in proefdiermodellen hebben laten zien dat naïeve $CD4^+CD25^+$ regulatoire T cellen de inductiefase van een immuunrespons controleren, terwijl effector/memory-achtige $CD4^+CD25^+$ regulatoire T cellen op de plek van een ontsteking aanwezig waren om de respons lokaal te onderdrukken.[21,22] Dus binnen de $CD4^+CD25^+$ regulatoire T celpopulatie zijn er cellen met een specifieke rol, zoals de preventie van antidonor immuunreactiviteit of het beperken van schade. $CD8^+$ regulatoire T cellen en IL-10 producerende regulatoire T cellen kunnen geïnduceerd worden na herhaalde stimulatie met antigenen in het bijzijn van respectievelijk IL-2 en IL-10.[23,24] Deze cellen kunnen dus gegenereerd worden na transplantatie. De condities voor het genereren van $CD8^+$ regulatoire T cellen zijn optimaal in het getransplanteerde hart (aanwezigheid van antigenen) tijdens alloreactiviteit (aanwezigheid van IL-2). Deze cellen kunnen dus een rol spelen in het controleren van een afweerreactie in het transplantaat. Daarentegen vonden we geen toename van IL-10 mRNA expressie tijdens een acute afstoting. Dit hoeft dus niet de plek te zijn waar de actie van IL-10 producerende regulatoire cellen plaatsvindt. Verder onderzoek naar de verschillende regulatoire T celpopulaties zal duidelijk maken waar en wanneer de actie van een bepaalde populatie plaatsvindt.

Dit proefschrift geeft nieuwe inzichten in de rol van regulatoire T cellen in immuunregulatie na harttransplantatie. Een belangrijke vraag is: kunnen deze nieuwe inzichten ook gebruikt worden voor klinische doeleinden? We postuleren dat fenotypische en functionele analyses van regulatoire cellen van grote waarde kunnen zijn voor het immunologisch monitoren van hartontvangers. Zo

vonden we een relatie tussen een verhoogde expressie van CD127 op slecht regulerende $CD4^{+}CD25^{bright+}$ T cellen en het optreden van een acute afstoting. Het analyseren van de expressie van deze marker met behulp van flowcytometrie kan dus mogelijk patiënten identificeren die een verhoogd risico hebben op acute afstotingen. Het voordeel van flowcytometrie is dat het een snelle, kwantitatieve en gevalideerde techniek is, wat het bruikbaar maakt voor het monitoren van harttransplantatiepatiënten. Als we patiënten met een verhoogd risico op acute afstotingen kunnen onderscheiden van degenen met een lager risico, kan er een meer individuele immunosupressieve behandeling toegepast worden. Dit zal dan leiden tot een verbeterde lange termijn overleving en kwaliteit van leven. Om te bevestigen dat CD127 een bruikbare marker is om acute afstoting te voorspellen zou een prospectieve studie uitgevoerd moeten worden.

Een klinische toepassing voor regulatoire T cellen waar veel interesse voor is is het gebruik van deze cellen voor celtherapie. Deze cellen kunnen *in vitro* gegenereerd worden door T cellen die geïsoleerd zijn van de ontvanger te manipuleren. Verschillende studies hebben de *in vitro* generatie van regulatoire T cellen uit cellen van zowel muis als mens beschreven.[25-28] Idealiter moeten deze cellen alleen immuunresponsen tegen donorantigenen onderdrukken. Dus voordat regulatoire T cellen gebruikt kunnen worden voor celtherapeutische doeleinden zal eerst de specificiteit voor donorantigenen gekarakteriseerd moeten worden. In dit proefschrift hebben we aangetoond dat de bron voor donorspecifieke regulatoire T cellen het transplantaat zelf is: cellen met donorspecifieke suppressieve activiteiten zijn *in vitro* gekweekt uit transplantaten tijdens een acute afstoting. Deze cellen kunnen dus theoretisch gezien potentiële kandidaten zijn voor celtherapie. Als deze cellen echter alleen gekweekt kunnen worden uit transplantaten tijdens een acute afstoting, betekent dit dat deze cellen alleen gebruikt kunnen worden als afstotingsbehandeling of voor de preventie van een nieuwe acute afstoting. Het gevaar van *ex vivo* kweken van regulatoire T cellen is dat het gedrag van deze cellen na het inspuiten in de ontvanger onbekend is. Behouden deze cellen hun suppressieve activiteiten? Wordt het gedrag van de cellen beïnvloed door de omgeving waarin zij zich bevinden? Migreren zij naar de plek waar zij hun suppressieve functie moeten uitvoeren? En wat is het effect van immunosupressieve medicijnen op de functie van gekweekte regulatoire T cellen? Al deze factoren moeten met zorg geëvalueerd worden in relevante experimentele proefdiermodellen, voordat het gebruik van regulatoire T cellen uit het transplantaat overwogen kan worden als celtherapie voor het behandelen van transplantatiepatiënten.

We concluderen dat verschillende regulatoire T celpopulaties die aanwezig zijn in het perifere bloed en het getransplanteerde hart een rol spelen in immunologische processen, zoals immunologische rust en acute afstotingen, in harttransplantatiepatiënten.

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Dankwoord
Curriculum vitae
PhD portfolio

Appendix

Dankwoord

Het is zover: mijn proefschrift is af! Zoals bij vele promovendi het geval is heeft mijn promotietraject de nodige pieken en dalen gekend. Beter gezegd: mijn promotieonderzoek begon in een klein dalletje, werd gaandeweg een hobbelig pad omhoog (met een enkele zijweg) en eindigde op een steile hoge 'snowy mountain top' (Volgens een wijze vriendin zijn dat de 'echte pieken'). En nu ik vol euforie op de top sta, wil ik graag alle mensen bedanken die mij geholpen hebben daar te komen. Want zonder hen was het mij echt niet gelukt!

In de eerste plaats wil ik mijn copromotor Dr. C.C. Baan bedanken. Beste Carla, bedankt voor alle steun en hulp in de afgelopen jaren. Jouw deur stond voor alles open: of het nu om 'de pieken' of 'de dalen' ging. Je enthousiasme voor het wetenschappelijk onderzoek werkt aanstekelijk. Ik heb veel geleerd op het transplantatielab, zowel over onderzoek doen als over mijzelf. Dankzij dit promotieonderzoek weet ik dat ik graag in het wetenschappelijk onderzoek verder wil.

Mijn promotor Prof.dr. W. Weimar. Beste Willem, bedankt dat jij mij de mogelijkheid hebt gegeven om promotieonderzoek op jouw afdeling te verrichten. Ik heb veel vrijheid gekregen in de opzet en ontwikkeling van het onderzoek. Daarbij heb ik van jou geleerd om altijd kritisch naar de resultaten te kijken. We hebben vele prikkelende discussies gevoerd over de artikelen, abstracts en presentaties. Dat heeft geleid tot een aantal mooie publicaties en meerdere 'wereldtournees'.

Graag wil ik ook de leden van de kleine commissie bedanken: Prof.dr. J.D. Laman, Prof.dr. F.H.J. Claas en Dr. A.H.M.M. Balk. Beste Jon, bedankt voor je kritische blik op mijn proefschrift en de subsidieaanvraag voor een postdoctoral fellowship in het buitenland. Uiteindelijk is de aanvraag (nog) niet ingestuurd, maar het heeft wel geleid tot een leuke, uitdagende plek als postdoc op jouw afdeling. Beste Frans, bedankt voor het lezen en beoordelen van mijn proefschrift. Beste Aggie, jouw suggesties op de manuscripten en het proefschrift zijn altijd welkom geweest. Ook bedankt voor je enthousiaste reacties op geaccepteerde artikelen en presentaties.

Dr. R.A. de Weger, Prof.dr. H.J. Metselaar en Prof.dr. A.J.J.C. Bogers, dank voor uw bereidheid plaats te nemen in de grote commissie. Dr. A.R. Bushell, thank you for taking place in the committee.

Verder wil ik de co-auteurs van mijn publicaties bedanken: Kadir Caliskan, Lex Maat en Pieter Zondervan. Beste Kadir, je leverde naast de klinische gegevens van de patiënten ook waardevolle aanvullingen voor de manuscripten. Beste Lex, bedankt voor je positieve e-mails, wanneer een artikel of abstract geaccepteerd was. Beste Pieter, jouw beoordeling van de afstotingsgraad van de biopten was onmisbaar voor dit onderzoek.

Uiteraard wil ik mijn collega-promovendi, met wie ik vele uren heb doorgebracht in het 'AIO-hok', bedanken. Thijs en Jeroen, we zijn ongeveer tegelijkertijd begonnen en het was lang de vraag wie als eerste zou promoveren. Ik ben dan toch echt de eerste, maar ik weet zeker dat jullie promoties niet lang op zich laten wachten. Jullie waren twee geweldige collega's met wie ik heel veel lol heb gehad. Jullie stonden ook altijd klaar met een luisterend oor en adviezen. Ik ga mijn linker- en rechterkant missen. Aan de mannen die het hok inmiddels verlaten hebben, Petros, Dennis en Martijn, bedankt voor de gezellige gesprekken, filosofische inslag en wekelijkse dosis lachtherapie. Varsha, jouw komst verdubbelde het aantal vrouwen en theedrinkers in het hok. Het was fijn om met iemand in het hok over vrouwendingen te kunnen praten. Meindert, ik vergeet nooit meer het Braziliaanse eten in Praag... Bedankt voor je hulp met Illustrator en Indesign. Annelies en Perikles, jullie ook bedankt voor alle gezelligheid.

Van het transplantatielaboratorium wil ik graag alle (oud-)collega's bedanken voor alle steun en gezelligheid: Nicole, Martin Ho, Nicolle, Wenda, Wendy, Annemiek, Thea, Joke, Rens, Karin, Corné, Lysian, Martin Hu, Len, Barbara, Cees, Saskia en Cécile. Ik had mij geen betere collega's kunnen wensen tijdens mijn promotietraject. Ik heb genoten van alle gezelligheid, vooral tijdens de koffiepauzes, lunches, borrels, labweekenden en uiteraard op het kweeklab, waar ik menig uur (zingend) heb doorgebracht. In het bijzonder wil ik graag Sander bedanken: jij was de enige die mijn zangkunsten op waarde wist te schatten. Daarnaast heb je mij ontzettend geholpen met het kweek- en PCR werk. Ik denk dat onze experimenten die niets met dit onderzoek te maken hadden een goede kans hadden gemaakt op de 'Ig Nobel prize'. Ik kom je vast nog wel tegen op de virtuele racebaan. Mariska, bedankt voor jouw hulp met het GL onderzoek. De dubbele PKH-kleuringen waren geen gemakkelijke opgave, maar we hebben er toch mooie resultaten mee behaald. Ronella, van jou heb ik de limiting dilution assay geleerd. Veel werk voor een paar meetpunten, maar wel één van de leukste technieken die er is. Ook bedankt voor je hulp met het kweken van de GL. Beste Jurjen, jij was vooral in het begin van het promotietraject mijn onmisbare hulp. Ik heb veel geleerd van onze brainstormsessies. Je hebt mij enorm geholpen met mijn eerste publicaties en het bedenken van vervolgstudies. Daarnaast was je ook een prettige uitlaatklep. Bedankt daarvoor. We gaan nu echt binnenkort een borreltje doen.

Binnen het Erasmus MC wil ik verder graag nog de dames van de D-vleugel en de dames van de polikliniek harttransplantatie bedanken.

Ik mag mijzelf gelukkig prijzen dat ik tijdens mijn promotie twee geweldige paranimfen naast mij heb staan: Heidi en Monique. Lieve Mo, naast onze liefde voor de wetenschap bleken we nog veel meer gemeen te hebben: yoga, shoppen, (vrouwen)films en een wijntje/cocktail op zijn tijd! We hebben de afgelopen jaren veel gedeeld, van hotelkamers tot lief en leed. Van goede collega ben je goede vriendin geworden! Bedankt voor alle hulp met de lay-out van dit proefschrift

en dat je mijn paranimf wilt zijn. Mijn allerliefste vriendinnetje Heidi, wij hebben de afgelopen jaren heel wat meegemaakt. Meestal met een lach, soms met een traan. Ik heb veel van jou geleerd, in het bijzonder over de begrippen 'doorzettingsvermogen' en 'lef' en wat 'echte vriendschap' inhoudt. Bedankt dat je ook bij mijn promotie weer aan mijn zijde staat. Ik hoop dat we met onze mannen nog vele uitstapjes gaan maken. Er moet immers nog een 'moose' gevonden worden… What's that coming over the hill?

Tot slot wil ik graag mijn familie, schoonfamilie en vrienden bedanken voor alle steun. De afgelopen tijd heb ik het vaak te druk gehad om mijn sociale leven op peil te houden. Ik hoop dat ik dat nu weer een beetje goed kan maken. Lieve papa en mama, het beste wat ouders voor hun kinderen kunnen doen is rotsvast in ze geloven. Jullie hebben mij altijd geleerd naar de sterren aan de hemel te reiken, maar daarbij ook met beide benen op de grond te blijven staan. Jullie hebben mij gemaakt tot wie ik nu ben en daarvoor kan ik jullie niet genoeg bedanken. Lieve Remy en Erwin, ik ben trots op jullie.

De allerlaatste woorden die ik in dit proefschrift schrijf zijn gericht aan mijn allergrootste steun en toeverlaat: Henno. Lieverd, ik denk niet dat het met een pen te beschrijven valt hoe dankbaar ik ben voor alles wat je de afgelopen jaren voor mij hebt gedaan. Jij bent mijn rots in de branding. Jouw eindeloze geduld (bij opmerkingen als: het is nu écht bijna af...), relativeringsvermogen (bij opmerkingen als: ik weet niet of ik het af ga krijgen...), schouder om op uit te huilen (bij opmerkingen als: ik krijg het nóóóit af...) en onuitputtelijk bron van enthousiasme (bij opmerkingen als: het is af!) waren van onschatbare waarde. Het maakt voor mij niet uit waar we volgend jaar terecht komen: bij jou ben ik thuis. Ik houd van je!

Curriculum Vitae

Ilona Esmeralda Dijke was born in Rotterdam on the 14th of November 1979. In 1998 she completed secondary school (VWO) at the Comenius College in Capelle aan den IJssel, and started the study Biological and Medical Laboratory Techniques at the Hogeschool Rotterdam in Rotterdam. For her graduation research she crossed the North Sea and worked at the Medical Research Council Toxicology Unit of the University of Leicester in Leicester, England. The research project concerned the identification and sequencing of genes involved in the development of the resistance phenotype in breast carcinoma cells. In December 2001 she obtained her Bachelor degree cum laude. After working as a research technician at the department of Neuro-Oncology of the Erasmus MC in Rotterdam from January 2002 to September 2003, she was looking for a new challenge. This challenge was found at the Department of Internal Medicine of the Erasmus MC where she was offered a position as PhD-student. Under supervision of Prof.dr. Willem Weimar and Dr. Carla Baan she worked on the project 'the immune regulation after heart transplantation' of which the results are described in this thesis. During her PhD training she travelled around the world to present her data at conferences. In 2005 she received the Genzyme Award for best presentation from the Dutch Transplantation Society and a travel grant from the European Society for Organ Transplantation. Since November 2008 she works as a postdoc on the project "European Framework Programme 6: foam cell function" under supervision of Prof.dr. J.D. Laman at the Department of Immunology of the Erasmus MC (coordinator of the project: Prof.dr. G. Pasterkamp - University Medical Center Utrecht). She lives with her husband Henno van Gemeren in Capelle aan den IJssel.

PhD Portfolio

Name PhD student:	Ilona Esmeralda (Esmé) Dijke
Erasmus MC Department:	Internal Medicine – Transplantation
PhD period:	September 2003 – September 2008
Promotor:	Prof.dr. W. Weimar
Copromotor:	Dr. C.C. Baan

1. PhD training

		Year
General academic skills		
- Basic radiation protection 5A/B		2003
- English biomedical writing and communication		2005
Research skills		
- Statistics: classical methods for data analysis (Nihes)		2004
- Methodology of patient-related research and guidelines for grant applications		2005
In-depth courses		
- Molecular immunology (Postgraduate School of Molecular Medicine)		2005
(Inter)national conferences		
- Annual Meeting NTV[1], Texel, the Netherlands	March 17-19,	2004
- International Transplantation Society Congress, Vienna, Austria	Sept 5-10,	2004
- Annual Meeting NTV, Kerkrade, the Netherlands	March 9-11,	2005
- Annual Meeting ISHLT[2], Philadelphia, USA	April 6-9,	2005
- American Transplant Congress, Seattle, USA	May 21-25,	2005
- ESOT[3] Congress, Geneva, Switzerland	Oct 16-19,	2005
- Annual Meeting NVVI[4], Noordwijkerhout, the Netherlands	Dec 8-9,	2005
- Annual Meeting NTV, Zeewolde, the Netherlands	March 15-17,	2006
- Annual Meeting ISHLT, Madrid, Spain	April 5-8,	2006
- World Transplant Congress, Boston, USA	July 22-27,	2006
- Annual Meeting NVVI, Noordwijkerhout, the Netherlands	Dec 7-8,	2006
- Annual Meeting NTV, Zeewolde, the Netherlands	March 28-30,	2007
- Annual Meeting ISHLT, San Francisco, USA	April 25-28,	2007
- American Transplant Congress, San Francisco, USA	May 5-9,	2007
- ESOT Congress, Prague, Czech Republic	Sept 30–Oct 3,	2007
- Annual Meeting NVVI, Noordwijkerhout, the Netherlands	Dec 20-21,	2007
- Annual Meeting NTV, Zeewolde, the Netherlands	March 26-28,	2008
- Annual Meeting ISHLT, Boston, USA	April 9-12,	2008
- American Transplant Congress, Toronto, Canada	May 31-June 4,	2008

1 Nederlandse Transplantatie Vereniging (Dutch Transplantation Society)
2 International Society for Heart and Lung Transplantation
3 European Society for Organ Transplantation
4 Nederlandse Vereniging voor Immunologie (Dutch Society for Immunology)

Seminars and workshops

- Clinical Review Symposium NTV, Utrecht, the Netherlands	Nov 25	2004
- Seminar 'New developments in medical immunology', Rotterdam, the Netherlands	Nov 23	2005
- MolMed[5] Day, Rotterdam, the Netherlands	Febr 1	2006
- Clinical Review Symposium NTV, Utrecht, the Netherlands	Nov 23	2006
- MolMed Day, Rotterdam, the Netherlands	Febr 6	2008

Presentations

- Alloantigen-induced cytotoxic hyporesponsiveness in immunosuppressed cardiac allograft recipients. International Transplantation Society Congress, 2004 — poster
- Donor-specific cytotoxic hyporesponsiveness is associated with IL-10 expression after clinical heart transplantation. Annual Meeting NTV, 2005 — oral
- Cytotoxic hyporesponsiveness is associated with high IL-10 mRNA expression in immunosuppressed cardiac allograft patients. Annual Meeting ISHLT, 2005 — oral
- Donor-specific cytotoxic hyporesponsiveness is associated with IL-10 expression after clinical transplantation. American Transplant Congress, 2005 — oral
- IL-10 producing T cells participate in donor-specific cytotoxic hyporesponsiveness after clinical heart transplantation. ESOT Congress, 2005 — oral
- Donor-specific cytotoxic hyporesponsiveness associated with IL-10 mRNA expression after clinical heart transplantation. Annual Meeting NVVI, 2005 — oral
- Donor-specific cytotoxic hyporesponsiveness associated with high IL-10 mRNA expression levels after clinical heart transplantation. MolMed day, 2006 — poster
- $FOXP3^+$ T cells regulate anti-donor responses in the graft after heart transplantation. Annual Meeting NTV, 2006 — oral
- $FOXP3^+$ T cells regulate anti-donor responses in the graft after heart transplantation. Annual Meeting ISHLT, 2006 — oral
- $FOXP3^+$ T cells regulate anti-donor responses in the graft after heart transplantation. World Transplant Congress, 2006 — poster
- $FOXP3^+$ T cells restrain anti-donor responses in the graft of cardiac allograft patients. Annual Meeting NVVI, 2006 — poster
- High FOXP3 mRNA expression levels in the graft, but not in the peripheral blood, are associated with acute rejection in heart transplant patients. Annual Meeting NTV, 2007 — oral
- Acute rejection is associated with high FOXP3 mRNA expression levels in the graft, but not in the peripheral blood, of heart transplant recipients. Annual Meeting ISHLT, 2007 — oral
- $FoxP3^+$ T cells can be expanded from rejecting human cardiac allografts. Annual Meeting ISHLT, 2007 — oral
- High FOXP3 mRNA expression levels in the graft, but not in the peripheral blood, are associated with acute rejection in heart transplant patients. American Transplant Congress, 2007 — oral
- The vast majority of $FoxP3^+$ T cells expanded from rejecting human cardiac allografts are $CD8^+CD28^+CD127^-$. American Transplant Congress, 2007 — poster

5 Molecular Medicine

- Differential expression of CD127 on $CD4^+FoxP3^+$ T cells between healthy controls and heart transplant patients. ESOT Congress, 2007 — oral
- High FOXP3 mRNA expression in the graft reflects anti-donor immune reactivity in heart transplant patients. ESOT Congress, 2007 — oral
- $CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells of rejecting heart transplant patients have inferior immune regulatory functions. Annual Meeting NVVI, 2007 — oral
- Donor-specific immune regulation by $CD8^+FoxP3^+$ lymphocytes expanded from rejecting human cardiac allografts. Annual Meeting NTV, 2008 — oral
- Inadequate immune regulatory function of $CD4^+CD25^{bright+}FoxP3^+$ T cells in patients who experience acute rejection after clinical heart transplantation. Annual Meeting NTV, 2008 — oral
- Inadequate immune regulatory function of $CD4^+CD25^{bright+}FoxP3^+$ T cells in patients who experience acute rejection after clinical heart transplantation. MolMed day, 2006 — poster
- $CD4^+CD25^{bright+}FoxP3^+$ regulatory T cells of rejecting heart transplant patients have inferior immune regulatory functions. Annual Meeting ISHLT, 2008 — oral
- Donor-specific immune regulation by $CD8^+$ lymphocytes expanded from rejecting human cardiac allografts. American Transplant Congress, 2008 — oral
- Inadequate immune regulatory function of $CD4^+CD25^{bright+}FoxP3^+$ T cells in patients who experience acute rejection after clinical heart transplantation. American Transplant Congress, 2008 — poster

2. Teaching activities

		Year
Lecturing		
- Lecture for second year medical students	June 15	2005
- Lecture for PhD-students/Postdocs on the PLAN research day	Nov 28	2008
Supervising practicals		
- 1st ESOT basic course in laboratory skills	Nov 29-Dec 1	2004
- 2nd ESOT basic course in laboratory skills	Okt 30-Nov 2	2006