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DISSERTATION

Titel der Dissertation

Immune-regulation of allogeneic stimulation by CTLA-4Ig: Propagating $CD4^+CD25^+FoxP3^+$ T cells in an IDO independent fashion

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Mag.rer.nat. Edda Veith

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1 Zusammenfassung/ Abstract

1.1 Zusammenfassung

Die hämtopoetische Stammzelltransplantation (HSZT) ist eine vielversprechende, kurative Behandlungsmöglichkeit für eine Vielzahl von Krankheiten, wie zum Beispiel Leukämien, malignen Lymphknotenerkrankungen und angeborenen Immundefekten. Das Ziel der HSZT liegt in der Wiederherstellung einer durch Spenderzellen vermittelnden Hämatopoese im Patienten (Transplantationsempfänger). Dieser Prozess, auch als Chimärismus bezeichnet, soll langfristig den zumindest teilweisen Ersatz des eigenen Immunsystems durch das des Spenders gewährleisten. Damit die transplantierten Stammzellen anwachsen können ohne abgestoßen zu werden, muss sich der Patient zuvor einer immunsuppressiven Behandlung mittels Radio- und/ oder Chemotherapie unterziehen. Jedoch ist die HSZT für den Patienten mit erheblichen Risiken verbunden und ihre Anwendung daher derzeit auf vital bedrohliche Erkrankungen limitiert, da es infolgedessen zu bedrohlichen Infektionskrankheiten und/ oder einer Allo-Immunreaktion („graft-versus-host“ – GvHD) kommen kann. Tatsächlich ist die GvHD, bei der die transplantierten allo-reaktiven T Lymphozyten des Spenders die Organe des Patienten als fremd erkennen und bekämpfen, eine häufig auftretende, ungewollte und gefürchtete Nebenerscheinung. Daher führt die Kombination aus Immunsuppression, verzögerter Wiederherstellung des Immunsystems durch neugebildete Immunzellen, Infektionen und GvHD zu einer hohen Morbiditäts- und Mortalitätsrate bei HSZT Empfängern. Abhilfe dafür kann eine T Zellpopulation leisten, die trotz Ausschaltung/ Reduktion ihres allo-reaktiven Potentials ihre Effektivität gegen pathogene Infektionserreger und maligne Empfängerzellen beibehält. Eine Möglichkeit besteht in der Anwendung des pharmakologisch hergestellten Fusionsproteins CTLA4-Ig. *In vitro* Studien haben gezeigt, dass CTLA4-Ig die antigen-spezifische Immunantwort unterdrückt (Anergie), indem es die CD28:CD80/86 Co-Stimulation blockiert. Weiters wurde postuliert, dass dessen Wirkung zur Ausbildung eines spezifischen regulatorischen Phänotyps Dendritischer Zellen (DZ), vermittelt durch die Aktivität des Tryptophan – metabolisierenden Enzyms Indolamin-2,3-Dioxygenase (IDO), führt. Auf Basis dieser Erkenntnisse wurde in der vorliegenden Studie die immuno-regulatorisch vermittelnde Rolle von CTLA4-Ig in einem allogenen *in vitro* Modell basierend auf Mauszellen untersucht, in dem allogene Balb/c T Zellen mit C57BL/6 DZ, generiert aus Milz und Knochenmark, co-kultiviert wurden. Es konnte gezeigt werden, dass die Präsenz von CTLA-4Ig während der Co-Kultur zu einer starken Unterdrückung der

allogenen T-Zell Antwort führte. Dieser inhibierende Effekt durch CTLA-4Ig konnte jedoch weder mit der Aktivierung des immuno-modulatorischen Enzyms IDO, noch mit der Induktion eines DZ regulatorischen Phänotyps in Verbindung gebracht werden sondern beeinträchtigte direkt die Interaktion von T-Lymphozyten und DZ in der Co-Kultur und betraf präferenziell die $CD4^+$ T- Zell Antwort. Weiters konnte in diesen $CD4^+$ T-Zell Populationen eine hohe $CD25^+CD62L^+FoxP3^+$ Expression, kompatibel mit einem regulatorischen Phänotyp, nachgewiesen werden. Dieser regulatorische Phänotyp konnte sogar nach Restimulation aufrechterhalten werden.

Zusammenfassend, CTLA-4Ig hemmt die allogene proliferierende T Zell Antwort auf eine IDO unabhängige Art und Weise und fördert die Ausbildung eines regulatorischen T Zell Phänotyps. Die Expansion regulatorischer T Zellen, vermittelt durch CTLA-4Ig, stellt möglicherweise eine neue therapeutische Alternative dar um Nebenwirkungen, die während einer Stammzelltransplantation auftreten, zu lindern.

1.2 Abstract

Hematopoietic stem cell transplantation (HSCT) has emerged as a promising curative treatment modality for a variety of diseases, including leukemia, lymphoma and congenital immunodeficiency syndromes. The ambition of a successful HSCT is to stably establish and maintain donor-type hematopoiesis in the recipient, termed chimerism. Successful introduction of a stable chimerism implies a profound and prolonged manipulation of the recipient immune system which is achieved by radiation and/ or chemotherapy. This immunosuppressive conditioning is necessary to mediate engraftment and to prevent rejection. Furthermore, it eradicates additional residual malignant diseases. Thus, during a prolonged period of immune suppression and dysregulation after HSCT, the patient is at high risk for developing severe infections or graft-versus-host disease (GvHD) since immune reconstitution is slow and functionally impaired. Due to the high frequency of allo-reactive T cells, the risk of GvHD is much higher than the capability to develop protective immunity against environmental pathogens during T cell reconstitution. All these facts contribute to a major extent to transplant related morbidity and mortality (TRM).

T cells specifically tolerant against recipient allo-antigens are critical to provide recipients of HSCT with T cell immunity without promoting GvHD. The chimeric protein CTLA-4Ig was previously described to induce antigen-specific T cell unresponsiveness (anergy) *in vitro* by blocking the CD28:CD80/86 costimulatory pathway. Furthermore, it was reported to affect

dendritic cell (DC) function via the induction of the immunosuppressive indoleamine 2,3 dioxygenase (IDO) pathway, thus promoting a DC regulatory phenotype. Consequently, we studied immuno-regulation mediated by CTLA-4Ig in an allogeneic setting using C57BL/6 splenic and bone marrow derived DCs as stimulators of allogeneic Balb/c T cells. We found that CTLA4-Ig potently dampened allogeneic T cell responses when present during T cell/DC co-culture. This dampening effect was neither related to the activation of the IDO pathway nor to the induction of a DC regulatory phenotype, but directly affected DC/T cell crosstalk and preferentially targeted the $CD4^+$ T cell response. Moreover, $CD4^+$ T cells recovered from those DC/T cell co-cultures yielded a T cell population with a high expression in $CD25^+CD62L^+FoxP3^+$ suggesting a CTLA4-Ig mediated regulatory phenotype. Remarkably, this regulatory phenotype was preserved upon restimulation.

In conclusion, CTLA4-Ig inhibits allo-stimulated T cells in an IDO independent fashion by modulating the allogeneic, proliferative T cell response while propagating a regulatory phenotype. Therefore, expanding regulatory T cells as mediated by CTLA-4Ig may open a new therapeutic window for successful T cell therapy during HSCT.

2 Introduction

2.1 Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) is a procedure in which pluripotent hematopoietic stem cells capable of reconstituting normal bone marrow functions are administered to a patient. In general, allogeneic HSCT is a powerful curative approach to treat patients suffering from congenital or acquired malignant and non-malignant diseases [1]. Selection of the type of transplantation depends on the type of malignancy, age of the recipient, availability of a suitable donor and the stage and status of the disease. In principle, HSCT aims to establish a long term and self-sustaining donor-type hematopoiesis in the recipient, so called chimerism [2]. The types of hematopoietic stem cells are categorized based on the source of progenitor cells used in the transplant. Currently, three sources of stem cells are distinguished: bone marrow, peripheral blood, or cord blood. Further, hematopoietic stem cells can be obtained from the patient himself (termed autologous transplant), from a genetically identical twin (syngeneic transplant) or from another human leukocyte antigen (HLA)-matched related or unrelated individual (allogeneic transplant) [3]. Each of these sources of cells has specific advantages and disadvantages, and each has found particular applications in the care of patients. For instance, patients receiving autologous transplants have a lower risk of life-threatening complications. Since there is no risk of GvHD in autologous transplantation, immunosuppressive therapies to prevent GvHD and graft rejection are not required. However, disease recurrence generally occurs less frequently in patients receiving allogeneic transplants than autologous ones. Furthermore, allogeneic transplants have the advantage to be free of contaminating tumor cells and may induce an immune graft-versus-leukemia effect based on donor-derived immunocompetent cells [3].

Prior to HSCT, recipients undergo an immunosuppressive conditioning by radiation and/ or chemotherapy with the purpose, to help eradicate the patient's disease prior to the infusion of hematopoietic stem cells, and to suppress immune reactions to allow for engraftment and to prevent rejection [4, 5]. Thereafter, donor bone marrow, or peripheral blood stem cells (PBSCs) from donors that have been treated with granulocyte colony-stimulating factor (G-CSF) are infused [6]. Nevertheless, immunosuppression also sows the seed for negative side effects as a consequence of an inferior reconstituted immune system in the immediate post-transplant period [7]. Hence, next to transplantation-associated toxicity and failure of engraftment [8], patients are highly susceptible for reactivation or exacerbation of

opportunistic infections. Further, graft recipients are prone to develop pathogenic allo-reactions, particularly involving the T cell system in the direction of graft-versus-host disease (GvHD). All these possible complications implicate a high transplant-related morbidity and mortality (TRM) [9, 10] (Figure 1).

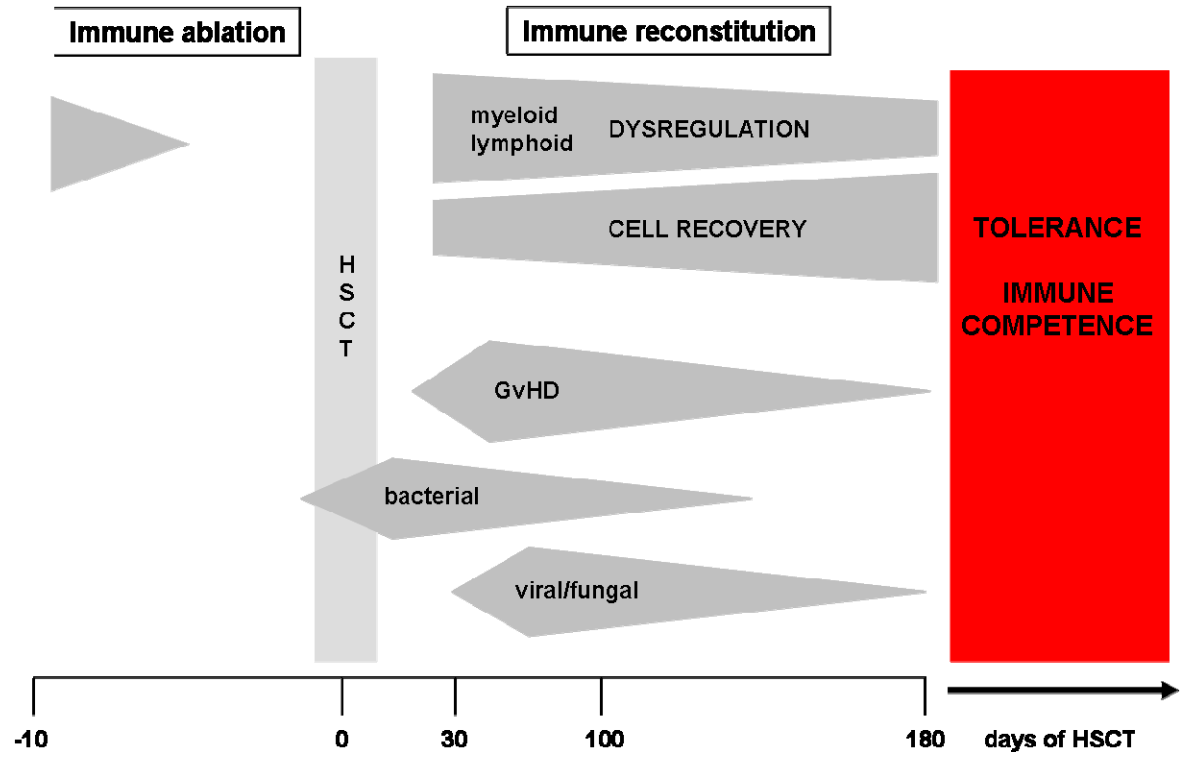


Figure 1 The immune system after HSCT.

During a prolonged period of immune suppression and dysregulation after HSCT the patient is at high risk for developing life-threatening opportunistic infections or GvHD since immune reconstitution is slow and functionally impaired (Jürgens B.; PhD Thesis University of Vienna; 2008).

2.2 Immune reconstitution following HSCT

The reconstitution of different cellular subsets of the immune system after an allogeneic HSCT occurs in a chronological manner. Cells representing the innate part of the immune system, e.g. monocytes, macrophages or natural killer cells, recover rapidly after HSCT [11]. Cells constituting the adaptive part of the immune system, take a longer time to quantitatively recover. Therefore, T- and B-cell responses will be incomplete for a prolonged post-transplant period. These days, a deficiency of B-cells within the first three months after transplantation can easily and effectively be replaced by pharmacologically prepared immunoglobulin [11]. T

cells, however, require several months after HSCT for quantitative reconstitution and even after T cell recovery, immunity is still imbalanced as a result of disturbed T cell function [11, 12]. Particularly in the first weeks after surgery, the lymphoid system of transplant recipients contain altered compositions of T cell populations [13]. Whereas, $CD8^+$ T cells propagate to normal or elevated levels three to six months after HSCT, $CD4^+$ T cells increase slowly to return to normal levels e.g. in children within less than one year, where T cell reconstitution occurs more rapidly than in adults [14]. $CD4^+$ T cell reconstitution is particularly slow in older patients because of poor thymic function [7] (Figure 2).

Since mainly alloreactive T cells expand instead of the immunocompetent ones [15] patients are at a much higher risk to develop GvHD than to regain T cell-mediated protective immunity. To overcome this problem, pharmacological immunosuppression is essential in the post-transplant period. Consequently, T cells remain incapable of re-establishing normal functions, including immune competence to environmental pathogens and self-tolerance.

To conclude, treatment-related circumstances of allogeneic HSCT result in profound and prolonged T cell deficiency. Impaired T cell function is a leading factor of TRM in the post-transplant period. Thus, the problem of T cell regeneration remains a main immunological challenge after HSCT.

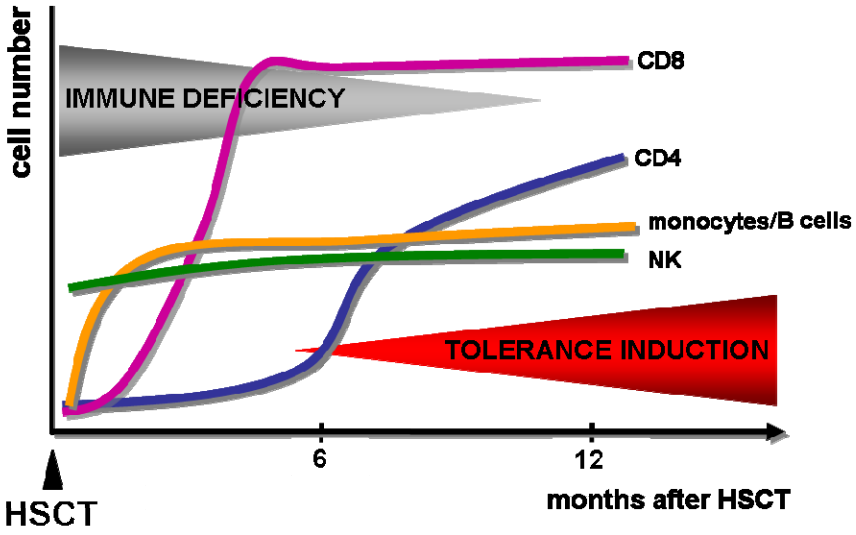


Figure 2 ***Reconstitution of different cellular subsets of the immune system after allogeneic HSCT.***

Natural killer cells (NK), monocytes and B cells return rapidly to pre-treatment levels, whereas especially $CD4^+$ T cells show a delayed recovery. Finally, a successful transplantation is characterized by reconstitution of full immunity and tolerance to the allogeneic graft (Hainz U.; PhD Thesis University of Vienna; 2005).

2.3 Patient's risks in the early post-transplantation period

2.3.1 Bacterial, viral and fungal infections

Bacterial infections are common during the early post-transplant period, but are generally well controlled by the appropriate use of antibiotics. Viral and fungal infections are less well treatable by drugs and are therefore one of the main clinical burden apart from GvHD [16]. Viral infections, in particular infections with cytomegalovirus (CMV) and Epstein-Barr virus (EBV), are often reactivated after allogeneic stem cell transplantation. Therefore several attempts have been undertaken to support HSCT recipients with a specific T-cell immunotherapy against these viral diseases. Such include the generation of CMV- or EBV-specific T effector cells in vitro and adoptively transferring these cells to the patients [17, 18]. However, since these T cells are only effective against target viral antigens, they are incompetent and/ or non-reactive against other infections, such as those mediated by fungi [18, 19].

2.3.2 Graft-versus-host disease (GvHD)

GvHD is a complex disease caused by donor T cells which react against host allogeneic antigens in the immunosuppressed patient [20, 21]. As mentioned above, the therapeutic approach of HSCT is based on the deletion of the recipient's hematopoietic system using chemotherapy and irradiation, and on its substitution by hematopoietic cells from a healthy donor. The donor graft contains both hematopoietic stem cells that are capable to durably reconstitute a complete hematopoiesis, and immunocompetent cells including T cells. This donor T cell compartment contains alloreactive cells which recognize host allo-antigens [22, 23]. Basically, the development of GvHD can be divided into three sequential steps or phases [24]. In the first step (phase I), antigen-presenting cells (APCs) of the host organism are activated by the underlying disease and the conditioning regimen. Damaged host tissues respond by producing "danger" signals, including (i) proinflammatory cytokines (e.g. tumor-necrosis factor (TNF)-α), (ii) chemokines and (iii) increased expression of adhesion molecules, MHC antigens and costimulatory molecules on host APCs [25, 26]. In the second step (phase II), donor T cells proliferate and differentiate in response to host APCs. This activation is augmented by the "danger" signals which were generated in phase I, at least partially, by increasing the expression of costimulatory molecules [26-28]. In the last step

(effector phase), cellular mediators (e.g. cytotoxic T lymphocytes and NK cells) and inflammatory mediators (e.g. TNF-α, interferon (IFN)-γ, interleukin (IL)-1 and nitric oxide) synergize to amplify local tissue injury and further promote inflammation and destruction of target tissues [27, 29].

In the clinical setting, GvHD is divided into acute and chronic forms. Acute GvHD is defined by occurring within 100 days post transplantation and is characterized by damage to the skin, liver and the gastrointestinal tract, whereas chronic GvHD has more diverse manifestations that can be similar to autoimmune syndromes [6]. Patients with acute GvHD are suffering from rash, jaundice, diarrhea, and gastrointestinal hemorrhage. When the epithelial cell damage is very extensive, the skin or lining of the gut are simply slough off and under these circumstances acute GvHD may be fatal [3, 30]. Acute GvHD is classified into grade I (mild), II (moderate), III (severe) and IV (very severe) which is determined by the extent of involvement of the three main target organs. Severe GvHD possesses a poor prognosis: 25% long term survival for grade III and 5% for grade IV [31]. Chronic GvHD normally occurs after day 100 post-transplantation. The emergence of chronic GvHD may be (i) progressive, when active or acute GvHD changes over to chronic; (ii) quiescent, when acute GvHD that was resolved completely is later followed by chronic GvHD or (iii) it may develop *de novo*. Besides, patients older in age are more prone to develop chronic signs of GvHD [32, 33]. Typical clinical signs of chronic GvHD are dry eyes, oral lesions, nail dystrophy, skin sclerosis as well as skin ulcers.

The incidence of acute GvHD is most often related to the degree of mismatches between HLA (human leukocyte antigen) proteins of the donor and the recipient [34]. HLA proteins are encoded by the major histocompatibility complex (MHC), are highly polymorphic and are mainly divided into two classes. Class I HLA (A, B, and C) proteins are expressed on nearly all nucleated cells of the body to a distinct extent. Class II proteins (DR, DQ and DP) are predominantly expressed on hematopoietic cells such as B cells, dendritic cells and monocytes, but their expression is inducible following inflammation or injury on many other cell types [29, 35]. In spite of HLA identity between a patient and a donor, nearly 40% of patients receiving HLA-identical grafts develop acute GVHD. The reasons for this are genetic differences lying outside the HLA loci, termed “minor” histocompatibility antigens [34].

Diverse strategies have been developed to support HSCT recipients with a specific T-cell immunotherapy in order to minimize unwanted and uncontrolled graft-versus-host immune reactions. Among these were clinical studies exploring T cell depletion (TCD) as a prophylaxis for GvHD. For the depletion of T cells three principal strategies were used: (i) negative selection of T cells *ex-vivo*, (ii) positive selection of $CD34^+$ stem cells *ex vivo*, and (iii) anti-T cell antibodies *in vivo* [36].

Most strategies concerning *ex-vivo* TCD-therapy revealed that the emergence of both acute and chronic GvHD was significantly reduced. However, high rates of graft failure, relapse of malignancy, infections, and Epstein-Barr virus-associated lymphoproliferative disorders were observed [37-40]. The treatment of patients with anti-T cell antibodies before transplantation (*in vivo* TCD) aims to reduce the host immune response and thus promotes engraftment. Additionally, donor T cells are down-regulated since the antibody is still circulating at the time of transplantation and thus GvHD is prevented [41, 42]. However, side effects of anti-T cell antibody infusions are common and TRM remains unchanged because of a higher risk for lethal infections. Although all these approaches may have, at least, some success they exclusively focus on the issue of elimination but not on regulating allo-reactivity. Nonetheless, both these processes seem to be required for the generation of true transplantation tolerance [43].

One further strategy is to generate a regulatory T cell population from the donor prior to transplant by an *ex vivo* expansion of a $CD4^+CD25^+$ T cell population. These T cells in fact, when being transfused, have been shown to prevent GvHD [44-50]. Still, the specificity of regulation of GvHD by *ex vivo* expanded regulatory T cells (Tregs) seems to be a critical issue. *In vitro* studies revealed that, after activation, Tregs display a bystander suppressive effect on all conventional T cells (generally immunosuppressive activity) [51, 52]. This bystander suppression of Tregs could limit their capacity to prevent GvHD because of impaired immune reconstitution during the first months after HSCT. Yet, when recipient-specific *in vitro* expanded Tregs were used, bystander suppression seemed to be limited (in contrary to polyclonally expanded Tregs) accompanied by significant immune reconstitution [47, 48].

2.4 Tolerance

In general, T cell tolerance is primarily achieved by the elimination of potentially self-reactive lymphocytes in the thymus through a mechanism termed negative selection (central tolerance). Nevertheless, some self-reactive lymphocytes may escape negative selection in the thymus and thus manage to enter peripheral tissues. Therefore, such self-reactive T lymphocytes are strictly controlled by peripheral tolerance including mechanisms. These mechanisms include the induction of anergy or apoptosis in self-reactive T lymphocytes either by regulatory T cells or regulatory APCs. The failure in inducing tolerance towards self-antigens is the result for several menacing autoimmune disorders [3, 53].

The ultimate goal of allogeneic HSCT is to introduce chimerism in the recipient long term and self-sustaining, i.e. to create tolerance against recipient type self-antigens. Transplantation tolerance is defined by (i) the maintenance of a chimeric state without a pathologic immune reaction against the allo-antigens (ii) in the absence of pharmacological immunosuppression (iii) while preserving intact immune reactions against environmental antigens.
As mentioned above, in HSCT T cells are the major effector component of the immune system and have the potential to direct alloreactivity towards rejection or GvHD or tolerance [54]. Therefore, it is a critical task of research in the field of HSCT to support the allo-HSCT recipient with T cells which are specifically tolerant against allo-antigens and retain their protective capability to control infections as long as the process of developing allo-antigen specific tolerance by nature is incomplete. The magic goal is to generate such T cells *ex vivo* and utilize these T cells for adoptive transfer strategies.

Transplantation tolerance essentially involves two critical processes, (1) the deletion of alloreactive T cells as a first step and (2) the development of anergy and regulatory activity of alloreactive T cells [43]. Based on these demands, two approaches hypothetically would excellently satisfy these criteria and are currently probed: (i) the utilization of the tolerizing potential of the enzyme indoleamine 2,3-dioxygenase (IDO) or (ii) inducing allo-antigen specific tolerance via co-stimulation blockade.

2.5 Biological and biochemical background of IDO activity

Tryptophan (TRP) is the least abundant of the essential amino acids in mammalians [55]. Since it cannot be synthesized by the human body from other components to sufficient levels, its availability depends on food intake. A small proportion of TRP (approximately 1%) is utilized for the synthesis of the neurotransmitter Serotonin. About 5% are used for protein synthesis and the remaining major proportion (~95%) is metabolized along the kynurenine pathway to end up in the production of the co-enzyme nicotinamide-adenine-dinucleotide (NAD)**.** The processing of TRP to kynurenines is mediated by two different enzymes: tryptophan 2,3-dioxygenase (TDO) and IDO (Figure 3). TDO is a homeostatic enzyme mediating TRP degradation exclusively in the liver, whereas ubiquitously-expressed IDO is not restricted to specific organs and tissues and governs the levels of TRP in the human body [56]. However, whereas TDO's activity is induced by TRP and metabolic steroids, IDO becomes activated in response to inflammatory signals. Therefore, IDO is detected, amongst others, in patients suffering from autoimmune diseases and in those recovering from transplantations [57-59]. Under such conditions, enhanced systemic IDO activity shifts the TRP equilibrium in the intercellular microenvironment towards lower TRP concentrations resulting in an accumulation of kynurenines influencing alloantigen-specific T cell responses [60].

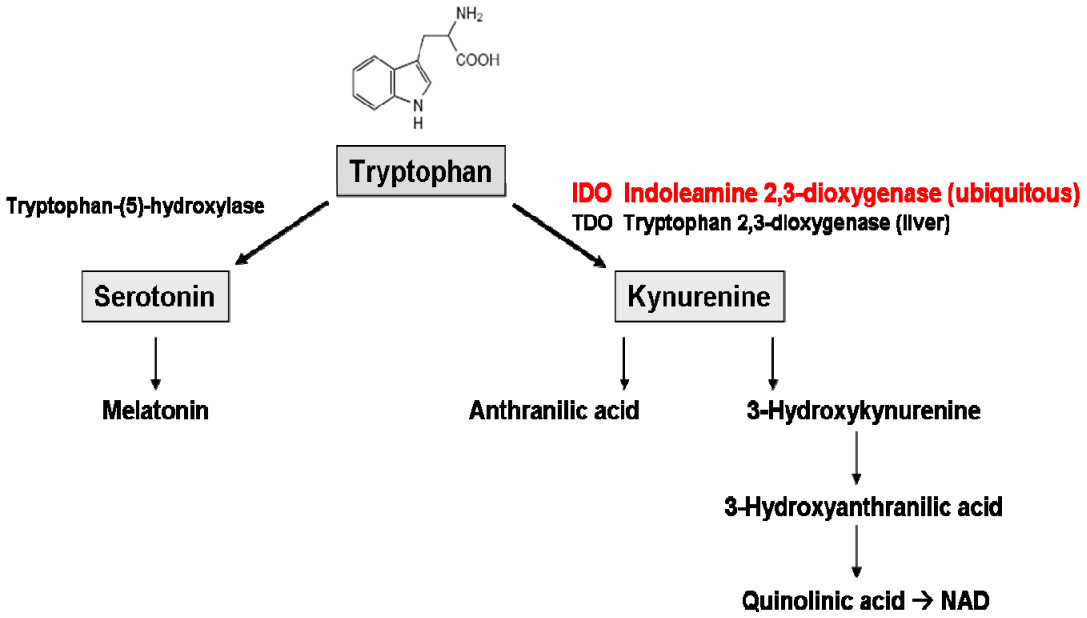


Figure 3 Pathways of tryptophan catabolism.

IDO and TDO initiate tryptophan catabolism by oxidative cleavage of the pyrrole ring of tryptophan.TDO activity occurs in the liver and is responsible for tryptophan degradationin steady state conditions. The enzymatic activity generates N-formylkynurenine that rapidly

converts into kynurenine. The downstream products of kynurenine shown in this graph have been described to possess immunosuppressive activity (Jürgens B.; PhD Thesis University of Vienna; 2008).

Originally, IDO activity has been described as a host defense mechanism. Enhanced tryptophan degradation did deprive pathogens, such as Chlamydia or Toxoplasma, from their access to tryptophan and exposed them to toxic tryptophan metabolites in order to terminate infections [61, 62]. By the late 1990s, a study by D. Munn et al [63] showed that IDO activity was critically involved in the immunologic acceptance of semi-allogeneic fetuses in a murine model. This study has since fuelled a new understanding of IDO's activity as a central pathway for dampening potentially dangerous immune reactions [60] including allo-antigen driven immunopathology.

IDO expression combined with actively metabolized TRP was found in multiple APC types such as monocytes/macrophages [64], dendritic cells [65, 66] and even non-classical APCs, e.g. bone marrow stroma cells [67]. Importantly, they utilize the IFN-γ inducible enzyme tryptophanyl-tRNA-synthetase [68], which catalyzes the association of tryptophan with its specific tRNA, resulting in a tryptophan-tRNA. This complex provides a reservoir of TRP protected from IDO-mediated degradation and protects IDO-inducible cells from TRP self-starvation. Some APCs may constitutively express IDO such as the murine $CD8^+$ lymphoid DC subset [69]. In other types of APCs, expression of IDO is inducible by agents that are generally considered pro-inflammatory e.g. lipopolysaccharide (LPS), TNFα, a DC maturation cocktail containing TNF-α, IL-1ß/IL-6 and prostaglandin E2 (PGE2), and, most prominently, IFN-γ [66, 70].

Since, IDO is induced by the same mechanisms and molecules that initiate immune activation [71, 72] it is thought to function as a feedback mechanism to counter-regulate or terminate potentially dangerous effector immune responses [73, 74]. Previous research suggested that B7 (CD80/ CD86)-engagement by the fusion protein CTLA-4Ig on murine DCs results in down-stream cellular signalling events leading to IDO activation that initiates the immunosuppressive pathway of tryptophan catabolism [74-76]. In detail a pathway was proposed in which CTLA-4Ig binding to CD80/86 of DCs elicits IFN-γ production which in an autocrine or paracrine fashion induces IDO to ultimately generate regulatory activity in DCs [71] (Figure 4). Further, it was reported that CTLA-4Ig functions *in vivo* in allogeneic islet transplant tolerance by triggering the production of functional IDO in APCs (Grohmann

et al., 2002). Subsequent studies proposed that in fact CTLA-4^{+}, CD4^{+}C25^{+} regulatory T cells might exert their effect by IDO induction in DCs [77].

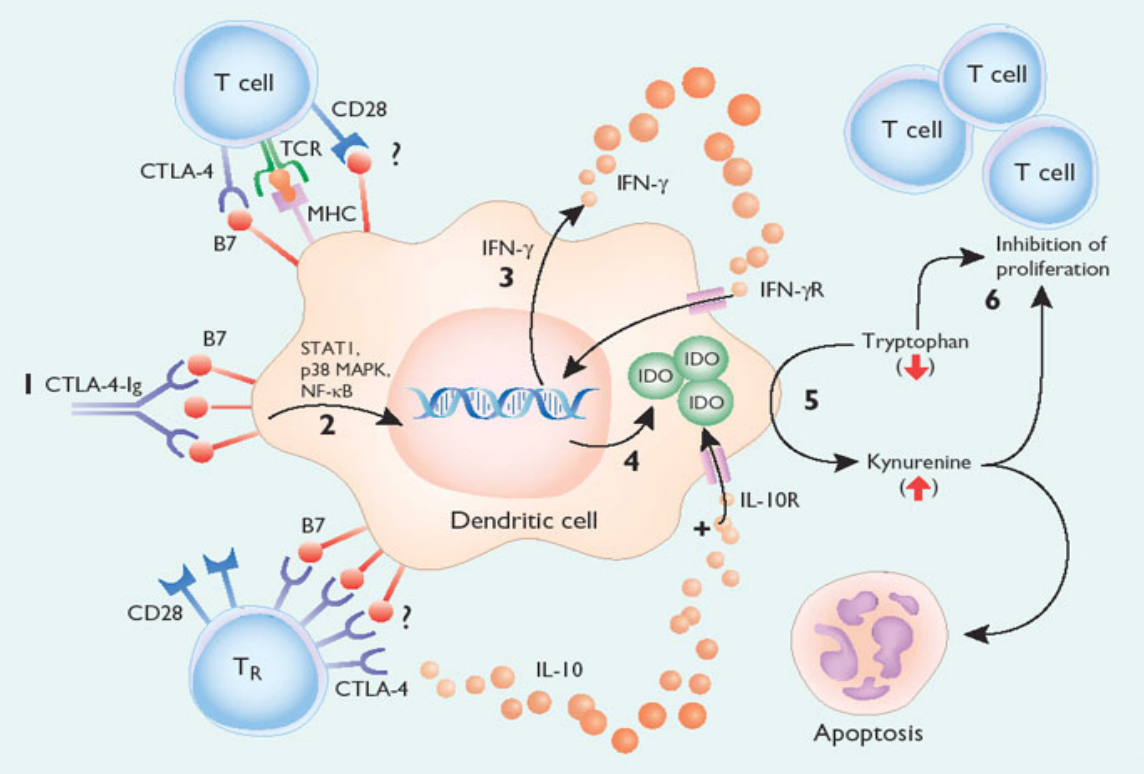


Figure 4 Model of tolerance induction by B7-dependent signaling in DCs.

CTLA-4-Ig-binding to the DCs induces Dc signaling via CD80/86 (B7-1/B7-2). In a signal transduction pathway that is dependent on STAT1, p38 MAPK and NFkB, the DC is stimulated to produce IFN-γ. IFN-γ acts in an autocrine or paracrine manner to promote upregulation of IDO which catalyses the degradation of TRP to kynurenines (Finger E.B. and Bluestone J.A.; Nature Immunology; 2002).

Nevertheless, it has to be kept in mind, that IDO expression alone does not necessarily indicate IDO activity. In fact, IDO activity is tightly regulated by post-translational mechanisms. They may include an alteration of the enzyme's heme-active binding site [78] possibly by an effect of nitric oxide (NO) [79, 80]. Pathways to counteract the effect of IDO on immune responses include CD40 ligation of lymphoid DC [81] by an IL-6 dependent mechanism [82].

2.6 Mechanisms of IDO-mediated transplantation tolerance

Allogeneic HSCT is intimately associated with a state of sustained immune activation, which invariably will emerge in HSCT recipients. Immune activation is driven by recognition of

major or minor histocompatibility antigens even in case of HLA-matched donors, but will also result from tissue damage caused by the conditioning regimens. This state of sustained immune activation will include the continuous secretion of pro-inflammatory cytokines (including IFN-γ) by APCs or activated T cells, which, in turn, will induce sustained IDO activity.

In the current model of IDO's immunosuppressive activity, IDO preferentially targets activated T cells [71]. Upon inflammation, IDO expression and activity is induced in APCs leading to enhanced tryptophan breakdown and accumulation of kynurenines in the microenvironment. Both features are influencing the immunoregulatory activity of alloantigen-specific T cells as follows. After the activation of antigen-specific T cells, the reduced levels of TRP and enhanced levels of kynurenines trigger the activated alloreactive T cells to undergo cell cycle arrest and apoptosis instead of mounting an effector immune response towards the alloantigen presented by the APC [60, 83, 84] (Figure 5). Therefore it was suggested that the T cell non-responsiveness induced by IDO-expressing APCs is alloantigen specific [85]. However, T cells that are not activated by the particular antigen presented by IDO-expressing APCs (resting T cells), may remain largely unaffected and thus should retain their immunological potential to induce adequate responses when subsequently challenged by normal APCs.

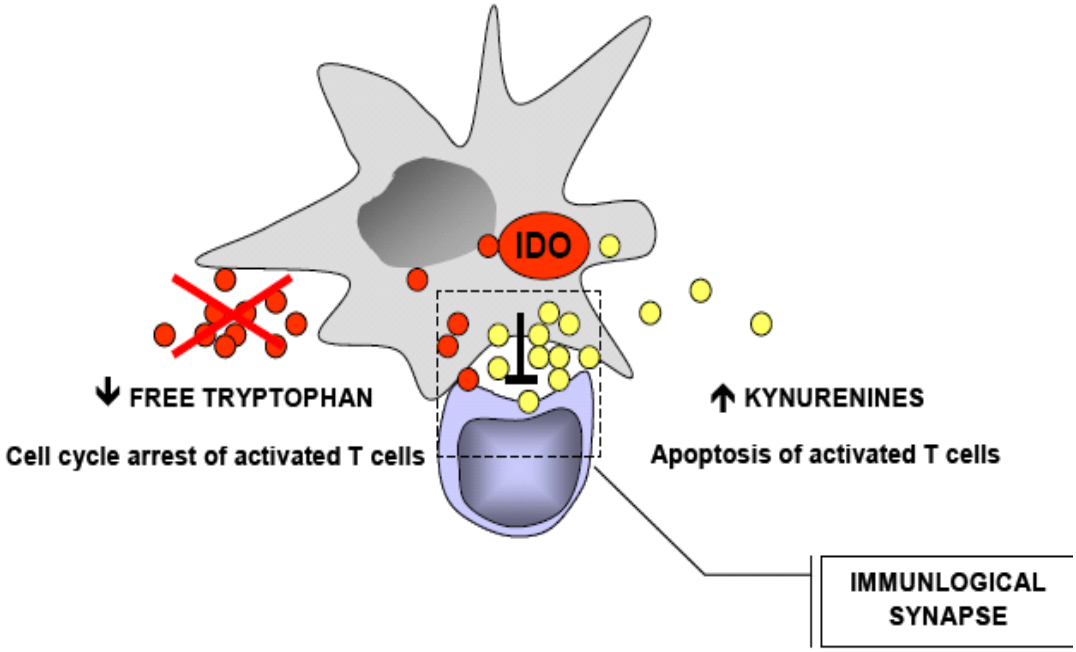


Figure 5 Hypothetical antigen-specific activity of IDO.

By concept, IDO competent antigen-presenting DCs exert their T cell suppressive effect particularly within the immunological synapse (dashed area), which is formed by a DC

derived MHC/antigen complex and a T cell receptor complex recognizing the antigen. The effects of IDO activity, namely low TRP concentration and high levels of TRP breakdown products (kynurenines) are focused in this microenvironment and therefore specifically affect antigen-specific T cells. These are arrested in cell cycle progression and are rendered susceptible to apoptosis. T cells that are not antigen-specific remain unaffected (Hainz U.; PhD Thesis University of Vienna; 2005).

Correspondingly, employing IDO activity may represent an approach to generate alloantigen-specific tolerance after HSCT [85] (Jürgens et al). It was shown that enhanced IDO activity is present in HSCT recipients [86] and possibly involved in modulation of GvHD [87]. Further, it has been shown that T cells exposed to IDO-expressing DCs acquire a T cell regulatory phenotype (high levels of expression of the CD25 cell surface molecule accompanied by intracellular expression of FOXP3) in murine [88] and human systems [85]. By these mechanisms IDO activity essentially meets the above mentioned criteria required for tolerance induction in transplantation [43] as it (i) selectively eliminates allo-reactive T cells by apoptosis and (ii) generates regulatory T cells.

Together, IDO is a potential candidate to mediate transplantation tolerance as T cells activated by alloantigens under conditions of enhanced tryptophan metabolism become susceptible to apoptosis, leading to a quantitative reduction of alloreactive T cells.

2.7 Co-stimulation and co-stimulation blockade

T lymphocytes play a central role in the initiation and regulation of the adaptive immune response to antigens, whether foreign or native. T cell activation is tightly regulated, including essentially the signaling via T cell co-stimulation receptors.
In general, naïve T cells require two signals for their full activation [89]. The first signal (Signal 1) is an antigen-specific one, provided by the T cell receptor when it recognizes and interacts with a specific antigen that is presented in MHC-antigeneic peptide-complexes on the surface of APC. The second signal (Signal 2), termed co-stimulatory signal, is supplied by the interaction between specific receptors (co-receptors) on the T cell and their ligands on the APC [90, 91]. Together, both signals synergize in activating a number of down-stream cellular signaling pathways leading to optimal cytokine production, proliferation, effector function and survival [92]. Co-stimulation alone in the absence of signal 1, generally leading to T cell receptor stimulation has no effect on the T cell. Furthermore, if a T cell receives signal 1 without a co-stimulation signal, T cell unresponsiveness or anergy (antigen specific unresponsiveness) is induced [93].

The blockade of signaling through co-stimulation receptors with the use of specific co-stimulation inhibitors, enables the selective prevention of T cell responses and has the potential of inducing tolerance to specific antigens in both autoimmunity and transplantation [94-97]. Hence, co-stimulation blockade has become of great interest in the field of transplantation being used as an immunosuppressive therapy. Co-stimulation blockers are main candidates for such clinical approaches. Some of them have proved successful in being used as maintenance therapy replacing current drugs that require frequent therapeutic drug monitoring and are associated with severe side effects and chronic toxicities [98].

Multiple co-stimulatory pathways are involved in the regulation of T cell activation which may function either to augment or attenuate antigen-specific T cell responses. These include (i) the CD28-CD80/86 pathway, (ii) the CD40/CD154 pathway and (iii) the LFA-1/ICAM pathway. Among these, co-stimulatory interactions between the homodimer CD28 and its ligands CD80 and CD86 of the B7 family have been reported as the most critical and certainly the best characterized one's [99, 100].

2.7.1 CD40/CD154 and LFA-1/ICAM pathways – a short overviw

The CD40/CD154 pathway was originally described to play a pivotal role for B cell activation and isotype class-switching, as well as for DC activation and maturation. Later on, this pathway was reported to contribute to T cell activation by upregulating MHC molecules and the co-stimulatory molecules CD80 and CD86 on APCs [101, 102]. More precisely, CD40 and its only known ligand CD154 (also termed CD40 ligand; CD40L) are members of the TNF and tumor necrosis factor receptor (TNFR) superfamily [103, 104]. Whereas CD40 is constitutively expressed on APCs (such as B cells, macrophages, DCs), CD154 is induced on the surface of T cells upon activation [103]. The interaction between CD40 and CD154 initially provides co-stimulatory signals to APCs rather than to T cells. Signaling via CD40 is mediated by specific adapter proteins, so called TRAFs (tumor necrosis factor receptor-associated factors) for which the cytoplasmic tail of CD40 contains the respective binding sites and leads to the activation of the transcription factor NFκB and to the induction of NFκB-responsive pathways [105, 106]. This in turn leads to an upregulation of MHC molecules and of the co-stimulatory molecules CD80 and CD86, enhanced DC survival and increased production of inflammatory cytokines such as IL-12 and TNF. Taken together, CD40 signaling results in mature APCs that are more powerful to promote effective T cell responses due to augmented antigen presentation and co-stimulation. Additionally, a poorly defined CD40L signal to the T cell might occur but the importance of this signal for the T cell remains largely unclear.

Lymphocyte function-associated antigen 1 (LFA-1) is a member of the integrin family and is constitutively expressed on all T cells at either low or high surface densities. Further it is also found on B cells, macrophages and neutrophils. LFA-1 is a ß2 integrin consisting of two chains, (i) a unique alpha chain (CD11a) and (ii) a beta chain (CD18) which is common to other ß2 integrins. LFA-1 interacts with ICAM-1 (intracellular adhesion molecule 1) which is expressed on APCs and thereby functions as an adhesion molecule [3]. Thus, LFA-1 plays an important role in stabilizing contacts between T cells and APCs and ensures optimal activation of T cells. In addition to its adhesive functions, LFA-1 delivers, through its interaction with ICAM-1, co-stimulatory signals that are important for T cell activation and cytotoxic T cell function [107, 108].

2.7.2 Molecular mechanisms underlying the CD28-CD80/86 pathway

The CD28 receptor is known to be constitutively expressed by a majority of $CD4^+$ T cells in mice and humans but was reported to be only expressed by approximately 50% of murine $CD8^+$ T cells [109]. It seems that those $CD8^+$ CD28 negative T cells might act independently of co-stimulation or use other co-stimulatory pathways to get fully activated [110]. The homodimer CD28 interacts through a specific recognition motif (MYPPPY) in its extracellular domain with CD80 and CD86 of APCs [111]. In contrast to CD28, the co-stimulatory molecules CD80 and CD86 are generally expressed, if at all, at very low levels on resting APCs, such as dendritic cells, macrophages and B cells. However, upon cellular activation CD80 and CD86 co-stimulatory molecules undergo remarkable upregulation. Both B7 molecules exhibit similar Ig-like extracellular domains and short cytoplasmic tails, while sharing only approximately 26% total sequence identity. Nevertheless, CD80 and CD86 have distinct but overlapping functions. While CD86 may be important in mediating initial T cell activation, CD80 may play a greater part in perpetuating the immune response [110, 112]. The interaction of CD28 with CD80/ CD86, together with signaling through the T cell receptor (signal 1), promotes the expansion (signal 2) of antigen-stimulated T cells and their differentiation into effector and memory cells. Additionally, CD28 signalling leads to enhanced production of cytokines (especially IL-2), upregulation of cell survival genes (such as *Bcl-x_L*) and enhanced cell cycle progression (Figure 6, middle panel). After T cell activation, the upregulation of a second receptor, termed cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is induced [113]. The homodimer CTLA-4 is structurally homologous to CD28 and also binds to CD80 and CD86 ligands via a MYPPPY recognition motif but with a higher affinity than CD28. Additionally, each CTLA-4 dimer is able to bind two different B7 homodimers, thereby forming a stable lattice-structure with B7 molecules [114]. This highly enhances the affinity (stability) of CTLA-4:B7 interactions [115]. However, this structure is not seen in the case of CD28, which can only interact with a single B7 dimer at a time [114]. Unlike CD28, CTLA-4 is a negative regulator for T cell activation and once upregulated, it successfully competes for CD80 and CD86 ligands and thus turns off T cell activation [116, 117]. T cell proliferation is downregulated by inhibiting IL-2 production and blocking cell-cycle progression (Figure 6, bottom panel). The important role of CTLA-4 as a negative regulator for T cell activation is reflected in the phenotype of CTLA-4–deficient mice. It was reported, that these mice develop severe lymphoproliferation and autoimmunity and die within a few weeks after birth. Because of CTLA-4 deficiency, T cells are prevented to

undergo cell cycle arrest. This leads to an increased accumulation of activated T cells which ultimately infiltrate vital organs causing animal death [118, 119].

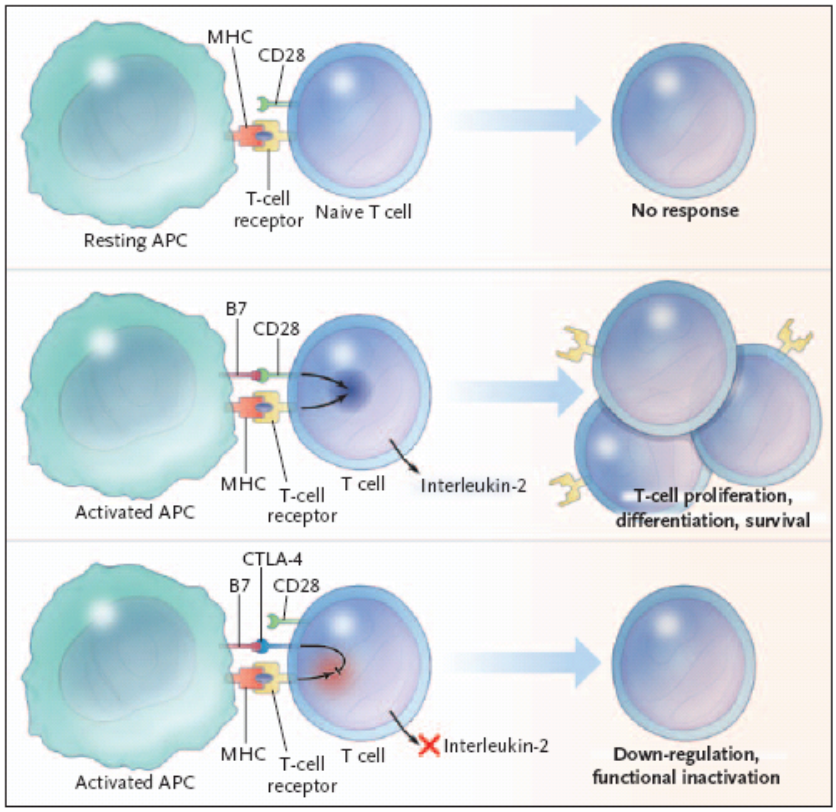


Figure 6 Roles of the B7-CD28/CTLA-4 pathway in the regulation of T cell activation

Resting APCs express low or no B7-1/B7-2 (CD80/ CD86) co-stimulatory molecules and are unable to activate naïve T cells (top panel). Upon activation APCs stimulate the expression of B7 co-stimulatory receptors. Interactions of B7 co-stimulatory molecules with CD28 receptors of the T cell lead to expansion and differentiation of naïve T cells (middle panel). The CTLA-4 receptor, which has a higher affinity to B7-1 and B7-2 co-stimulatory molecules than CD28 is upregulated on activated T cells. Interactions of B-7 molecules of activated APCs with the CTLA-4 T cell receptor inhibit T cell responses (bottom panel) (Sharpe A.H.; New England Journal of Medicine; 2006).

2.7.3 Therapeutics designed to inhibit CD28-mediated co-stimulation

Throughout the 1990s several attempts have been undertaken to inhibit CD28-mediated co-stimulatory signalling with the purpose to induce tolerance to treat autoimmune disease as well as to prevent graft rejection or graft-versus- host disease.

One strategy of blocking CD28-mediated signals resulted in the development of anti-CD80/ CD86 monoclonal antibodies with the intent to induce anergy *in vitro* or tolerance *in vivo*. For example in a human *in vitro* model, allo-anergy was induced in donor T cells by ex-vivo stimulation with allogeneic recipient APCs in the presence of humanized anti-CD80 and anti-CD86 monoclonal antibodies [120]. These antibodies were generated to bind both CD80 and CD86 co-stimulatory molecules with similar avidity and kinetics leading to an inhibition of allogeneic responses of both $CD4^+$ and $CD8^+$ T cells. In addition, the ability of $CD4^+$ and $CD8^+$ T cells to respond against viral infections (such as human herpes virus) was preserved. Furthermore, transplantation of such *ex-vivo* generated allo-anergized T cells revealed promising success in a phase 1 clinical study of haploidentical bone marrow transplantation with an acceptable engraftment and a lower outcome of viral infections and acute GvHD [121]. Furthermore, the use of such anti-CD80/ anti-CD86 antibodies has also proved successful to induce tolerance in solid organ transplantations as reviewed in Ford and Larsen 2009 [122]. Accordingly, it was shown in a non-human primate model that renal allograft rejection was delayed even in the absence of additional conventional therapies (calcineurin inhibitors (CNIs) or steroids) [123, 124]. This promising observation was further confirmed in a phase I clinical trial, where these monoclonal antibodies were safely applied as part of a combination immunosuppressive therapy [125].

Another concept to block CD28-mediated co-stimulatory signaling is to selectively target the CD28 receptor. This approach was assumed to be very beneficial in inhibiting immune responses since it was designed to specifically block CD28-mediated signaling while leaving inhibitory signals, triggered by the interaction of CTLA4 and CD80/CD86, intact. Using monoclonal antibodies targeting the CD28 receptor *in vitro* lead to a direct blockade of CD28 and could effectively inhibit allogeneic T cell proliferative responses in a mixed lymphocyte reaction. Furthermore, short- term administration of anti-CD28 mAbs was reported successful in rodent models of renal transplantation in preventing allo-graft rejection [126]. Accordingly, co-stimulation blockade using a specific anti-CD28 mAb in a fully MHC-mismatched mouse model of BMT successfully inhibited the expansion of alloreactive donor T cells and thus prevented GvHD [127]. However, most antibodies to CD28 are agonistic or superagonistic and therefore, when bound to the specific receptor, are able to initiate CD28-mediated signaling into the cell even in the absence of a TCR-signaling complex (Signal 1). Correspondingly, the application of a specific superagonistic anti-CD28 mAb in a human phase I clinical trial caused a systemic inflammatory response in six healthy volunteers

resulting in a multiorgan failure, while supporting the expansion of regulatory T cells in preclinical studies [122].

2.7.4 The fusion protein CTLA-4Ig: potent inhibitor of CD28-mediated T cell co-stimulation

Another strategy to counteract CD28-induced T cell co-stimulation is mediated by recombinant CTLA-4Ig [96, 128, 129]. In general, CTLA-4Ig is structurally based on natural CTLA4 as it is composed of human or mouse CTLA4 fused to human or mouse IgG, respectively. The resulting chimeric protein is soluble, forms homodimers and acts as a CD28 analogue featuring a much higher affinity to CD80/ CD86 present on APCs than CD28. Therefore, CTLA4-Ig effectively interferes with the binding of CD28 to B7 ligands, which results in the inhibition of CD28-mediated T cell co-stimulation leading to T cell anergy (Figure 7). Further specific modifications to CTLA-4Ig have been introduced by the company "Bristol Myers Squibb" by fusing the extracellular domain of CTLA4 to human Fcγ1 (Fc portion of IgG1) – known under the conventional name "abatacept", or "Orencia".

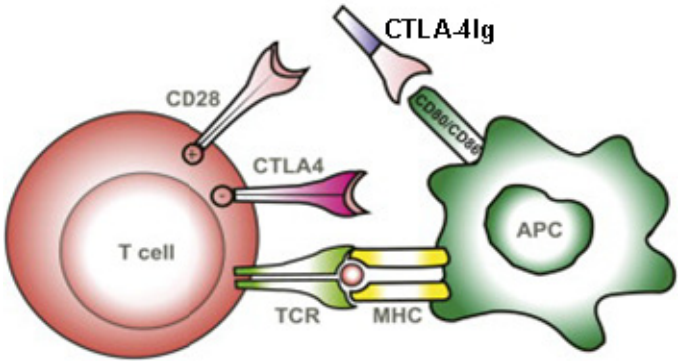


Figure 7 ***CTLA-4Ig as a potent inhibitor of CD28-mediated T cell co-stimulation***
(Vincenti F.; J Allergy Clin Immunol; 2008)

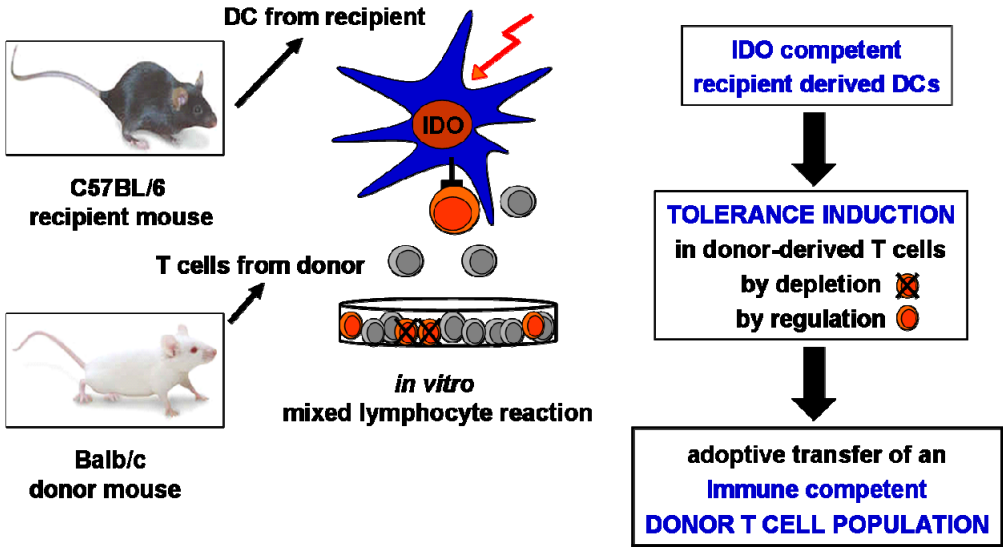
In clinical models, CTLA-4Ig was successfully tested in the treatment of autoimmune diseases including rodent models of encephalomyelitis, multiple sclerosis, autoimmune diabetes, collagen-induced arthritis, autoimmune glomerulenephritis and in several transplantation models, reviewed in [130]. In these transplantation models, application of recombinant CTLA-4Ig supported engraftment and graft survival, the induction of donor-recipient chimerism and donor-specific tolerance, while reducing GvHD in the absence of immunosuppressive regimen [130]. Furthermore, treatment with CTLA-4Ig prevented the development of anti-donor antibody and T cell responses and resulted in a long-term survival

of islet, cardiac, and renal allografts in murine models, reviewed in [122]. As “abatacept” has been successfully used as an immunosuppressive regimen in a clinical trial for the auto-immune disease rheumatoid arthritis it was approved by the US “Food and Drug Administration” (FDA), reviewed in [131].

Unexpectedly, the positive effects of administered chimeric CTLA-4Ig during transplantation could not be reproduced in studies using the more robust model of allogeneic renal transplantation and pancreatic islet transplantation in non-human primates resulting in only modest prolongation in allograft survival [132, 133]. One explanation was proposed due to the fact that “abatacept” is leaky in its ability to suppress CD28-mediated T cell stimulation since it features a lower avidity to CD86 compared with CD80 [134]. Therefore two additional mutations have been included to synthesize recombinant “belatacept” (originally termed “LEA29Y”, Bristol-Myers Squibb) that shows increased binding affinity to both B7 molecules providing more potent immunosuppressive properties required for transplantation [135, 136]. However, one has to mention that “belatacept” just showed better results than “abatacept” in non-human primate studies while being inefficient in targeting murine B7 as reviewed in Pree 2006 [137].

3 Working hypothesis

This project addresses our continuing interest in the generation of allo-antigen tolerized T cells, particularly in the context of HSCT. The capability to *ex vivo* generate donor T cell populations, which are tolerant towards recipient allo-antigens but retain immune activity against pathogenic microorganisms, may provide recipients of HSCT with immunological broadly competent T cell immunity without aggravating the risk of GvHD as long endogenous T cell reconstitution is insufficient. Based on the conceptual view of IDO and the promising findings made by utilizing co-stimulation blockade and the recently proposed link between the two, we explored the following hypothesis: Immunological tolerance can be achieved using CTLA-4Ig treated and IDO competent DCs as stimulators of allogeneic T cells.



We address this hypothesis in a murine model by co-culturing Balb/c derived donor T cells in an *in vitro* cell culture (MLR) with C57BL/6 derived recipient DCs that were induced to express functional IDO before co-culture. By concept, high IDO activity will deplete the essential amino acid TRP and accumulate TRP metabolites, such as kynurenines in the microenvironment, thus inducing tolerance in allo-reactive T cells either by depletion (induction of apoptosis in activated T cells) or by regulation (inhibition of allogeneic T cell proliferation or promotion of Tregs) while leaving resting T cells unaffected. Such *in vitro* manipulated T cells can be safely transferred to recipient mice of HSCT and are supposed to effectively transfer T cell immunity while not inducing GvHD.

4 Materials and Methods

4.1 Mouse strains

Female C57BL/6 (H-2^b), Balb/c (H-2^d) and DBA/2J (H-2^d) mice were purchased from Charles River Laboratories (Sulzfeld, Germany). All mice were maintained under specific pathogen-free conditions at the Biomedical Research Institute, Medical University of Vienna (Vienna, Austria) and were used at 6 to 10 weeks of age. All experiments were performed according to a protocol approved by the local review board of the Medical University of Vienna and were accomplished in accordance with national and international guidelines of laboratory animal care.

4.2 Cell culture

Isolated mouse cells were cultured in IMDM medium (Invitrogen, Carlsbad, USA) containing 2 mM L-glutamin and 25 mM Hepes supplemented with 10% (v/v) heat-inactivated fetal bovine serum (PAA clone; PAA Laboratories, Pasching, Austria), 100 U/mL penicillin/ 100 µg/mL streptomycin (PAA-Laboratories) and 50 µM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO) - hereafter termed complete medium, in a humidified incubator containing 5% CO_2 at 37°C.

4.2.1 Reagents for cell culture

Murine recombinant IFN-γ was purchased from BD Pharmingen. hCTLA-4Ig (abatacept) consisting of the extracellular domain of human CTLA-4 linked to a fragment (hinge-CH2-CH3 domains) of the Fc portion of human IgG1 [138, 139] was generously provided by Bristol-Meyers, Squibb Pharmaceuticals (Princeton, NJ). Recombinant mouse cytolytic CD152/Fc chimera, a soluble dimeric fusion protein engineered by fusing the extracellular domain of mouse CD152 (CTLA-4) to a mouse mutant IgG2a Fc domain was purchased from Sigma and in this study termed CTLA-4Ig(S).

4.3 Techniques of cell preparation and stimulation

4.3.1 DC generation

DCs were generated from total splenic cells or from bone marrow precursor cells of 6 - to 10 week old female C57BL/6 wt mice. The quality of DC generation was monitored microscopically every other day for a typical DC morphology. A classical DC phenotype, defined by expression levels of the cell surface markers CD11c, CD11b, MHC class I and II, CD80 and CD86, was determined by flow cytometric analysis.

Generation of splenic DCs

Splenic DCs were obtained from collagenase-treated spleens (Collagenase type IV from *clostridium histolyticum*, Sigma-Aldrich). Purification of DCs was performed according to previously described protocols [74, 81, 82, 140-143] as well as according to personal recommendations of Fallarino F. The two purification procedures used and described below, led to comparable results when cells were assayed in parallel in *in-vitro* experiments.

Preparation of splenic DCs by gradient centrifugation followed by adherence

Spleens (normally 5) were collected in a cell culture Petri dish (90x20mm; IWAKI, Japan) and air dried for a short time (1-2 min). For collagenase treatment 100 U/mL and 400 U/mL collagenase were prepared in 1x HBSS (Sigma). 1 mL of the 100 U/mL collagenase solution was injected into each spleen and effluent cells were collected with a syringe. Cell culture plates were washed with 5 mL IMDM complete medium to neutralize collagenase and spleens were then overlaid with 5-10 mL of the 400 U/mL collagenase solution. Collagenase-treated spleens were digested for 30 min at 37°C. After the incubation period, cell culture plates including the spleens were washed with IMDM complete medium to neutralize collagenase and digested spleens were smashed with a plunger and made into a single cell suspension by pressing through a 70µm sterile cell sieve (Falcon; DB Labware, Franklin Lakes, NJ). Total spleen cells were recovered by centrifugation at 1300 rpm for 8 min, the cell pellet was resuspended in MACS buffer containing 2 mM EDTA (pH 7.2) (Miltenyi) to disrupt DC-T cell complexes and cells were recovered by another centrifugation step. Cells were suspended in a 50% (p = 0,609) isoosmotic Nycodenz solution (Sigma-Aldrich), overlaid with medium and centrifuged at 3000 rpm for 15 min at 4°C. The low density fraction at the interface was collected and washed several times. The recovered cells were resuspended in IMDM complete

medium, collected in a cell culture Petri dish and allowed to adhere for 1.5-2 hours at 37°C. After the adherence step, the cell culture plates were thoroughly washed several times to remove non-adherent cells. The remaining adherent cells were provided with fresh IMDM complete medium and incubated for an addition of 18 h at 37°C to allow DCs to detach. The recovered cells were routinely 80 +/-10% positive for $CD11c^+$ expression and consisted of 90-95% $CD8\alpha^-$ and 5-10% $CD8\alpha^+$ cells.

Purification of splenic DCs by Magnetic Cell Sorting (MACS)

DCs were purified according to CD11c expression using positive selection columns in combination with CD11c MicroBeads (MACS; Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's protocol. Briefly, collagenase-treated spleens were digested for 30 min at 37°C and made into a single cell suspension as described above. Total spleen cells were recovered by centrifugation at 1300 rpm for 8 min. The cell pellet was resuspended in sterile cold Erythrocyte lysis buffer (consisting of 0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA.; pH 7.2-7.4) for 2-5 min at room temperature (RT) to remove red blood cells and cell clusters were disintegrated by vigorous pipetting. After another centrifugation step (1300 rpm; 8 min) the cell pellet was resuspended in cold MACS buffer (pH 7.2) and 1mg/ 500µL labeling volume mouse IgG (Sigma-Aldrich) was added to the cell suspension to block Fc receptor-mediated magnetic labeling followed by addition of CD11c MicroBeads (100µL/ 10^8 total cells) for 15 min at 4°C. Then, the magnetically labelled cell suspension was loaded onto a MACS column, which was placed in the magnetic field of a MACS separator. The magnetically retained $CD11c^+$ cells were eluted as the positively selected cell fraction. The recovered cells were routinely >90% $CD11c^+$ and consisted of 90-95% $CD8\alpha^-$ and 5-10% $CD8\alpha^+$ cells.

Isolation of $CD8\alpha^+$ DC subpopulations was performed in a two-step procedure using the $CD8^+$ Dendritic Cell Isolation Kit (MACS; Miltenyi Biotec) according to the manufacturer's instructions. In brief, single cell suspensions of collagenase treated spleens were prepared as described above. First, single cell suspensions were depleted of T cells, B cells, and NK cells by indirect magnetic labeling with a cocktail of biotin-conjugated Abs against CD90.2 (Thy1.2), CD45R (B220) and CD49b (DX5) and Anti-Biotin MicroBeads followed by separation over a MACS column. In a second step, the flow-through fraction containing the pre-enriched dendritic cells was labelled with CD8a (Ly-2) MicroBeads and $CD8\alpha^+$ DCs were

isolated by positive selection. The recovered cells were >90% positive for $CD11c^+$ and $CD8a^+$.

4.3.2 Generation of bone marrow derived dendritic cells (BMDCs)

DCs were generated from mouse bone marrow according to an established protocol [144] with minor modifications. Briefly, femora and tibiae of 6- to 10 week old female C57BL/6 wt mice were dislodged and remaining tissue and muscles were removed with sterile gauze. The bones were sterilized with 70% (v/v) EtOH in a cell culture Petri dish (90 x 20mm; IWAKI) for 1 minute, air dried and put into IMDM medium (Invitrogen). Both ends of the bones were cut with sterile scissors, and bone marrow was flushed with IMDM medium from femora and tibiae using a syringe and a 27G ¾ needle (0.40 x 20 mm; Sterican; B.Braun, Bethlehem, PA, USA). The bone marrow cells were centrifuged at 1300 rpm for 8 min. and the pellet was resuspended in sterile cold Erythrocyte lysis buffer (pH 7.2-7.4) for 2-5 min at RT. The bone marrow was washed twice, and cell pellets were gently resuspended in IMDM complete medium. The cell number was determined on a Coulter Particle and Size Analyzer (Coulter Electronics Ltd., UK) and cells were plated in 6-well culture plates (IWAKI) at a concentration of $2{,}5x10^6$ per well (5 mL/well). Immature DCs were generated by culture in complete IMDM medium supplemented with 5 ng/mL recombinant mouse IL-4 (BD Pharmingen) and 3 ng/mL recombinant mouse GM-CSF (BD Pharmingen) for 6 days. Half of the medium (including all supplements) was replaced every 2^{nd} day to remove non-adherent granulocytes, whereas clusters of developing DCs remained loosely attached. On day 6 of the culture, non-adherent and loosely adherent DC aggregates were gently harvested for analysis or stimulation procedures. Immature BMDCs were highly positive for CD11c and CD11b (> 80%) and showed low expression levels of MHC class I/II and CD80/CD86.

4.3.3 DC activation

Splenic DCs were plated in 24-well culture plates (IWAKI) at a concentration of $1x10^6$/mL per well and exposed to the indicated concentration of 40 µg/mL CTLA-4Ig (IgG1a or IgG2a tails) or 200 U/mL IFN-γ for 24h. At the end of the stimulation period cells were harvested, phenotypically analyzed and used for subsequent experiments.

Bone marrow derived immature DCs were plated in 24-well culture plates (IWAKI) at a concentration of $1x10^6$/mL per well and exposed to a previously optimized concentration of

100 ng/mL LPS (Calbiochem; *Escherichia coli* O111:B4) with or without 20 ng/mL IFN-γ [144] for 48h hours. At the end of the activation period cells were harvested, phenotypically analyzed and used for subsequent experiments. This DC activation resulted in the upregulation of the surface markers MHC class I/II and CD80/86.

4.3.4 $CD3^+$ T lymphocyte isolation

$CD3^+$ T cells were isolated from spleens of wt Balb/c mice by magnetic cell sorting (negative selection) using the Pan T Cell Isolation Kit (MACS; Miltenyi Biotec) according to manufacturer's instructions. Briefly, spleens were made into a single cell suspension as described above. Total spleen cells were recovered by centrifugation at 1300 rpm for 8 min and red blood cells were lysed in a hypotonic buffer (pH 7.2-7.4) for 2-5 min at RT as mentioned above. Cells were washed, recovered by centrifugation and resuspended in cold MACS buffer (pH 7.2). Non-T cells were indirectly magnetically labelled with a cocktail of biotin-conjugated Abs against CD11b (Mac-1), CD45R (B220), DX5 and Ter-119 as well as Anti-Biotin MicroBeads. The magnetically labelled non-T cells were depleted by retaining them on a MACS column, placed in the magnetic field of a MACS separator. Unlabelled T cells passed through the column and could be collected. This procedure resulted in greater than 95% enrichment of the targeted cell population. The purity and viability of the enriched T cells was evaluated by flow cytometry.

4.3.5 T cell stimulation and mixed lymphocyte reaction

Highly purified total $CD3^+$ T cells (1×10^5) were co-cultured with allogeneic DCs (1×10^4) for 3 to 6 days in 96-well round bottom plates (NUNC; Thermo Fisher) in triplicates in a total volume of 200 µL IMDM complete medium per well (mixed lymphocyte reaction; MLR). In some experiments, DC/T cell co-cultures were performed in 24-well plates (IWAKI) in a 1:10 DC to T cell ratio (1×10^5 DC and 1×10^6 T cells) in a total volume of 1.1 mL IMDM complete medium per well. Where indicated, CTLA-4 Ig (100µg/mL; IgG1a or IgG2a fusion) was added to the MLR on day 0 and thus for the whole culture period [145].
For DC independent proliferation assays, $CD3^+$ T cells (1×10^5) were cultured in 96-well round bottom plates coated with 3 µg/mL anti-CD3 (145-2C11) and 1 µg/mL anti-CD28 (37.51) (BD Biosciences) in triplicates in a total volume of 200 µL per well for 48 hours.

T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) (Sigma) according to a previously described protocol [146]. Briefly, T cells were washed with 1 x PBS and resuspended in 1 x PBS containing 0.1%BSA at a concentration of $1x10^7$ cells/ mL. Cells were stained with 5µM CFSE for 10 min at 37°C followed by short blocking with fetal bovine serum. Afterwards, cells were washed twice with IMDM complete medium and stored on 37°C until further use. Proliferative response was assessed by flow cytometric analysis of CFSE dilution. Where indicated, the "FlowJo Proliferation Platform" (Tree Star, Inc.) was used to analyze T cell proliferation, where "Percent divided" is defined as the percentage of T cells in the starting population that divided (assuming that no cells died during the culture). Percentage of inhibition was calculated by comparing the percentage of CFSE-negative T cells in co-cultures performed in the absence of CTLA-4Ig to co-cultures in which CTLA-4Ig was present according to the following formula: Percentage of inhibition = [1- (percentage of $CFSE^-$ T cells in co-cultures with CTLA-4Ig / percentage of $CFSE^-$ T cells in co-cultures without CTLA-4Ig)] x 100.

4.4 Techniques of analyses

4.4.1 Flow cytometry

Flow cytometric analyses were performed by using a FACSCalibur or a BD LSR II flow cytometer (BD Biosciences) and the list mode data were analyzed using either DivaCell (BD Biosciences) or FlowJo (Tree Star, Inc.) software. Cell staining procedure was performed according to standardized methods in our laboratory. In brief, 1 x 10^5 cells were washed in 1 x PBS (Invitrogen), recovered by centrifugation (1300 rpm; 8 min) and resuspended in 30µL purified anti-CD16/32 (mouse, clone 2.4G2, BD Pharmingen) diluted 1:100 in 1 x PBS for 10 min at 4°C to block unspecific binding via Fcγ receptors. Tested amounts of the respective fluorochrome-coupled mAbs were added and samples were incubated for 30 min at 4°C. After an additional washing step with 1 x PBS (1300 rpm; 8 min), the supernatant was discarded and the cells were immediately analyzed by flow cytometry. The viability of the cells was determined by addition of DAPI (4',6 Diamidino-2-phenylindoldihydro-chlorid) shortly before measurement. Appropriate isotype control antibodies were included in the analyses.
The following FACS antibodies were used: unconjugated anti-CD16/32 (2.4G2), FITC-anti-H-2D[b] (KH95), PE-anti-I-A[b] (AF6-120.1), PE-Cy™7-anti-CD11c (HL3), APC-Cy™7-anti-CD11b (M1/70), PerCP-anti-CD8a (53-6.7), APC-anti-CD3 (145-2C11), PerCP-anti-

CD4 (RM4-5), APC-Cy7-anti-CD8a (53-6.7), PE-Cy™7-anti-CD25 (PC61), FITC-anti-H-2D[d] (34-2-12), PE-anti-B220 (RA3-6B2), APC-anti-CD8a (53-6.7) (all from BD Biosciences), PerCP/Cy5.5-anti-CD80 (16-10A1), Alexa Fluor® 700-anti-CD86 (PO3), FITC-anti-CD62L (MEL14), APC-anti-CD90.2 (30-H12) (all from BioLegend, San Diego, CA). For intracellular staining of FoxP3 in T cells, the Alexa Fluor 647 anti-mouse/rat/human FOXP3 Flow Kit (BioLegend) was used according to the manufacturer's protocol.

4.4.2 Enzyme-linked Immunosorbent Assay (ELISA)

For examination of IFN-γ cytokine secretion, cell culture supernatants of DCs were analyzed by ELISA technique with the use of the BD OptEIA™ Mouse IFN-γ ELISA Set (BD Biosciences). Cell culture supernatants were centrifuged (1300 rpm; 5 min) to remove any particulate material and samples were stored at -20°C or immediately used. The assay procedures were performed exactly as recommended by the manufacturer's protocol.

4.4.3 Immunoblotting

IDO protein expression in DCs was investigated by immunoblot analysis. For the preparation of protein extracts from different dendritic cell cultures, cells (5 x 10^5-1 x 10^6) were harvested, washed twice in cold 1 x PBS and lysed by addition of 50 µL per 1 x 10^6 cells radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150mM NaCl, 1mM EDTA) supplemented with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics GmbH, Germany) for 10 min at 4°C. After a centrifugation step, SDS sample buffer (20% Glycerin; 0.125M Tris-HCl; 6% 2-mercaptoethanol; 3% SDS; bromphenole-blue) was added to the supernatant containing the whole cell lysates and proteins were denaturated for 5 min at 95°C. After cooling on ice, the samples were either used immediately for analyses or frozen at -20°C until use. Protein samples were fractionated on a 10% SDS-polyacrylamide gel for 90 min at 60 mA and transferred to a nitrocellulose membrane (Millipore Corp., Bedford, MA, USA) using a tank blot unit (BioRad Laboratories, Hercules, CA, USA), strictly working with cold buffers and blotting on ice at 300 mA for 60 min. As molecular mass standard a pre-stained protein marker (BenchMark™ Pre-Stained Protein Ladder (6-180 kDa); Invitrogen) was used. In order to control the efficiency of blotting, membranes were reversibly stained with 1x PonceauS Red (Sigma). After ordinary washing with A.d and 1 x PBST (0.1% Tween),

membranes were blocked for 1 hour at RT (PBS; 0.2% Tween 20; 5% non-fat dry milk) and probed with the respective antibodies. For IDO protein detection a rabbit anti-mouse IDO polyclonal antibody (diluted 1:2500 in 1x PBST with 0.1% Tween, 5% BSA, 0.1% NaN_3, 4°C O.N.) was kindly provided by O. Takikawa (National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Japan) [147, 148] and a mouse monoclonal anti-mouse glyceraldehyde-3-phosphate dehydrogenase (GapdH) antibody (diluted 1:4000 in 1x PBST with 0.1% Tween, 5% BSA, 0.1% NaN_3, 1h RT) was purchased from Ambion (Austin, TX, USA) and used as an internal control. Detection of antibody binding was performed using the Odyssey Infrared Imaging System (Odyssey Classic; LI-COR Biosciences, Lincoln, Nebraska USA) according to the manufacturer's instructions and the respective fluorescent secondary antibodies: Goat Anti-Rabbit IgG, DyLight™800 Conjugated and Goat Anti-Mouse IgG, DyLight™680 Conjugated (Pierce Biotechnology/ Thermo Fisher Scientific, Rockford, IL., USA; diluted 1:20.000 in LI-COR blocking buffer; 20min RT in the dark).

4.4.4 RNA isolation and semiquantitative reverse transcription PCR

Expression levels of IDO transcript in DCs were determined by semiquantitative reverse transcription-PCR procedure (RT-PCR). Total cellular RNA was isolated from cell populations and RT-PCR was performed by reverse transcription of 2µg total RNA. In brief, total RNA was isolated from cells with the use of TRIZOL reagent (Invitrogen) according to the recommendations of the manufacturer and stored at -80°C or immediately used for cDNA synthesis. RNA was reversed transcribed with 200 Units Moloney-murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) and 100 pmol random hexamers (GE Healthcare, Vienna, Austria) at 42°C for 1 h. RT-PCR was performed using Hot Start Taq polymerase (Qiagen, Vienna, Austria) according to the manufacturer's instructions with an initial activation step at 95°C for 14 min. Cycling conditions were as follows: denaturation at 95°C for 30 sec, annealing at 60°C for 30 sec, and elongation at 72°C for 1 min carried out for 35 cycles, followed by a final extension at 72°C for 7 min. Amplification of the house keeping gene GapdH was used as internal PCR control. Oligonucleotides (MWG Biotech AG, Ebersberg, Germany) used for amplification of the murine IDO or of the murine GapdH and PCR product sizes are listed in Table 1. Amplification products were size-fractionated by agarose gel electrophoresis on a 1% agarose gel, stained with ethidium bromide and quantified by scanning densitometry (Gel-Doc 1000, Molecular Analyst Software, Biorad).

Gene	Sense primer	Antisense primer	Amplicon size(bp)
IDO	CGA CAT AGC TAC CAG TCT GGA GAA AG	GCG AGG TGG AAC TTT CTC ACA GAG	441
GapdH	ACC ACA GTC CAT GCC ATC AC	TCC ACC ACC CTG TTG CTG TA	452

Table 1 ***Oligonucleotides and PCR product sizes for the determination of IDO and control gene expression***

4.4.5 Quantification of kynurenine, tryptophan and nitric oxide

IDO enzymatic activity was determined by measuring the levels of tryptophan and kynurenine in the cell culture supernatants by high-pressure liquid chromatography (HPLC) as previously described [86, 149, 150] in cooperation with D. Fuchs (Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Austria). Briefly, tryptophan was detected by its natural fluorescence at 286 nm excitation and 366 nm emission wavelengths. 3-Nitro-L-tyrosine, used as an internal standard, and kynurenine were determined by ultraviolet absorption at 360 nm. An albumin-based external standard mix was prepared and included 50 µM tryptophan (Serva, Heidelberg, Germany), 10 µM kynurenine (Sigma-Aldrich), and a frozen serum pool. Upon the addition of 25 µL of 2 M trichloroacetic acid (Merck, Darmstadt, Germany) the reaction vials were immediately vortexed and centrifuged at 12 000g (13 000 rpm) for 6 min at RT to precipitate protein. The concentration of the components was calculated according to peak heights and was compared with 3-nitro-L-tyrosine as a reference standard.

The production of nitric oxide (NO) in murine DCs produced by active iNOS (inducible nitric oxide synthase) into the cellular microenvironment was indirectly evaluated by measuring levels of nitrate and nitrite in the cell culture supernatants. The iNOS activity was determined in cooperation with D. Fuchs (Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Austria) as previously described (Maloney, E. M., O. St Claire Morgan, et al. (2000). Briefly, concentrations of total nitrite and nitrate in cell supernatants were measured by Griess reaction: 75 µl A.d. and 125 µl Griess reagent were added to 50 µl of samples and the mixture was incubated for 10 min at RT. A 96-well-reader/photometer was used for determination of the absorption at 560 nm.

4.5 In vivo experiments

4.5.1 Procedure of HSCT and adoptive T cell transfer

Recipient C57BL/6 mice (female, 8-10 weeks old) received myeloablative irradiation therapy (10.0 Gy) as a conditioning regimen. A conventional source of x-ray irradiation was used (250 kV, tube 20x20 cm, 3.1 mm copper-, 0.8 mm tin-, 0.25 mm chromium- and 1.02 mm aluminum filter) at a dose rate of 0.5 Gy per minute. This type of irradiation has been routinely used for similar procedure [151] at the Department for Radiation Therapy, Medical University Vienna, Austria. Donor bone marrow cells (BMCs) were obtained from femora and tibiae of age-matched (6- to 10 week old) Balb/c donor mice as described above (chapter 1.3.2.;Generation of BMDCs). Approximately 1 x 10^7 unseparated BMCs were infused into the tail vein of C57BL/6 recipients within 24 hours of myeloablation (d0, i.v.). This procedure generally allowed for rapid and uncomplicated engraftment. The Balb/c to BL/6 donor-recipient strain combination is one of the most stringent models as it crosses MHC mismatches plus minor histocompatibility antigen mismatches [152].
For the induction of GvHD in recipient C57BL/6 mice different tested concentrations (5 x 10^6 or 1 x 10^7) of $CD3^+$ T cells isolated of Balb/c donor derived total splenocytes as described above (chapter 4.3.4.; $CD3^+$ T cell isolation) were adoptively transferred to HSCT recipients within 24 hours of myeloablation (d0, i.v.).

4.5.2 GvHD monitoring

The monitoring of the animals for GvHD included (i) daily scoring of clinical GvHD for ~ 25-40 days according to the commonly used scoring system for murine bone marrow transplantation (BMT), (see below) (ii) the monitoring of the extent of chimerism (iii) the histopathological evaluation of autopsy material for the presence of GvHD in target organs.

GvHD scoring

The survival and appearance of mice were monitored daily and body weight was measured weekly. GvHD scoring included the assessment of the criteria as previously described [153] and illustrated in Table 2.

criteria	*grade 0*	*grade 1*	*grade 2*
weight loss	< 10%	10% - 25%	>25%
posture	normal	hunching noted only at rest	severe hunching, impairs movement
activity	normal	mild to moderately decreased	stationary unless stimulated
fur texture	normal	mild to moderate ruffling	severe ruffling/poor grooming
skin integrity	normal	scaling of paws/tail	obvious areas of denuded skin

Table 2 ***Assessment of clinical GvHD in transplanted animals*** *(Cooke et al.; Blood 1996)*

Assessment of chimerism

Blood was taken from the tail vein of recipient C57/BL6 mice following heating of the animals. The amount of blood taken did not exceed 1% of the body weight of the animals (~0.1-0.2 mL). Multilineage chimerism in a representative BMT recipient was analyzed by flow cytometry. $CD4^+$ and $CD8^+$ T cells, B cells (anti-B220) and myeloid cells were assessed for recipient chimerism in regular intervals by flow cytometry using the appropriate strain specific anti-HLA antibodies as described previously [151]. All antibodies used for chimerism studies are listed in chapter 4.4.1. Flow cytometry.

Histopathological analysis of tissue samples

At the end of the observation period HSCT recipients were sacrificed by cervical dislocation. Spleen, liver, gut, skin and lymphnode samples were examined by histopathology for typical signs of GvHD in collaboration with Fritz Wrba (Medical University of Vienna) in a blinded fashion. Four micrometers sections were cut from paraffin-embedded tissue fixed in 4.5% formalin (with a buffer pH of 7.5). Sections were stained with hematoxillin-eosin and Giesma according to standard protocols and were analyzed by an experienced pathologist.

4.6 Statistical analysis

All statistical analyses were performed using the Student *t* test (paired, 2-tailed). A *P* value below 0.05 was considered to indicate statistical significance. Survival curves were analyzed using the Kaplan-Meier algorithm.

5 Results

5.1 Impact of CTLA-4Ig and IFN-γ on tryptophan catabolism in murine splenic DCs

Murine splenic DCs have been reported to express IDO protein and tryptophan metabolizing activity upon exposure to CTLA-4Ig and were reported to suppress allogeneic T cell responses in an IDO dependent manner [74, 154]. These finding led to the assumption that binding of CTLA-4Ig to CD80/86 of the DC can initiate, via "reverse" signaling, the upregulation of functional IDO leading to tryptophan catabolism that is sufficient to inhibit proliferative allogeneic T cell responses [75]. Similarly, IFN-γ was found capable to induce strong IDO protein expression in splenic DCs where IDO metabolic activity appeared to be restricted to the $CD8\alpha^+$ subset [69, 155]. However, some other studies showed that the immunmodulatory effect of CTLA-4Ig was independent of IDO activity [156, 157].

To pin down the effect of CTLA-4Ig on the induction of a DC regulatory phenotype through IDO in allogeneic stimulation, we treated splenic $CD11c^+$ DCs with two different types of CTLA-4Ig fusion proteins (CTLA-4Ig (S); abatacept) or IFN-γ for 24h. Subsequently, the phenotype and capacity to acquire IDO expression and tryptophan metabolizing enzymatic activity were examined in these stimulated DCs.

Highly enriched splenic $CD11c^+$ DCs displayed a mature phenotype, indicated by the presence of MHC class I and MHC class II molecules, and high levels of expression of the co-stimulatory molecules CD80 and CD86 [158, 159]. The exposure of DCs to CTLA-4Ig resulted in reduced detectability of CD80/CD86 co-stimulatory molecules as previously reported [75], suggesting that the fusion protein bound properly. However, abatacept affected particularly the CD80 molecule while leaving expression levels of CD86 largely unchanged. This finding is consistent with previous observations as CTLA-4Ig, binding both CD80 and CD86 but with a lower affinity to the latter one, was reported being less potent in inhibiting CD86- than CD80-dependent co-stimulation [135, 136]. Consistent with the specificity of CTLA-4Ig, no effect was observed on the expression levels of MHC class I and MHC class II molecules. No effect on CD80/CD86 expression was observed upon stimulation with IFN-γ (Figure 8).

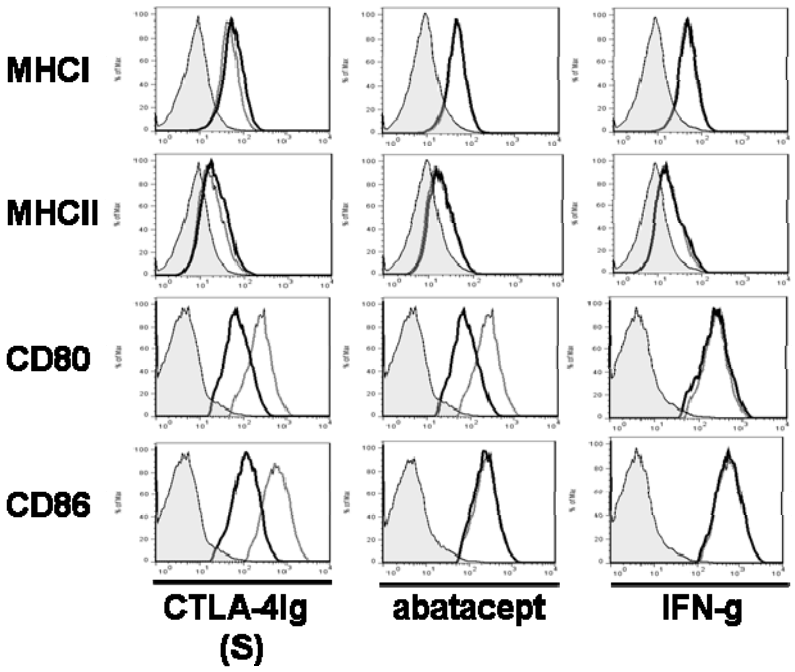


Figure 8 Phenotypical analysis of splenic CD11c$^+$ DCs.

DCs were stimulated with two different types of CTLA-4Ig fusion proteins (CTLA-4Ig(S), abatacept) or IFN-γ for 24h and DC phenotype was analyzed by FACS. Expression levels of cell-surface molecules (MHCI, MHCII, CD80/CD86) typically associated with DC maturation are depicted (shaded histograms indicate isotype control; open black histograms, DCs after stimulation; open grey histograms, untreated DCs). One representative of 3 similar experiments is shown.

Subsequently, splenic DCs were analyzed for expression of IDO-specific transcript by RT-PCR. Consistent with previous reports [154], in the absence of CTLA-4Ig or IFN-γ, considerable expression of IDO mRNA was observed in splenic DCs in contrast to Baf3 cells, used as a negative control. However, this baseline level of IDO mRNA transcript was not upregulated upon stimulation with either CTLA-4Ig (CTLA-4Ig(S) and abatacept) or with IFN-γ (Figure 9A). Additionally, IDO expression was investigated by immunoblot analysis. In line with other studies, baseline levels of IDO protein were detected in freshly isolated splenic DCs even in the absence of a stimulus [75, 76, 154]. IDO expression in human IDO competent DCs [85] was used as positive control and purified T-lymphocytes served as a negative control. Similar to IDO mRNA expression, we did not find an increase of basal IDO protein expression levels upon stimulation with either CTLA-4Ig nor by stimulation with IFN-γ (Figure 9B). The failure of CTLA-4Ig and IFN-γ to enhance IDO transcription and expression above baseline level was confirmed by analysis of IDO enzymatic activity measured as tryptophan degradation and accumulation of kynurenine in the cell culture

supernatant. Neither CTLA-4Ig (CTLA-4Ig (S) and abatacept) nor IFN- γ caused a decrease of tryptophan levels and no increase of kynurenine levels was detectable in the cell culture supernatant at the termination of the activation period. The concentrations of kynurenine in untreated control cultures and in stimulated conditions consistently remained < 1 µmol/L (Figure 9C) in contrast to human DCs stimulated by a combination of LPS/IFN-γ [85]. Even excess doses of CTLA-4Ig up to 100 µg/mL or IFN-γ up to 3500 U/mL did not induce DCs to metabolize tryptophan (Table 3).

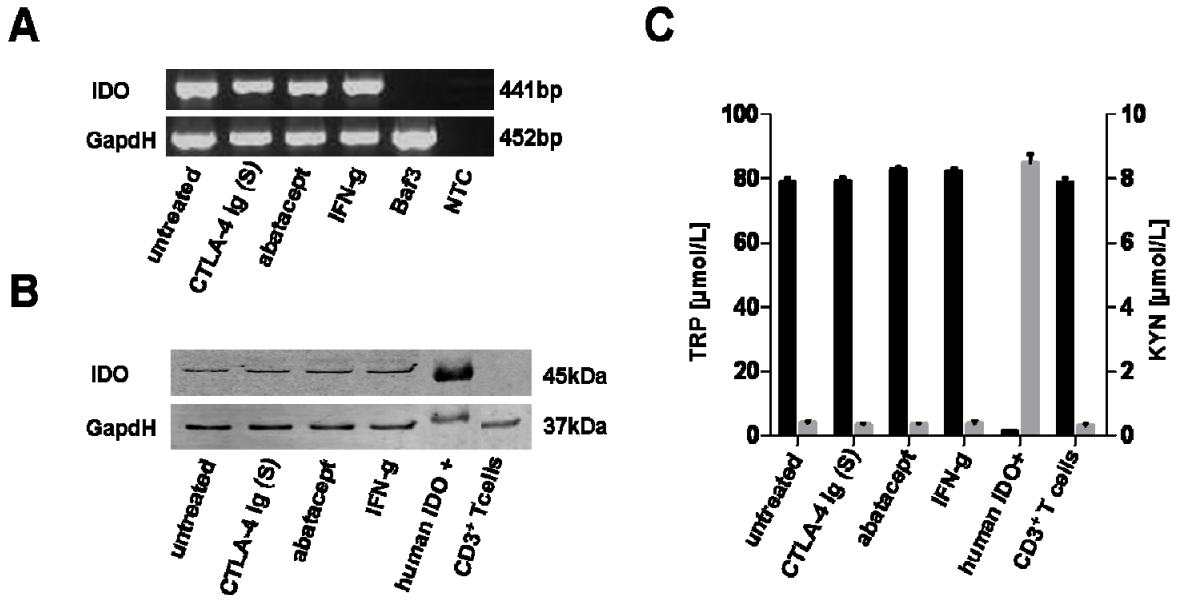


Figure 9 ***IDO expression and enzymatic activity is not induced in CTLA-4Ig or IFN-γ stimulated splenic CD11c$^+$ DCs.***

(A) *IDO mRNA expression was analyzed by RT-PCR using total RNA from untreated, CTLA-4Ig or IFN-γ treated DCs. Total RNA of murine Baf3 cells was used as negative control and GapdH as internal standard; NTC (non template control) indicates that no cDNA was added to the amplification mix during RT-PCR.* ***(B)*** *IDO protein expression in the same DCs as in (A) analyzed by immunoblotting. Human DCs stimulated by LPS/IFN-γ for 48h (human IDO+) as described [85] were used as positive and murine CD3$^+$ T cells were used as negative controls. GapdH served as internal standard.* ***(C)*** *The concentrations of tryptophan (TRP; black bars, left scale) and kynurenine (KYN; grey bars, right scale) in cell culture supernatants of the same DCs as in (A) were determined by HPLC. Error bars indicate the range of a total of 6 consecutive experiments.*

treatment	TRP [µmol/L]	KYN [µmol/L]
0 µg/mL CTLA-4Ig	80.2	0.32
20 µg/mL CTLA-4Ig	83.5	0.28
40 µg/mL CTLA-4Ig	77.8	0.30
100 µg/mL CTLA-4Ig	79.6	0.26
0 U/mL IFN-γ	77.7	0.26
200 U/mL IFN-γ	83.8	0.27
500 U/mL IFN-γ	81.6	0.27
1400 U/mL IFN-γ	80.0	0.27
3500 U/mL IFN-γ	84.3	0.28

Table 3 ***Tryptophan (TRP) and kynurenine (KYN) concentrations measured by HPLC*** *in cell culture supernatants of CD11c$^+$ splenic DCs stimulated for 24h with increasing concentrations (0-100 µg/mL) CTLA-4Ig (abatacept) or IFN-γ (0-3500 U/mL).*

Due to the fact that exposure of splenic CD11c$^+$ DCs to CTLA-4Ig or IFN-γ did not induce enzymatic activity of IDO, we next investigated whether IDO activity would be inhibited in those stimulated DCs. One possible mechanism for inhibition of IDO enzymatic activity is the accumulation of NO, produced by the IDO antagonist iNOS, into the cellular microenvironment of DCs [72]. Thus DCs were stimulated with two different types of CTLA-4Ig fusion proteins or IFN-γ for 24h as described above and iNOS enzymatic activity was analyzed by measuring NO concentrations in the cell culture supernatants. However, no significant increase of NO levels was detectable in the cell culture supernatants of both, CTLA-4Ig or IFN-γ stimulated DCs at the termination of the activation period. NO levels measured by HPLC in untreated control cultures and in stimulated conditions consistently remained <1 µmol/L (Figure 10). Hence, iNOS activity is not induced in splenic DCs upon stimulation with CTLA-4Ig or IFN-γ and is not involved in the incapability of these stimulated DCs to acquire functional IDO activity.

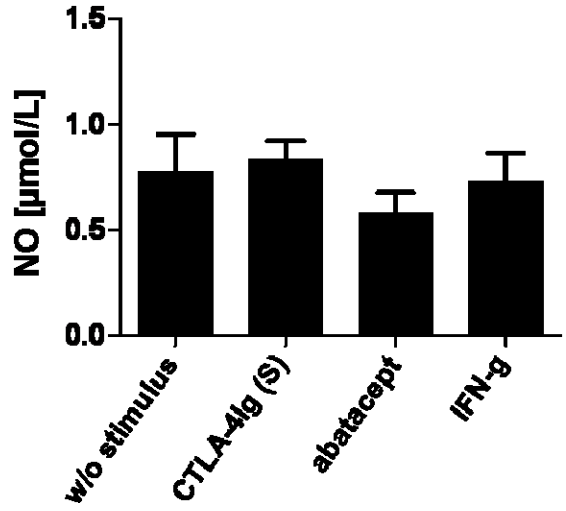


Figure 10 iNOS enzymatic activity is not induced in stimulated splenic CD11c$^+$ DCs. *DCs were stimulated for 24h with CTLA-4Ig (CTLA-4Ig(S); abatacept) or IFN-γ and the concentrations of nitric oxide (NO; black bars) in cell culture supernatants were determined by HPLC. Error bars indicate the range of a total of 6 consecutive experiments.*

In a separate set of experiments, we specifically enriched total splenic CD11c$^+$ DCs for their minute (~5%) CD8α$^+$ population and examined whether this particular DC subpopulation acquired IDO competence through CTLA-4Ig or IFN-γ as reported previously [154, 160]. However, like the total splenic DCs, the CD8α$^+$ DCs, while displaying baseline levels of IDO protein expression, did not acquire the capability to metabolize tryptophan and produce kynurenines upon stimulation with CTLA-4Ig or IFN-γ. The concentrations of kynurenine in untreated control cultures and in stimulated conditions consistently remained < 1 µmol/L (Table 4).

treatment	TRP [µmol/L]	KYN [µmol/L]
w/o stimulus	80.6	0.42
CTLA-4Ig (S)	80.5	0.28
abatacept	77.7	0.28
IFN-γ	76.6	0.26

Table 4 Tryptophan (TRP) and kynurenine (KYN) concentrations measured by HPLC *in cell culture supernatants of CD8α$^+$ splenic DCs stimulated for 24h with 40 µg/mL CTLA-4Ig (CTLA-4Ig(S); abatacept) or 200 U/mL IFN-γ. Results of 1 experiment, representative of 2 experiments, are shown.*

Finally, we examined whether CTLA-4Ig exposure *in vivo* would induce IDO enzymatic activity in splenic DCs as previously reported [74, 75]. Therefore, 100µg of CTLA-4Ig(S) or isotype-matched purified IgG2a were injected intraperitoneally (i.p.) into C57BL/6 mice. Splenic $CD11c^+$ DCs were recovered 24h after treatment of mice with CTLA-4Ig or purified IgG2a and IDO enzymatic activity was analyzed in cell culture supernatants. Anyhow, even CTLA-4Ig exposure *in vivo* did not induce functional IDO activity in splenic DCs. Kynurenine concentrations consistently remained < 1 µmol/L in both, supernatants of DCs recovered from CTLA-4Ig(S) or IgG2a treated mice (Figure 11).

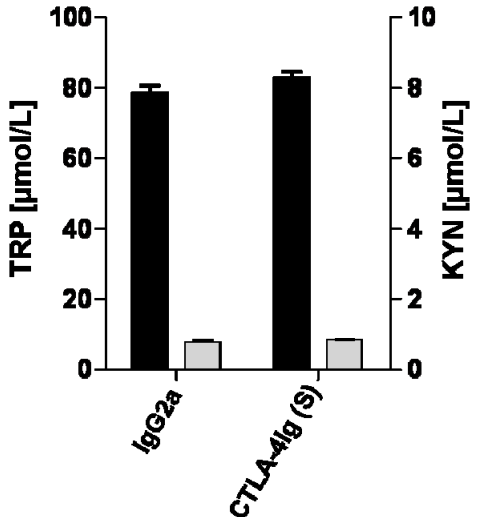


Figure 11 CTLA-4Ig exposure in vivo to induce IDO enzymatic activity in splenic DCs. *C57BL/6 mice received 100µg CTLA-4Ig (S) or isotype-matched purified IgG2a (i.p.). Splenic $CD11c^+$ DCs were isolated 24h after treatment of mice with CTLA-4Ig or IgG2a and cultured in IMDM complete medium until the next day. Tryptophan (TRP; black bars, left scale) and kynurenine (KYN; grey bars, right scale) concentrations in cell culture supernatants were determined by HPLC. Error bars indicate the range of a total of 3 consecutive experiments.*

Taken together, these findings suggest that in the present study neither CTLA-4Ig nor IFN-γ were able to induce IDO expression or enzymatic activity in splenic C57BL/6 DCs.

5.2 CTLA-4Ig primes splenic DCs for IFN-γ production but fails to initiate IDO competence

Since we could not detect any IDO activity in splenic DCs upon stimulation with CTLA-4Ig fusion proteins, we decided to verify whether CTLA-4Ig would have an effect on DCs at all. Therefore, we next sought to test whether CTLA-4Ig had an effect on DC IFN-γ production, as CTLA4-Ig binding to co-stimulatory molecules CD80/CD86 was reported to elicit IFN-γ production by DCs finally inducing IDO activity in an autocrine and/ or paracrine fashion [71, 76].

Thus, we measured IFN-γ release in the supernatants of CTLA-4Ig (abatacept; CTLA-4Ig(S)) treated versus untreated DCs. In these experiments we found that the exposure of DCs to both types of CTLA-4Ig fusion proteins caused a slight increase of IFN-γ secretion by C57BL/6 splenic DCs (Figure 12A). However this increase did not result in detectable tryptophan consumption or kynurenine production (Figure 12B).

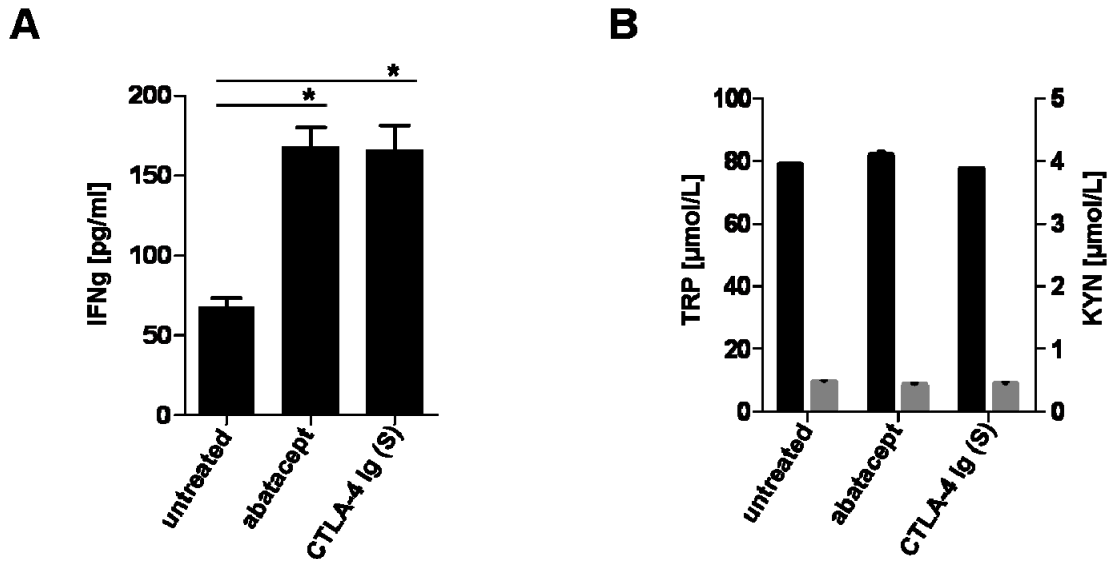


Figure 12 Induction of IFN-γ release in C57BL/6 splenic CD11c$^+$ DCs by CTLA-4Ig.

(A) *The amount of IFN-γ release in cell culture supernatants of splenic DCs treated with CTLA-4Ig (CTLA-4Ig(S); abatacept) for 24 h was analyzed by ELISA technique. The asterisk indicates significant differences between CTLA-4Ig treated and untreated DCs (*P < 0.05). Error bars indicate the range of a total of 3 consecutive experiments.* ***(B)*** *Cell culture supernatants of DCs stimulated as in (A) were analyzed for IDO activity by HPLC at the end of the 24h stimulation period in parallel. Tryptophan (TRP; black bars, left scale) and*

kynurenine (KYN; grey bars, right scale) concentrations in µmol/L are depicted. Error bars indicate the range of a total of 3 consecutive experiments.

Similar findings were obtained with splenic CD11c$^+$ DCs obtained from DBA/2J mice [76] (Figure 13). These findings finally excluded that the lack of IDO induction in DCs, exposed to CTLA-4Ig, was limited to the C57BL/6 strain.

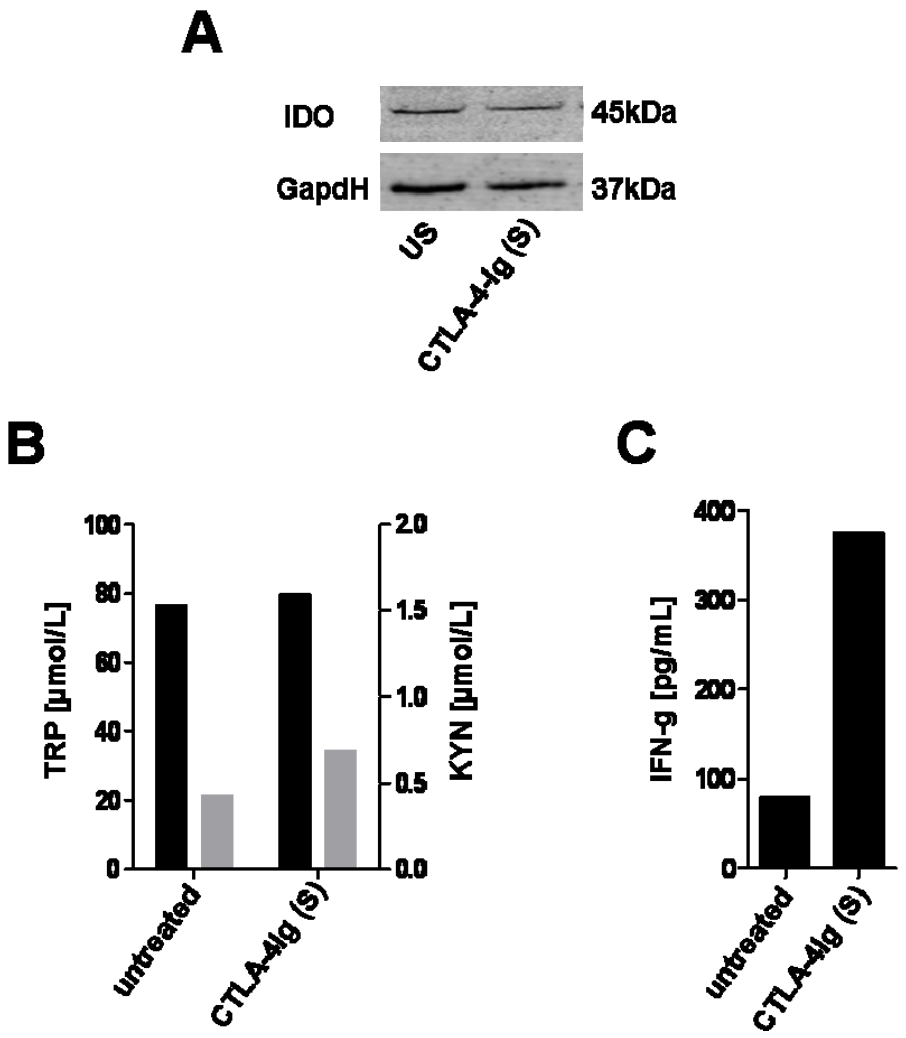


Figure 13 The effect of CTLA-4Ig on splenic CD11c$^+$ DCs obtained from DBA/2J mice. ***(A)*** *Splenic DCs were exposed to CTLA-4Ig (S) for 24h and IDO protein expression was examined by immunoblot analysis.* ***(B)*** *Concentrations of tryptophan (TRP; black bars, left scale) and kynurenine (KYN; grey bars, right scale) were examined by HPLC in cell culture supernatants of the same DCs as in (A).* ***(C)*** *Cell culture supernatants of the same DCs as in (A) were examined for IFN-γ release by ELISA technique. Results of 1 experiment, representative of 2 experiments, are shown.*

In summary, the two types of CTLA-4Ig fusion proteins tested caused DCs to produce IFN-γ but consistently failed to induce DCs to acquire IDO expression and activity.

5.3 Exposure of splenic DCs to CTLA-4Ig or IFN-γ does not confer a DC regulatory phenotype

From the recently reported effect of CTLA-4Ig on DCs it was assumed that CTLA-4Ig conferred a regulatory DC phenotype [75]. To explicitly address whether CTLA-4Ig or IFN-γ affect the stimulatory capacity of DCs *per se*, we devised an experiment, in which the DC population was pre-exposed to CTLA-4Ig or IFN-γ and washed before stimulating allogeneic T cells. Like in the experiments above, C57BL/6 splenic DCs were exposed to the different types of CTLA-4Ig fusion proteins (abatacept; CTLA-4Ig(S)) or IFN-γ for 24 hours or left untreated. At the end of the 24-hour pre-stimulation period the DCs were thoroughly washed and co-cultured with Balb/c T cells (mixed lymphocyte reaction, MLR) and the amount of allogeneic T cell responses was determined by CFSE dilution.

C57BL/6 derived splenic DCs were identified as potent stimulators of allogeneic Balb/c T cells, resulting in a proportion of approximately ~ 70% (71 ± 1.9%, mean ± SEM, n=3) of $CFSE^-$ T cells after a 6 day co-culture period (Figure 14). Strikingly, a pre-exposure of these DCs to CTLA-4Ig or IFN-γ did not reduce the capacity of the DCs to subsequently stimulate allogeneic T cells. In fact, C57BL/6 DCs induced comparable allogeneic T cell proliferation irrespective of whether they were left untreated or pre-exposed to CTLA-4Ig (abatacept; CTLA-4Ig(S)) or IFN-γ (Figure 14). This experimental outcome indicated that DCs pre-exposed to CTLA-4Ig or IFN-γ did not acquire a regulatory phenotype *in vitro*.

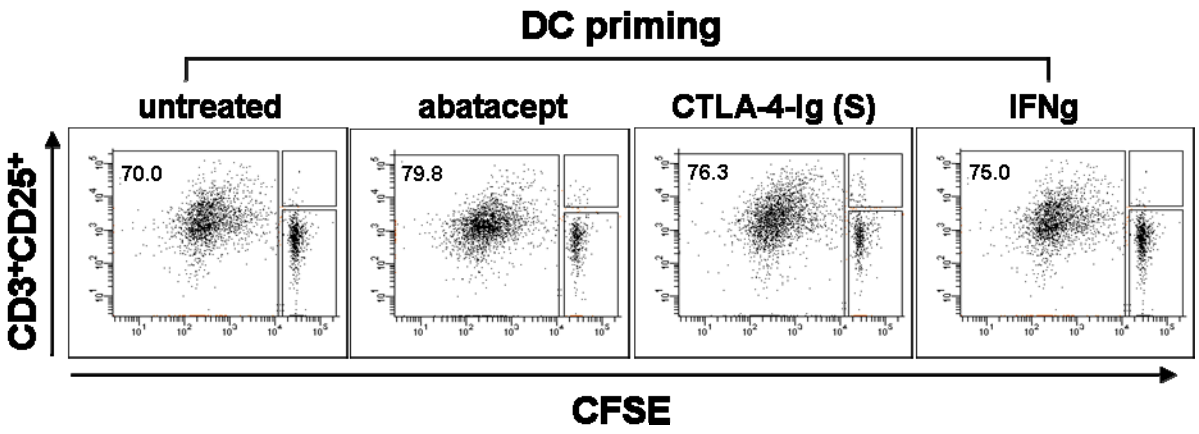


Figure 14 CTLA-4Ig and IFN-γ do not induce a DC regulatory phenotype.

C57BL/6 splenic CD11c$^+$ DCs were stimulated with CTLA-4Ig fusion proteins (abatacept; CTLA-4Ig) or IFN-γ for 24h. Thereafter, cells were thoroughly washed and co-cultured with Balb/c CD3$^+$ T cells. T cell proliferative responses were determined at the end of the culture period (day 6) by CFSE dilution and are depicted as per cent CFSE negative cells. Results of one experiment, representative of 3 experiments, are shown.

5.4 LPS/IFN-γ matured BMDCs fail to acquire IDO competence and to dampen allogeneic T cell responses in co-cultures

Recently, our group demonstrated that human monocyte-derived DCs acquire sustained IDO competence upon activation with a combination of LPS and IFN-γ for 48h and that these IDO competent DCs downregulated allogeneic T cell responses *in vitro* [85]. Furthermore, in an *in vitro* study that investigated murine BMDCs in the context of IDO mediated immunosuppression, IDO expression was found to be upregulated in BMDCs upon exposure to LPS or IFN-γ [161].

Thus, we activated C57BL/6 bone marrow derived immature DCs (iDCs) with LPS in the presence or absence of IFN-γ for 48h and examined the phenotype and their capacity to acquire IDO competence. iDCs displayed very low expression levels of MHC class I /II molecules and of the co-stimulatory molecules CD80/CD86. DC activation with LPS alone or in combination with IFN-γ (LPS/IFN-γ) for 48h resulted in the upregulation of the surface markers MHC class I/II and CD80/CD86 in comparison to iDCs and DCs cultured for 48h without maturation stimuli (unstimulated) (Figure 15A). Next, BMDCs were analyzed for constitutive IDO transcription by RT-PCR. Some basal level of expression of IDO mRNA was detected in iDCs and 48-hour untreated DCs. As reported previously in Jung et.al. 2007 [161], this baseline level of IDO mRNA expression was slightly upregulated upon exposing DCs to LPS or LPS/IFN-γ for 48h (Figure 15B). Similarly, the baseline levels of IDO protein expression detectable by immunoblot analysis were slightly enhanced after exposing the DCs to LPS or LPS/IFN-γ (Figure 15C). However, BMDCs did not acquire tryptophan metabolizing activity upon stimulation with LPS/IFN-γ. The concentrations of kynurenine measured by HPLC in the supernatants of iDCs, untreated and stimulated BMDCs remained < 1 μmol/L contrary to human DCs stimulated by a combination with LPS/IFN-γ (Figure 15D).

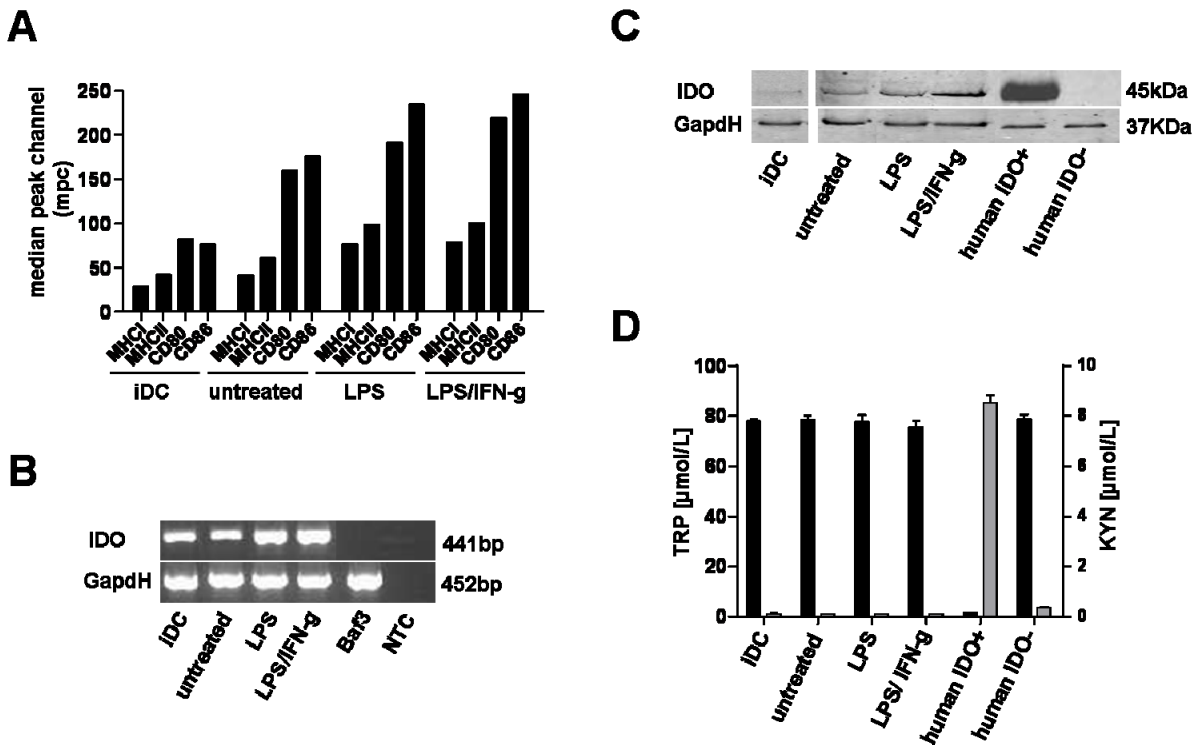


Figure 15 IDO expression and enzymatic activity is not induced in C57BL/6 BMDCs upon exposure to LPS/IFN-γ.

(A) *Phenotype of immature BMDCs (iDCs) and LPS or LPS/IFN-γ matured (48h) BMDCs analyzed by flow cytometry. Expression levels of cell-surface molecules (MHCI, MHCII, CD80/CD86) typically associated with DC maturation are illustrated (black bars; median peak channel). One representative of 3 similar experiments is shown.* ***(B)*** *IDO mRNA expression of the same DCs as in (A) as analyzed by RT-PCR. Total RNA of murine Baf3 cells was used as negative control and GapdH as internal standard; NTC, non template control.* ***(C)*** *IDO protein expression in the same DCs as in (A) analyzed by immunoblotting. Human DCs stimulated by LPS/IFN-γ for 48h (human IDO+) or 4h (human IDO-) as described [85] were used as positive and negative controls. GapdH served as internal standard.* ***(D)*** *Concentrations of tryptophan (TRP; black bars, left scale) and kynurenine (KYN; grey bars, right scale) in cell culture supernatants of the same DCs as in (A) as determined by HPLC. Error bars indicate the range of a total of 6 consecutive experiments.*

To examine, whether LPS/IFN-γ matured BMDCs confer a DC regulatory capacity, DCs were pre-exposed to LPS/IFN-γ before a co-culture with allogeneic T cells. Briefly, C57BL/6 iDCs were stimulated with LPS or by combination with LPS/IFN-γ for 48h or left untreated. At the end of the 48-hour stimulation period DCs were thoroughly washed and co-cultured with Balb/c derived T cells. The capacity to stimulate allogeneic T cell responses was determined

by CFSE dilution at the end of the culture period. C57BL/6 BMDCs were potent stimulators of allogeneic Balb/c T cells, resulting in ~ 65% (64.7 ± 2.2%, n=3) $CFSE^-$ T cells after a 6 day co-culture (Figure 16). However, a pre-exposure of these DCs to LPS/IFN-γ did not reduce the stimulatory capacity of the DCs. In fact, both LPS and LPS/IFN-γ stimulated BMDCs rather boosted allogeneic T cell responses as compared to untreated BMDCs (Figure 16).
Together, 48-hour matured BMDCs did not acquire functional IDO activity and were not able to dampen allogeneic T cell responses *in vitro*.

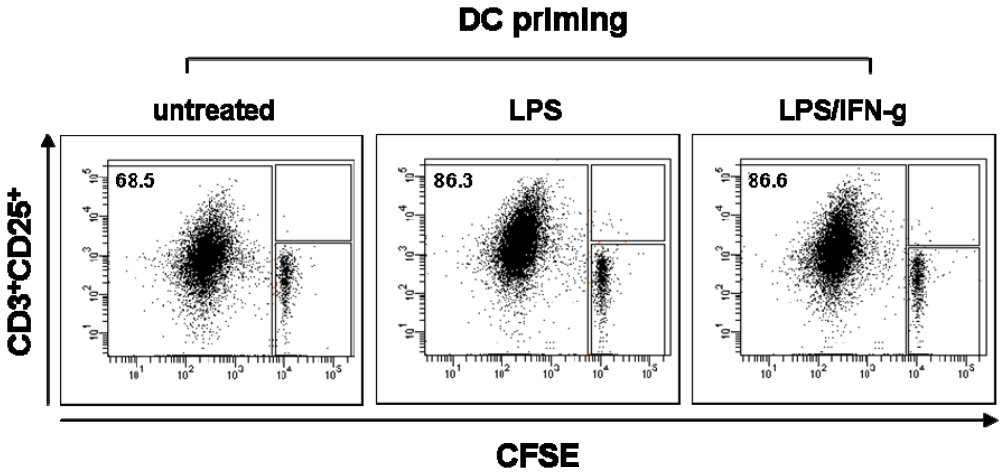


Figure 16 C57BL/6 BMDCs exposed to LPS/IFN-γ for 48h do not dampen allogeneic T cell responses.

C57BL/6 BMDCs were stimulated with LPS in the presence or absence with IFN-γ for 48h. Subsequently, cells were thoroughly washed and co-cultured with Balb/c $CD3^+$ T cells. T cell proliferative responses were determined by CFSE dilution after 6 days and are expressed as per cent CFSE negative cells. Results of one experiment, representative of 3 experiments, are shown.

5.5 CTLA-4Ig directly affects the interaction of stimulator and responder cells in co-cultures

CTLA-4Ig was reported to potently inhibit allogeneic T cell proliferation when added at the time at which DCs interacted with allogeneic T cells, suggesting that CTLA-4Ig directly prevented the activation of the co-stimulatory pathway signals [145]. This observation is in line with the original concept of CTLA-4Ig to block the interaction of CD80/CD86 with their counter-receptor CD28.

When CTLA-4Ig was added at the initiation of the DC/ T cell co-culture (C57BL/6 splenic DCs were used as stimulators of Balb/c $CD3^+$ T cells) and was present during the MLR, we found, as expected, a significant reduction of allogeneic T cell proliferative responses. CTLA-4Ig being present throughout the MLR inhibited T cell proliferation in a dose dependent manner with 100 µg/mL resulting in an inhibition of proliferation of 68% ± 0,5% (mean ± SEM) by day 3 of the MLR [145], in three consecutive experiments ($P<0.001$) (Figure 17). Of note, similar effects of CTLA-4Ig were observed when BMDCs were used as stimulators of allogeneic T cells (see below Figure 21).

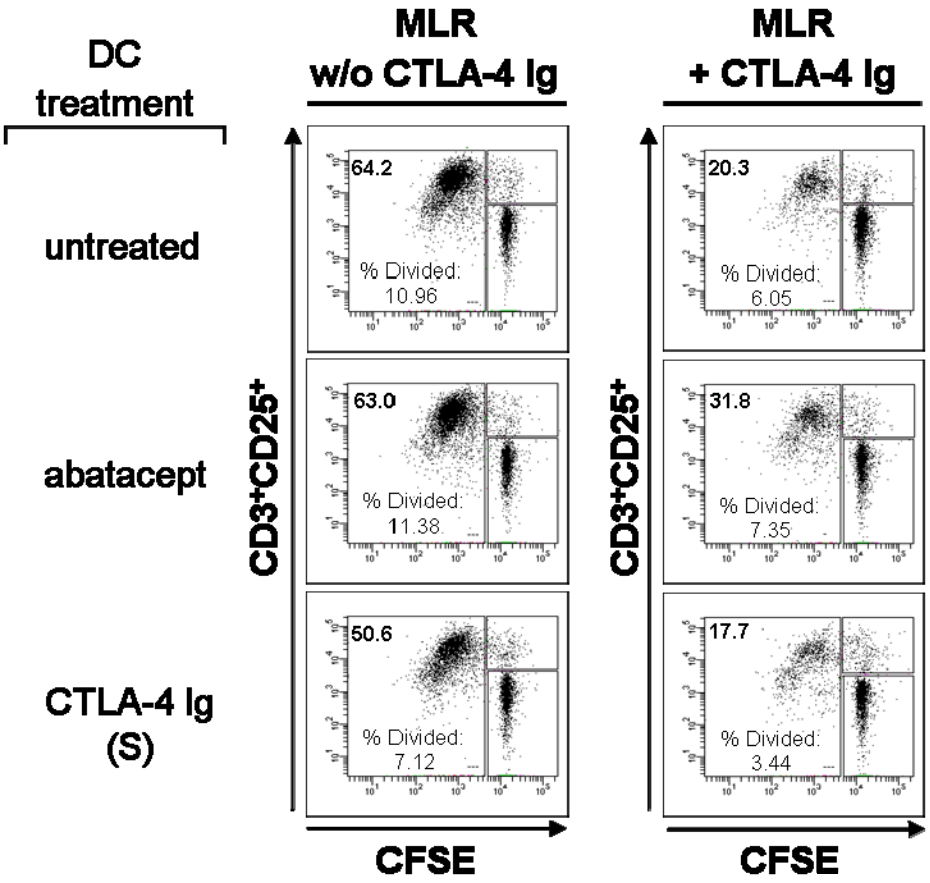


Figure 17 CTLA-4Ig impairs the interaction of stimulator and responder cells in MLRs. *C57BL/6 splenic DCs were stimulated with CTLA-4Ig fusion proteins (abatacept; CTLA-4Ig) or IFN-γ for 24h. At the end of the stimulation period, DCs were thoroughly washed and co-cultured with Balb/c $CD3^+$ T cells. CTLA-4Ig (abatacept; 100µg/mL) was added to the co-cultures at day 0 and thus was present for the total period of the MLR (right panel) or co-cultures were left untreated (left panel). T cell proliferative responses were analyzed by CFSE dilution after 3 days and are depicted as per cent CFSE negative cells or as the per cent divided cells (see Materials and Methods). Results of 1 experiment, representative of 3 experiments, are shown ($P < 0.01$).*

To exclude that CTLA-4Ig would reduce T cell responses independently of binding to co-stimulatory molecules on DCs, we performed DC independent T cell proliferation assays. For this purpose, we stimulated $CD3^+$ T cells using plate bound anti-CD3/ anti-CD28 in the presence or absence of increasing concentrations of CTLA-4Ig. In these experiments, CTLA-4Ig did not reduce T cell proliferation, even in excess concentrations, (Figure 18A) and had no toxic effect on T cells (Figure 18B).

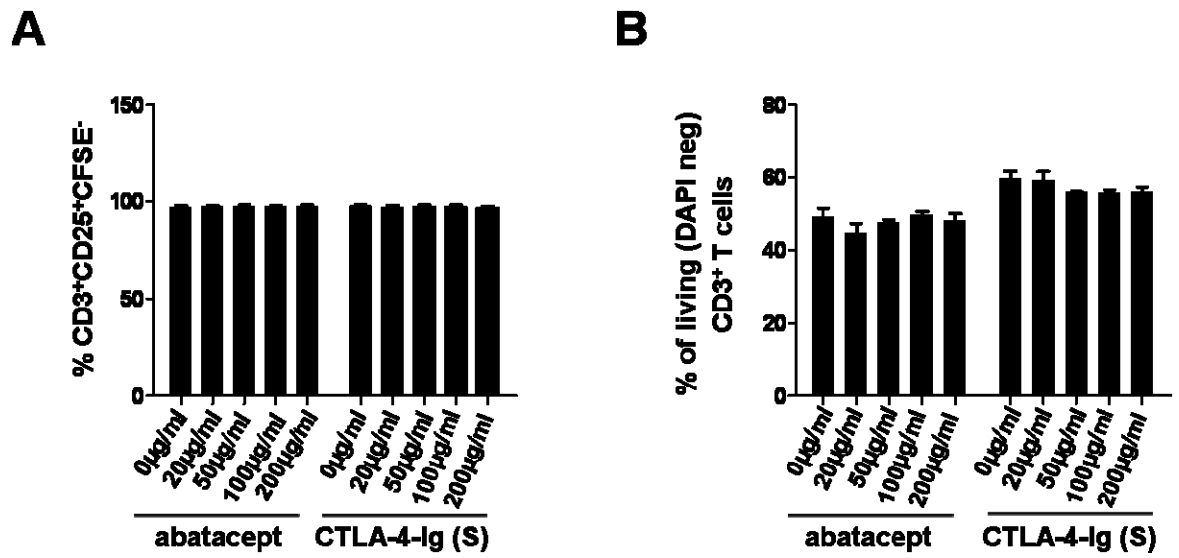


Figure 18 CTLA-4Ig does not affect per se the proliferative capacity of T cells.

(A) *Purified Balb/c $CD3^+$ T cell were stimulated with plate bound anti-CD3/ anti-CD28 for 48h in the presence or absence of increasing concentrations (0-200µg/mL) of CTLA-4Ig (abatacept; CTLA-4Ig (S)). T cell proliferation was assessed by CFSE dilution.* ***(B)*** *$CD3^+$ T cells of (A) were analyzed for per cent of living (DAPI negative) cells. Results of 1 experiment, representative of 2 experiments, are shown.*

Together, our findings strongly suggest that the immunmodulatory agent CTLA-4Ig neither promotes regulatory activity in DCs nor affects *per se* the proliferative capacity of T cells but directly interferes with the interaction of stimulator and responder cells in DC/ T cell co-cultures.

5.6 The immunmodulatory effect of CTLA-4Ig preferentially affects the $CD4^+$ T cell response and preserves a $CD4^+CD25^{high}$ phenotype

Next, in order to examine the effect of CTLA-4Ig on the major T cell subsets in allogeneic responses, MLRs were performed in the presence or absence of CTLA-4Ig (abatacept) as described above. After 3 days of co-culture proliferative responses were examined separately in $CD4^+$ and $CD8^+$ T cell populations by CFSE dilution.

As expected, both $CD4^+$ and $CD8^+$ T cells underwent considerable cell division by ~ 50% when stimulated with allogeneic DCs in the absence of CTLA-4Ig (Figure 19, black bars). In contrast and consistent with previous results [136, 162] we found that particularly $CD4^+$ T cells were inhibited to proliferate when CTLA-4Ig was present throughout the co-cultures (79 ± 0.5%, mean inhibition ± SEM), whereas the proliferation of $CD8^+$ T cells was largely spared form the effect (9 ± 1%, mean inhibition ± SEM) (Figure 19, grey bars).

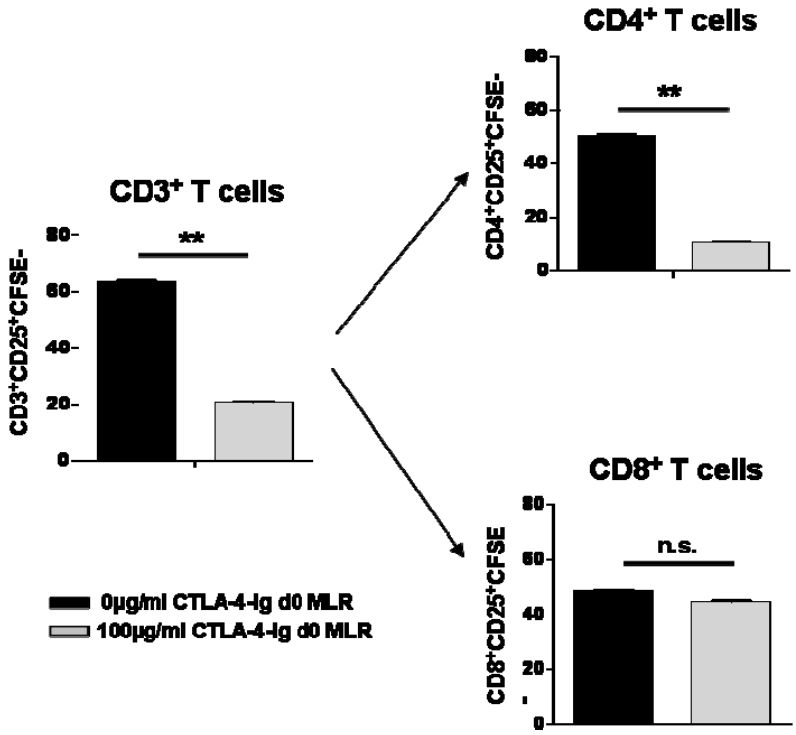


Figure 19 CTLA-4Ig preferentially affects the $CD4^+$ T cell population in an MLR.

*MLRs were performed with untreated splenic DCs and allogeneic Balb/c $CD3^+$ T cells in the presence or absence CTLA-4Ig (abatacept). After 3 days of co-culture the percent viable $CD3^+CD25^+$ T cells (left panel) were separated into $CD4^+CD25^+$ (upper right) and $CD8^+CD25^+$ (lower right) T cell populations by flow cytometry and individually examined for CFSE dilution. **Significant ($P < 0.001$) differences of CTLA-4Ig treated and untreated co-*

cultures; n.s. indicates not significant. Error bars indicate the range of a total of 3 consecutive experiments.

Additionally, we found that at the end of the culture period by day 6 a proportion of $CD4^+$ T cells with a CD25 high phenotype was preserved in those co-cultures performed in the presence of CTLA-4Ig (abatacept; CTLA-4Ig(S)) in contrast to co-cultures in the absence of CTLA-4Ig. This $CD4^+CD25^{high}$ preserved T cell population represented 40% of all living $CD4^+CD25^+$ T cells (Figure 20).

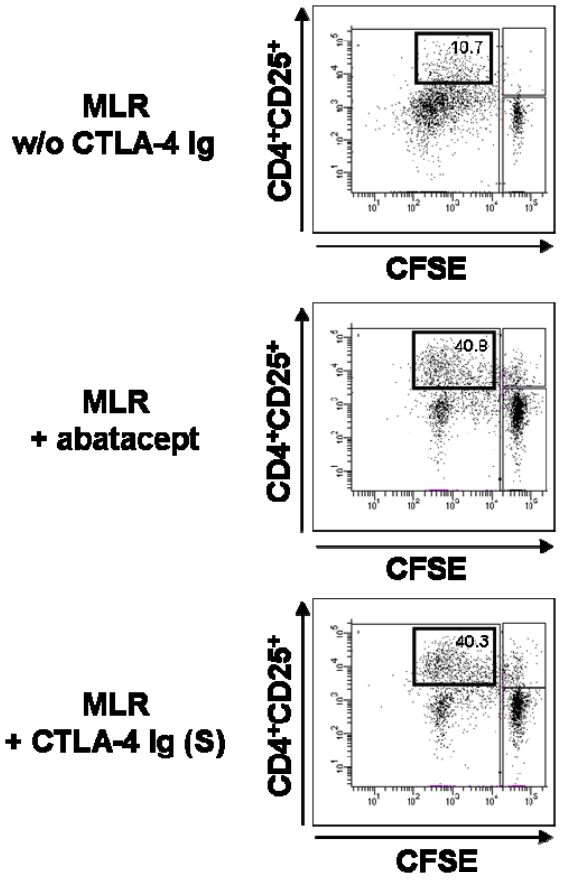


Figure 20 In an MLR, CTLA-4Ig preserves a $CD4^+CD25^{high}$ phenotype.

Proliferative responses of $CD4^+$ T lymphocytes in response to allogeneic splenic DCs, which were left untreated, in the presence or absence of CTLA-4 Ig (abatacept or CTLA-4Ig (S) were analyzed by CFSE dilution. At the end of the culture period (day 6) cells were gated for viable $CD4^+$ T cells. The proportion of $CD4^+CD25^{high}$CFSE-negative T cells of total $CD4^+CD25^+$CFSE-negative T cells is depicted (black boxes). Results are from 1 of 3 representative experiments ($P < 0.05$).

Thus, our findings suggest that CTLA-4Ig, preferentially affecting the $CD4^+$ T cell response when present during co-cultures, preserves a $CD4^+CD25^{high}$ putatively regulatory phenotype.

5.7 The presence of CTLA-4Ig during co-cultures propagates T cells displaying a $CD4^+CD25^+FoxP3^+$ phenotype

T cell expression of the CD25 molecule may indicate cellular activation or regulatory cells [163]. To distinguish whether the $CD4^+CD25^+$ T cell population induced by CTLA-4Ig represents regulatory cells, we next examined the T cells having undergone allogeneic stimulation in the presence of CTLA-4Ig for expression of the key molecules associated with murine regulatory T cells, FoxP3 and CD62L [164, 165].

In this set of experiments, BMDCs were generated from C57BL/6 mice and were used as stimulators of allogeneic Balb/c $CD3^+$ T cells in the presence or absence of CTLA-4Ig (abatacept). The allogeneic T cell response, determined by CFSE dilution, was inhibited by 50% in co-cultures where CTLA-4Ig was present throughout the MLR as compared to co-cultures that were left untreated (Figure 21A).

For characterization of a regulatory phenotype, T cells of the differently treated co-cultures were recovered and analyzed by flow cytometry. As a positive control we used $CD4^+CD25^+$ naturally occurring regulatory T cells (nTregs) isolated from lymph nodes of Balb/c mice which usually represent 5-10% of total $CD4^+$ T cells and have been reported to constitutively express FoxP3. As depicted in Figure 21B (upper row) nTregs showed high expression levels of the surface markers CD25 and CD62L and as well highly expressed intracellular FoxP3. Similar to these nTregs, the $CD4^+CD25^+$ T cell population having been exposed to CTLA-4Ig during the MLR highly expressed intracellular FoxP3 and the surface marker CD62L, suggesting a regulatory phenotype. In contrast, in $CD4^+CD25^+$ T cells recovered from the MLR in which CTLA-4Ig was absent during the co-culture, the proportion of cells expressing CD62L and intracellular Foxp3 was low (Figure 21B).

Hence, CTLA-4Ig, in addition to quantitatively down-regulating the allogeneic proliferative $CD4^+$ T cell response when present in a MLR, supported the development of regulatory T cells.

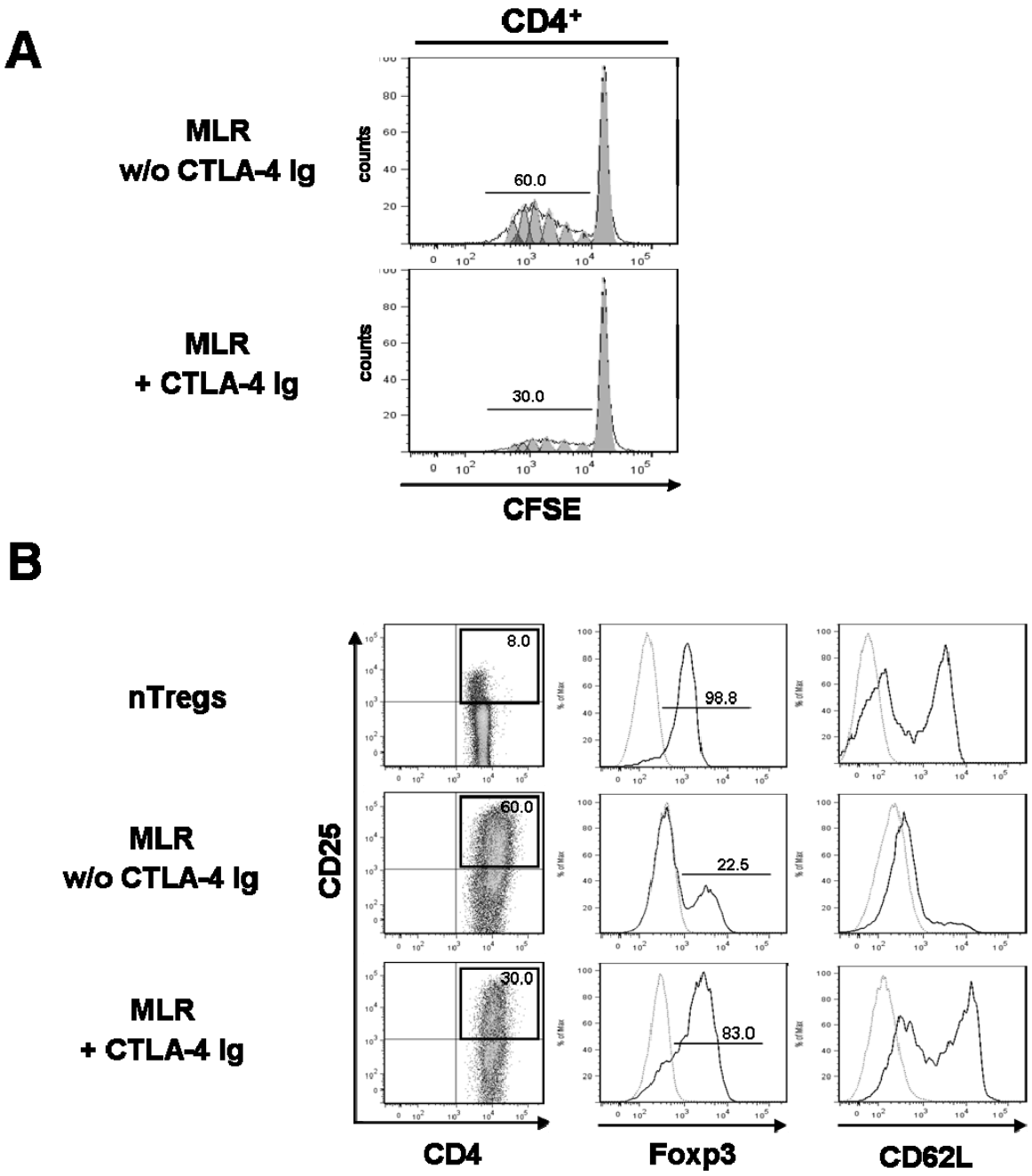


Figure 21 ***$CD4^+CD25^+$ T cells recovered from an MLR performed in the presence of CTLA-4Ig harbor a regulatory phenotype.***

(A) *C57BL/6 BMDCs were used as stimulators of Balb/c derived $CD3^+$ T cells in the absence or presence of CTLA-4Ig (abatacept). Proliferative responses of $CD4^+$ T cells were analyzed by CFSE dilution at the end of the co-culture period.* ***(B)*** *MLRs performed as in (A) but without staining T cells with CFSE were harvested and the $CD4^+CD25^+$ T cell population from co-cultures in the absence or presence of CTLA-4Ig was examined for the expression of the key cellular markers indicating a regulatory phenotype, intracellular FoxP3 and cell surface CD62L. Dotted grey lines indicate isotype controls. Balb/c $CD4^+CD25^+$ cells isolated*

from lymph nodes and representing naturally occurring regulatory T cells (nTregs) were used as positive control. Results of one experiment, representative of 2 experiments are shown.

5.8 The effect of CTLA-4Ig on allogeneic $CD4^+$ T cells is sustained upon restimulation

Co-stimulation blockade by CTLA-4Ig has been associated with the induction of sustained T cell unresponsiveness [166].

To test whether the effect of CTLA-4Ig to dampen allogeneic $CD4^+$ T cell responses was sustained or transient, a second round MLR was performed. Briefly, $CD3^+$ T cells of the first MLR, performed in the presence or absence of CTLA-4Ig, were harvested, stained with CFSE and restimulated in a subsequent MLR (2^{nd} round MLR) with the same stimulator cells but in the absence of CTLA-4Ig. Both, $CD4^+$ and $CD8^+$ cell populations having undergone stimulation by allogeneic DCs in the absence of CTLA-4Ig showed a rapid proliferative response upon restimulation (Figure 22A, upper panel). However, when the first MLR was performed in the presence of CTLA-4Ig, $CD4^+$ T cells were still inhibited to mount a full proliferative response in 2^{nd} round MLR. Particularly, the per cent divided cells as calculated by the FlowJo program (see Materials and Methods chapter 4.3.5) was reduced by 50% (per cent divided cells 44.8% in cells derived from MLR in the absence of CTLA-4Ig versus 22.5% in cells being derived from MLRs performed in the presence of CTLA-4Ig). Similar to the findings in the first MLR, the $CD8^+$ T cell population was less affected by CTLA-4Ig, the per cent divided cells in the 2^{nd} round MLR being 55.5% and 42.6% whether the first MLR was performed in the absence or the presence of CTLA-4Ig, respectively (Figure 23A, lower panel). Remarkably, in addition to the quantitative reduction of proliferation, the expression levels of CD25 and FoxP3 were retained in $CD4^+$ T cells upon restimulation when the first MLR was performed in the presence of CTLA-4Ig. In contrast, $CD4^+$ T cells having undergone allogeneic stimulation in the absence of CTLA-4Ig revealed a CD25 low FoxP3 low phenotype (Figure 22B).

Together, we here demonstrate that the inhibitory effect of CTLA-4Ig on allogeneic stimulation is sustained in the $CD4^+$ T cell population and that a T cell regulatory phenotype is preserved upon restimulation.

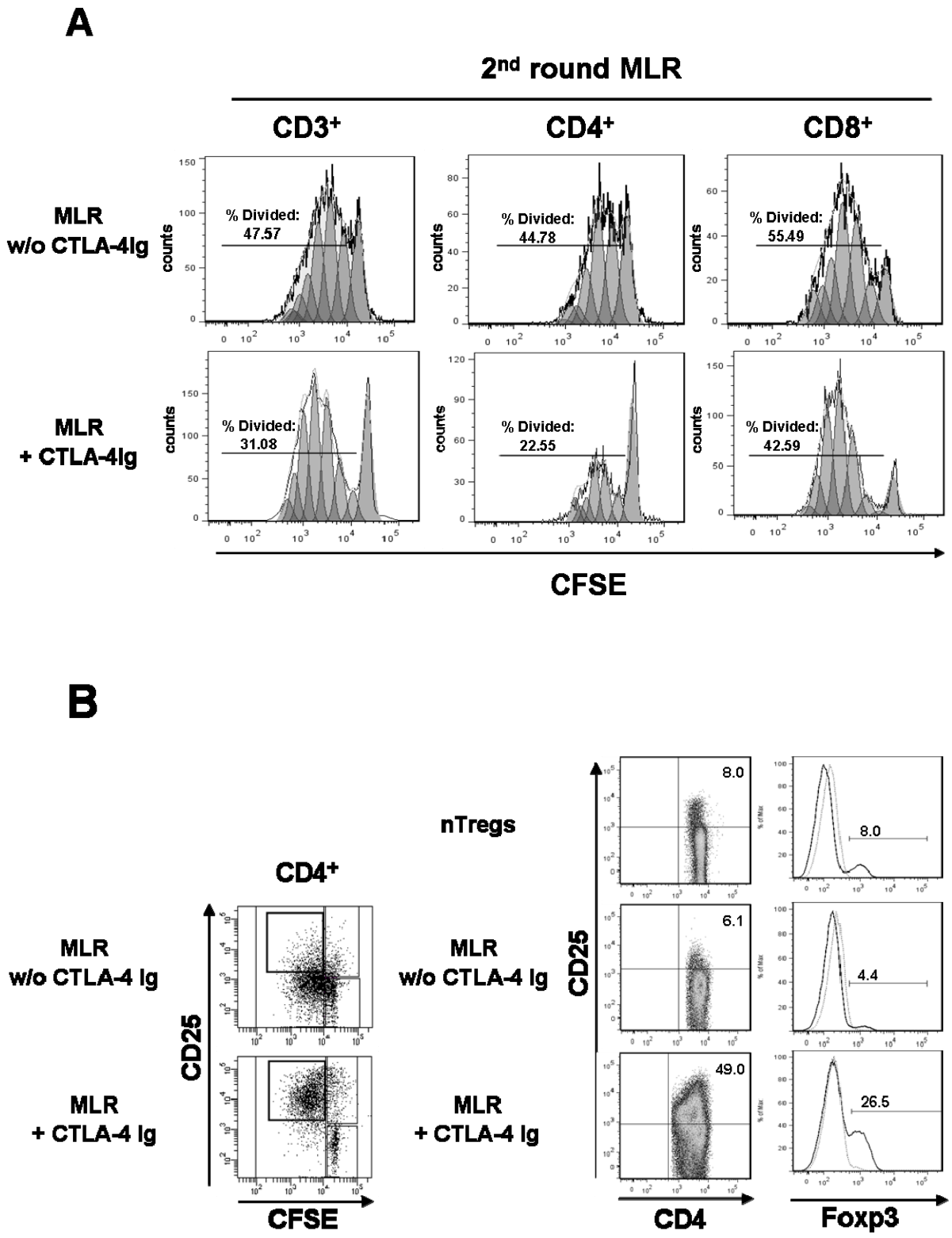


Figure 22 ***The effect of CTLA-4Ig on CD4$^+$ allo responses is preserved in a second round MLR.***

(A) *T cells were recovered from first MLRs which were performed with BMDCs as stimulators of Balb/c derived CD3$^+$ T cells in the presence or absence of CTLA-4Ig (abatacept). Thereafter, recovered cells were washed, stained with CFSE and were*

restimulated with the same allo-antigens (freshly generated C57BL/6 BMDCs) but in the absence of CTLA-4Ig (2^{nd} round MLR). Proliferative responses to 2^{nd} stimulation were analyzed by CFSE dilution, including the determination of the per cent divided cells (see inserted numbers) using FlowJo proliferation platform. ***(B)*** *T cells of the 2^{nd} round MLR from (A) were recovered and $CD4^+$ T cells were examined for intracellular expression of FoxP3. Dotted grey lines indicate isotype controls. Balb/c $CD4^+CD25^+$ nTregs isolated from lymph nodes were used as a positive control. Results of one experiment, representative of 2 experiments are shown.*

5.9 Establishment of an in vivo HSCT/ GvHD model

5.9.1 Preliminary data and future perspectives

As mentioned before, in HSCT, T cells play a central role in immune reconstitution but have the potential to induce GvHD [54]. Thus, a very important task of research in the field of HSCT is to support the allo-HSCT recipient with T cells which are tolerant against recipient allo-antigens to protect the recipient from GvHD while maintaining T cell immunity against pathogens. One possible approach is to generate such T cells *ex vivo* and use these T cells for adoptive transfer strategies.

In our murine model of HSCT, we hypothesize that those donor T cells of allo MLRs, which were exposed to CTLA-4Ig, will protect recipient mice from developing GvHD when adoptively transferred in the recipient strain. In contrast to this, T cells of the allo MLRs performed in the absence of CTLA-4Ig are supposed to induce GvHD in the recipient strain after adoptive transfer.

Therefore, we first established an *in vivo* HSCT/ GvHD mouse model to study whether the hypothetically tolerized T cells are able to protect recipients from GvHD. Briefly, for murine allogeneic HSCT, host conditioning for successful BMC engraftment was mediated by myeloablative irradiation (10 Gy) of recipient C57BL/6 mice one day prior to BMT. Subsequently within 24h, mice were transplanted with a conventional dose of approximately 10 x 10^6 Balb/c donor derived BMCs.

This procedure allowed for rapid and uncomplicated engraftment. Lineage-specific chimerism of transplanted animals was determined by flow cytometry of peripheral white blood cells which were stained with the appropriate donor strain-specific anti-HLA antibody and specific cell lineage markers. Three weeks after transplantation donor BMCs successfully engrafted in 6 out of 6 recipient C57BL/6 mice as depicted in Figure 23.

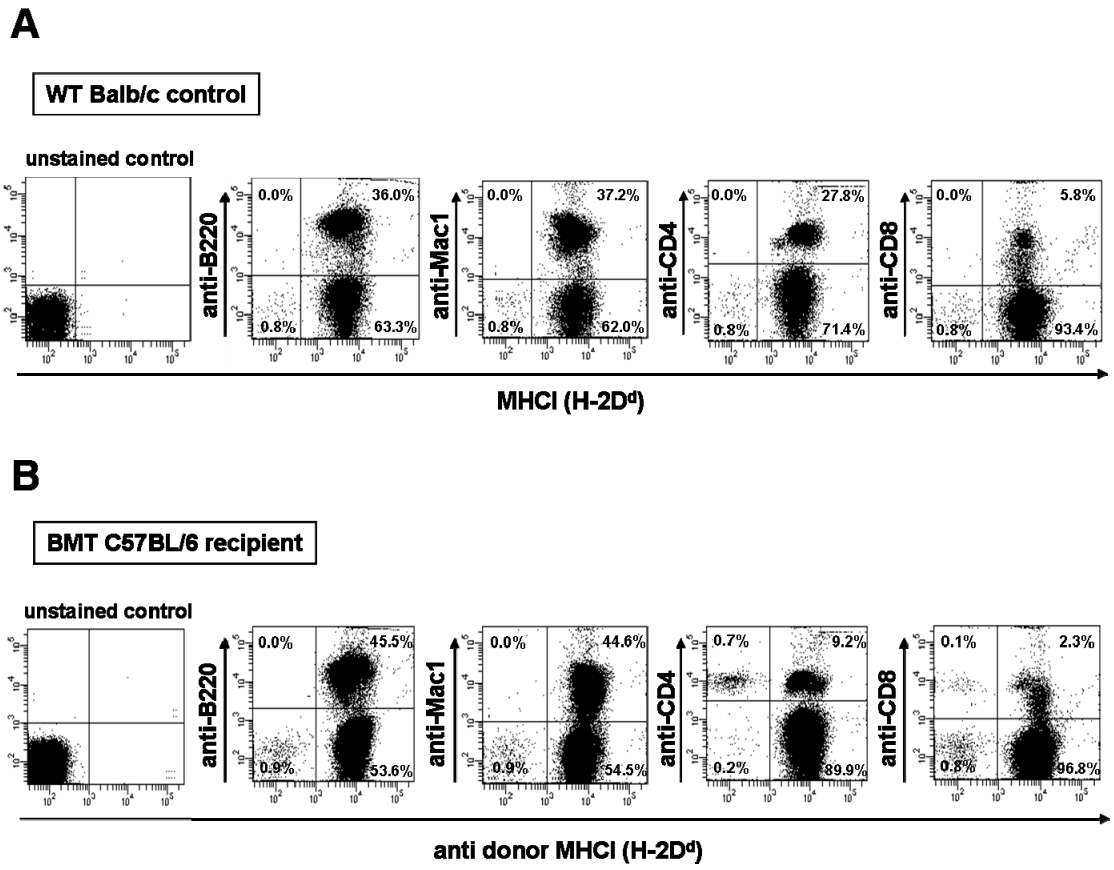


Figure 23 Lineage specific chimerism in a representative C57BL/6 bone marrow transplantation (BMT) recipient.

Flow cytometric analyses of peripheral white blood cells of ***(A)*** *Balb/c mice and* ***(B)*** *BMT recipient C57BL/6 mice stained with the appropriate donor strain-specific anti HLA antibody (H-2D^d) and specific cell lineage markers against CD4$^+$ and CD8$^+$ T cells, B cells (ant-B220) and myeloid cells (anti-MAC1) three weeks after BMT. One representative example out of 6 Balb/c and C57BL/6 BMT recipients is depicted.*

To define the appropriate donor T cell amount to successfully induce GvHD in C57BL/6 recipient mice, we followed protocols published by Cohen et.al. 2002 [46]. In brief, CD3$^+$ T cells were isolated of total splenocytes derived from donor Balb/c mice as described above. Thereafter, these donor T cells (10 x 10^6 or 5 x 10^6) were adoptively transferred together with 10 x 10^6 donor derived BMCs to C57BL/6 HSCT recipients (2 groups of 6 mice) within 24h of myeloablation via the tail vein. Additionally, to confirm successful engraftment one group of C57BL/6 recipients received donor derived BMCs (10 x 10^6) without donor T cells. The survival and appearance of recipient mice were monitored daily and assessed according to a

GvHD scoring procedure previously described by Cooke et. al.1996 [153] and body weight was determined weekly.

C57BL/6 recipient mice transplanted with BMCs and 5 x 10^6 T cells derived from Balb/c donors developed classical signs characteristic of clinical GvHD from ~ day 14 post transplantation (p.t.) onwards and died within 30 days p.t. (Figure 24; red line). These signs included (i) weight loss (15-25%) (Figure 25, red bars), (ii) kyphotic posture leading to impaired movement and reduced activity, (iii) moderate to severe ruffled, poor groomed and scaly fur texture and (iv) declined skin integrity (scaling of paws, ears and around eyes) indicating a grade 2 GvHD (Figure 25). GvHD was further confirmed by histopathology (not shown). C57BL/6 mice transplanted with Balb/c derived BMCs and an overdose of 10 x 10^6 T cells died very rapidly within 2 weeks p.t. without developing the above mentioned typical signs for GvHD except for weight loss (Figure 24, green line; Figure 25 green bars). C57BL/6 mice of the control group transplanted with donor derived BMCs survived more than 35 days and developed no signs related to GvHD (Figure 24, blue line; Figure 25, blue bars).

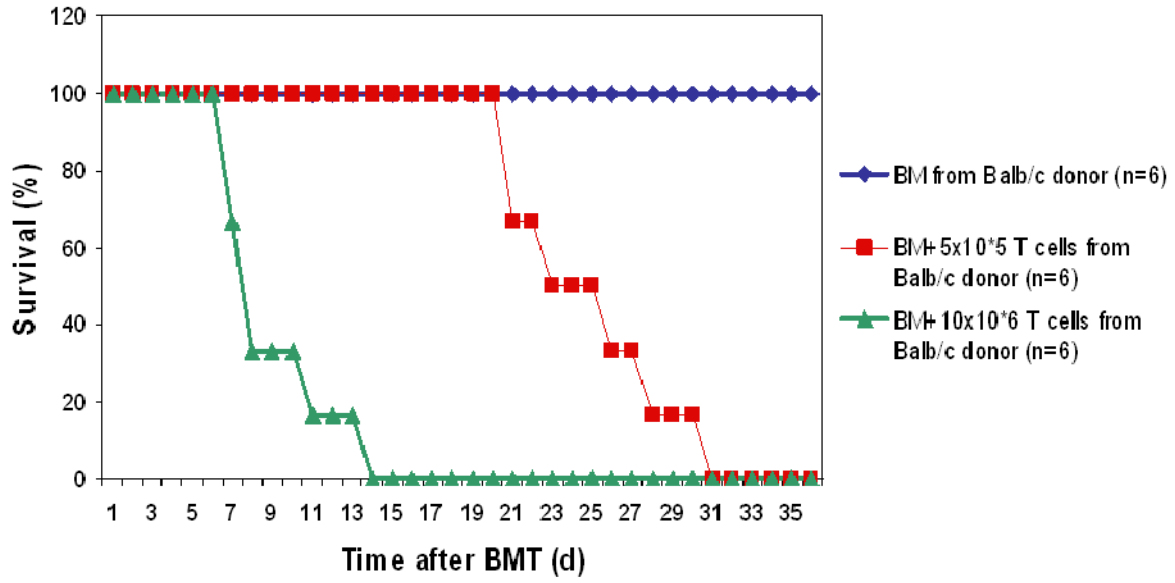


Figure 24 Survival curves after HSCT and allogeneic T cell transfer.

C57BL/6 recipient mice received a myeloablative irradiation dose of 10 Gy one day prior to bone marrow transplantation (BMT). Within 24 hours after lethal irradiation, 10 x 10^6 bone marrow cells (BMCs) (group 1; blue line), 10 x 10^6 BMCs + 5 x 10^6 $CD3^+$ T cells (group 2; red line) or 10 x 10^6 BMCs + 10 x 10^6 $CD3^+$ T cells (group 3; green line) were injected into recipients (n=6 per group) via the tail vein. The survival of recipients was monitored daily. Survival curves were analyzed using the Kaplan-Meier algorithm. On representative out of 2 consecutive experiments is shown.

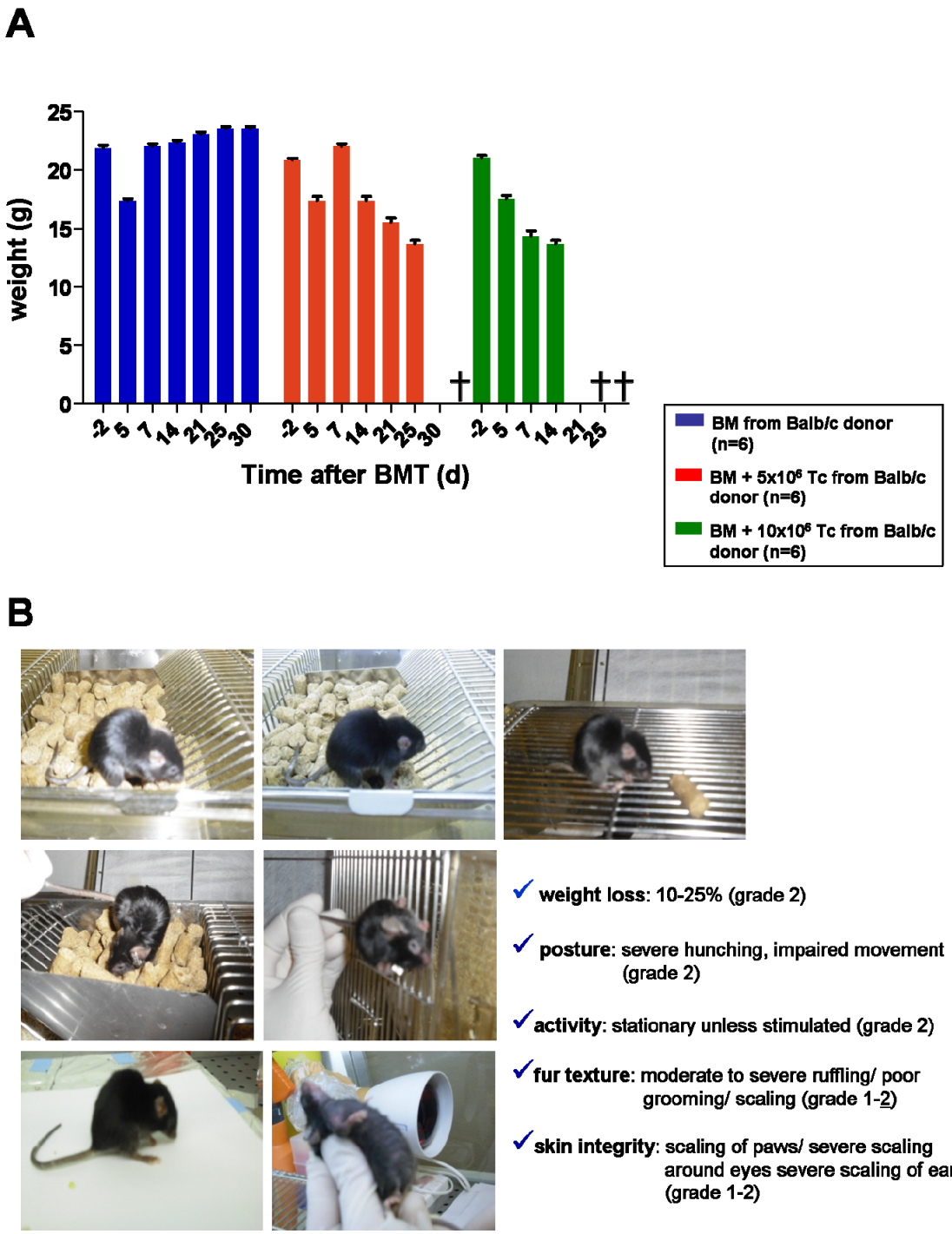


Figure 25 Assessment of GvHD in C57BL/6 recipient mice.

(A) *C57BL/6 recipient mice were lethally irradiated (10 Gy), separated into 3 groups (n=6) and received 10 x 10^6 Balb/c donor derived bone marrow cells (BMCs) (group 1) in combination with 5 x 10^6 (group 2) or 10 x 10^6 (group 3) $CD3^+$ T cells from Balb/c donors by tail vein injection. The body weight of recipients was determined weekly.* ***(B)*** *Appearance of recipient mice from (A) was monitored daily and evaluated according to a GvHD index as describe in Cooke et. al.1996 [153]. (n=2).*

To summarize, we successfully established an *in vivo* HSCT/ GvHD mouse model. First, we defined the minimal myeloablative irradiation dose and the rescuing cell dose of donor BMCs. Second, we defined the optimal donor T cell amount to induce GvHD in C57BL/6 recipients followed by a successful evaluation of grade 2 GvHD in transplanted animals.
Further experiments to directly test the tolerogenic capacity of the hypothetically tolerized T cells of allo MLRs performed in the presence of CTLA-4Ig are in progress.

6 Summary and Discussion

In the mammalian immune system T cells are inclined to recognize and initiate immune responses towards allo-antigens. Therefore, a key task in transplantation research, including both solid organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT), is to develop approaches to achieve a state of allo-specific tolerance. Allo-specific tolerance implies that T cells do not mount pathogenic immune reaction towards the allogeneic organ but preserve their protective activity towards environmental pathogens. Thus, the generation of allo-specific tolerized T cells may be critical to improve transplantation outcomes as to overcome the detrimental effects of pathogenic immune reactions in host-versus-graft and graft-versus-host direction prevailing in SOD and HSCT, respectively.

In numerous previous studies co-stimulation blockade has been shown to represent an effective means of generation of anergic T cells.

The major contribution of the present study to advance the understanding of the cellular mechanisms of CTLA-4Ig mediated inhibition of allogeneic T cell responses is by showing that CTLA-4Ig (i) does not require IDO activity, nor leads to the induction of a DC regulatory phenotype, but (ii) acts through directly impairing DC/T cell interaction and (iii) affects $CD4^+$ T cell responses not only quantitatively but also qualitatively by fostering the emergence of regulatory T cells.

CTLA-4Ig has been originally developed based on the concept that specific binding to CD80 and CD86 molecules expressed on APCs interrupts CD28 mediated stimulatory signaling in T cells [135, 167]. CD28 mediated signaling stimulates key pathways indispensible for an efficacious T cell response, such as cell proliferation, increased cell survival and a marked increase of cytokine secretion directing T cell differentiation towards a Th1 phenotype. In contrast, a blockade of this pathway has been linked to the emergence of anergy, with low IL-2 production and enhanced T cell death involving both passive cell death and activation induced cell death as underlying mechanisms [168]. These effects of CTLA-4Ig resulting from blocking the CD28:CD80/86 pathway have been solidly evidenced in numerous *in vitro* and *in vivo* studies [96, 166, 167, 169].

In 2002, Grohmann et al. [75] proposed a novel concept for the mechanism of CTLA-4Ig mediating tolerance. In their model, CTLA-4Ig binding to CD80/CD86 molecules would not only inhibit CD80/86 interaction with CD28 but provide a ‘reverse’ signal to DCs resulting in the production of IFN-γ and the induction of IDO [170]. IDO is the major and rate limiting enzyme of tryptophan metabolism in the mammalian organism apart from the liver [60]. It

metabolizes tryptophan along the kynurenine pathway. The resulting tryptophan depletion and kynurenine accumulation in the intercellular microenvironment interfere with the T cell proliferative capacity and promote apoptotic decline. Thus, IDO activity in DCs, has been shown to possess powerful immune regulatory and tolerance inducing capacity [171]. The concept of Grohmann et al. led to the assumption that binding of CTLA-4Ig to CD80/86 of the DC inducing tryptophan catabolism is critical to mediate the tolerogenic capacity of CTLA-4Ig [130]. The induction of IDO activity by CTLA-4Ig was therefore proposed to induce a regulatory DC phenotype. The model proposed by Grohmann et al. was confirmed by another study, where specific subsets of splenic DCs were identified to mediate potent IDO-dependent T cell suppression *in vitro* following CTLA-4Ig exposure *in vivo* [74]. Yet, in their experimental system CTLA-4Ig-mediated IDO upregulation or IDO-dependent T cell suppression did not require IFN-γ signals as demonstrated by the use of IFN-γ-receptor deficient mice. The reason for the different role of IFN-γ in IDO upregulation remained unsolved. The authors assumed that differences in experimental approaches, including mouse stain-specific factors could be a possible explanation for such disparities [74]. However, the role of CTLA-4Ig in inducing IDO activity in allogeneic stimulation has since been controversially discussed. Pree et al. showed that CTLA-4Ig, while being essential for the development of mixed chimerism in a murine model of non-myeloablative BMT, did not involve tryptophan metabolism and its effect was not reversed by the IDO inhibitor 1-methyl tryptophan (1-MT) [156]. In another study, CTLA-4Ig expressed in a murine DC cell line promoted tolerogenesis without inducing IDO expression [172]. Likewise, Davis and coworkers showed that abatacept did not induce IDO mRNA and kynurenine production in human monocyte-derived DCs [157].

IFN-γ is considered as one of the most prominent IDO inducers, which increases the transcriptional activity of the IDO promoter region *via* effects on signal transducer and activator of transcription (STAT1) and interferon regulatory factor 1 (IRF-1) [173, 174]. IDO induction mediated by IFN-γ signaling was reported to be involved in human [65, 66] and murine DCs [82, 154, 155]. The control of IDO transcription is complex and IDO activity is as well tightly regulated at the posttranslational level and thus, IDO protein can be expressed without functional activity as reviewed in Mellor et al. 2004 [171]. IFN-γ was found capable to induce strong IDO expression in murine splenic $CD11c^+$ DCs, yet, IDO metabolic activity seemed to be preferentially limited to the $CD8\alpha^+$ subset [81, 82, 175]. Anyhow, it was reported that IFN-γ treatment of both $CD8\alpha^+$ and $CD8\alpha^-$ splenic DC subsets resulted in comparable upregulation of IDO mRNA and protein expression [154]. Another study showed

that it was also possible to induce IDO enzymatic activity and a DC regulatory phenotype in $CD11c^+$ splenic DCs without separation into $CD8a^+$ and $CD8a^-$ subsets [75].

The present study comprehensively addressed the issue of IDO induction through CTLA-4Ig or IFN-γ, including the use of different mouse strains, different CTLA-4Ig fusion protein preparations, and broad assessment of IDO expression on mRNA and on protein level and enzymatic activity, ultimately seeking for CTLA-4Ig or IFN-γ mediated tolerance induction via IDO. However, the composite findings unequivocally showed that CTLA-4Ig immunoregulatory activity occurred in an IDO independent fashion. In effect, while we confirmed previous findings of CTLA-4Ig inducing C57BL/6 derived DCs to produce IFN-γ [76] CTLA-4Ig did not induce the IDO pathway in splenic murine DCs. DCs which were pre-exposed to CTLA-4Ig before being used to stimulate allogeneic T cells retained their ability to subsequently fully stimulate T cell responses. This observation might be explained by the rapid dissociation rate of CTLA-4Ig from CD80 and CD86 [134]. Yet, it clearly argues against the view that CTLA-4Ig induces a DC regulatory phenotype. Similarly, IDO expression and enzymatic activity was not induced in splenic DCs upon stimulation with IFN-γ. In addition, a pre-exposure of splenic DCs to IFN-γ did not confer a DC regulatory phenotype *in vitro*.

Recently a human study demonstrated that monocyte-derived DCs acquire sustained IDO competence upon activation with a combination of LPS and IFN-γ for 48h and that these IDO competent DCs downregulated allogeneic T cell responses *in vitro* [85]. However, in the murine system, only little information is available about IDO activity in BMDCs. In 2007, Jung et al. suggested that IDO expression in murine C57BL/6 BMDCs could be either induced upon stimulation with LPS or IFN-γ. Their findings indicated that LPS-induced IDO expression was controlled by an IFN-γ independent pathway and was mediated by PI3K and JNK. In contrast, induction of IDO expression by IFN-γ stimulation was reported to be dependent on JAK signaling [161]. At this point, one has to keep in mind that IDO protein expression in DCs does not necessarily mean that IDO is enzymatic active as reviewed in Mellor et al. 2003 [176]. In line with the findings of Jung et al. we found increased IDO mRNA and protein expression levels when C57BL/6 BMDCs were stimulated with LPS or in combination with LPS/IFN-γ. However, in contrast to the human system where IDO activity was routinely measured in terms of tryptophan degradation and kynurenine accumulation in the supernatant of 48h-LPS/IFN-γ matured DCs, murine BMDCs did not acquire functional IDO activity and were not able to dampen allogeneic T cell responses *in vitro*.

One possibility for the lack of IDO activity in murine DCs upon exposure to either CTLA-4Ig or IFN-γ or LPS in combination with IFN-γ that came into our mind was the inhibition of IDO activity by the IDO antagonist iNOS. It was reported that NO produced by iNOS inhibits IDO activity [72]. Interactions between tryptophan depletion and NO production were first described in studies by Thomas et al. demonstrating that IDO activity in IFN-γ primed human mononuclear phagocytes was inhibited by chemical NO donors [177]. Another study revealed that NO negatively modulated transcription of the IDO gene in mouse macrophages [178]. Further, IDO protein expression and enzymatic activity in extracts of IFN-γ stimulated rat cells were found to be inhibited by endogenous and exogenous NO, while transcription of *Indo*, the gene coding for IDO, was not affected [80]. Further studies accomplished by the same group revealed that IDO is degraded by NO in the proteasome [80]. In the present study, we did not find enhanced NO levels measured in the supernatants of the differently stimulated DCs. Therefore our results indicate that iNOS activity was not involved in the incapability of the stimulated DCs to acquire functional IDO activity.

The definitive explanation of the discrepant findings in the diverse studies addressing an association of CTLA-4Ig or IFN-γ and IDO induction remains unsolved at this point. As previously debated, seemingly trivial differences in experimental procedures [85, 179, 180] or CTLA-4Ig preparations (e.g., in the original study by Grohmann et al. CTLA-4Ig was fused to an IgG3 subclass [75]) may underlie the discrepancies. Irrespectively, the present findings strongly indicate that the immunomodulatory effect of CTLA-4Ig is not strictly dependent of the IDO pathway.

In fact, CTLA-4Ig was, as expected from previous reports [145], able to potently inhibit allogeneic T cell proliferation when added at the time at which DCs interacted with allogeneic T cells. This suggests that CTLA-4Ig directly prevented the activation of the co-stimulatory pathway signals, which is in line with the original concept of CTLA-4Ig via blocking the interaction of CD80/86 with their counterpart CD28.

As well consistent with previous reports, we found the $CD4^+$ T cell population to be more affected as compared to the $CD8^+$ T cell population [162, 181]. In addition to inhibiting $CD4^+$ cell proliferation quantitatively, CTLA-4Ig present during DC/T cell co-cultures also promoted the $CD4^+CD25^+$ T cell population to display a regulatory $FoxP3^+CD62L^+$ phenotype. FoxP3 expression in mice is viewed as the master transcription factor controlling development and function of Tregs [164, 182, 183]. In the murine system, $CD4^+CD25^-$ T cells when stimulated *in vitro* do not express FoxP3 [164, 182] contrary to the observations

made in the human system [85, 184, 185]. Thus, the stable FoxP3 expression of the $CD4^+$ T cell population as propagated by CTLA-4Ig which is retained upon a second stimulation indicates the emergence of *bona fide* regulatory T cells.

The role of Tregs cells in the context of CTLA-4Ig mediated co-stimulation blockade is puzzling. In principle, the homeostasis of Tregs has been proposed to be dependent on CD28 signaling. Therefore, CTLA-4Ig as it blocks CD28 mediated signal transduction has been suspected to negatively interfere with Treg survival and to even lead to exacerbation of autoimmune diseases [186]. Furthermore, CTLA-4Ig by its binding to CD80/86 may also affect the interaction of CD80/86 with CTLA-4, thus preventing the transmission of the counterstimulatory signals to T cells [135]. Contrary to this hypothetical view, some reports suggested that CTLA-4Ig leads to an augmentation of Tregs [187], possibly by a conversion of $CD4^+CD25^-$ cells to $CD4^+CD25^+$ T regulatory cells [188]. Others reported CTLA-4Ig treated DCs to acquire the ability to prevent collagen-induced arthritis and this efficacy of CTLA-4Ig was associated with an increase of $CD4^+CD25^+FoxP3^+$ cells. This DCs' ability occurred without involving IDO, and suggested a role for CTLA-4Ig rendering DCs able to enhance the emergence of Treg cells [189]. Co-stimulation blockade, in contrast, failed to enlarge the $CD4^+CD25^+FOXP3^+$ Treg population in a human study of patients having received kidney transplantation, [190], pointing at the previously recognized profound difference of CTLA-4Ig in human and rodent models [133, 191]. In the long term, however, CTLA-4Ig did not negatively interfere with Treg survival [192]. Finally, a specific role for $CD4^+$ Treg cells for tolerizing the $CD8^+$ T cell population was proposed [167].

While published reports preclude a definitive conclusion, the finding of this study, i.e. CTLA-4Ig propagating Tregs, warrant further testing. The generation of Treg populations through CTLA-4Ig *ex-vivo* might be beneficial for adoptive transfer strategies after transplantation to ameliorate pathogenic alloreactivity in the host-versus-graft as well as in the graft-versus-host direction. Such an approach is currently extensively studied in our laboratory.

In summary, the findings of this study support the view that of co-stimulation blockade by CTLA-4Ig effects potent immunoregulatory activity in an IDO independent fashion. By direct interference with the interaction of APCs and T cells, CTLA-4 affects predominantly the $CD4^+$ T cell population through two cellular mechanisms, inhibition of proliferation and allowing the emergence of a $CD4^+$ T cell population harbouring a regulatory phenotype.

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CTLA-4Ig propagating $CD4^+CD25^+FoxP3^+$ regulatory T cells in an indoleamine 2,3-dioxygenase independent fashion

Edda Veith (1), Nina Pilat (2), Dietmar Fuchs (3), Thomas Wekerle (2), Andreas Heitger (1)

(1) Children's Cancer Research Institute, Division Transplantation Immunology, Vienna, Austria
(2) Division of Transplantation, Department of Surgery, Medical University Vienna, Austria
(3) Division of Biological Chemistry, Biocenter, Innsbruck Medical University, Austria

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Corresponding author
Andreas Heitger, M.D.
Children's Cancer Research Institute
Zimmermannplatz 10
A – 1090 Vienna, Austria
Phone: +43 1 40 470 4070
FAX: +43 1 470 64070
E-mail: andreas.heitger@ccri.at

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Abstract

Immunosuppressive CTLA-4Ig fusion proteins block the CD28:CD80/86 costimulatory pathway but have also been reported to affect DC function via the induction of the immunosuppressive indoleamine 2,3 dioxygenase (IDO) pathway, thus promoting a DC regulatory phenotype. We here probed immunoregulation by CTLA-4Ig isotypes, including abatacept, in an allogeneic setting using C57BL/6 derived splenic and bone marrow derived DCs as stimulators of allogeneic Balb/c derived T cells. CTLA4-Ig potently dampened allogeneic T cell responses when present during T cell/DC co-culture. Although slightly inducing DC IFN-γ secretion, the dampening effect was neither related to activating the IDO pathway nor to the induction of a DC regulatory phenotype. Instead, CTLA-4Ig appeared to directly interrupt the DC/T cell crosstalk, preferentially affecting the $CD4^+$ T cell population. Moreover, $CD4^+CD25^+$ T cells recovered from DC/T cell co-cultures performed in the presence of CTLA4-Ig displayed $CD62L^+$ $FoxP3^+$ expression, compatible with a regulatory phenotype. Remarkably, this regulatory phenotype was preserved upon restimulation. In conclusion, CTLA-Ig affected allo-stimulated T cell responses quantitatively and qualitatively but independently of IDO. Expanding T regulatory cells as propagated by CTLA-4Ig may possess therapeutic potential.

Introduction

A major task in transplantation research, including solid organ transplantation (SOT) and hematopoietic stem cell transplantation (HSCT), is to achieve a state of allo-specific tolerance. T cells play a key role in the regulating transplantation immunology. Allo-specific T cell tolerance implies that T cells do not mount pathogenic immune reactions towards allogeneic organs but preserve protective activity towards environmental pathogens. Thus, the generation of allo-specific tolerized T cells may be critical to improve transplantation outcomes.

T cells in order to mount appropriate responses upon antigen recognition need to receive costimulatory signals. Costimulatory signaling involves a multitude of costimulatory molecules present on APCs interacting with their appropriate receptors on T cells to finally optimize T cell proliferation and cytokine secretion (reviewed in [193]). Previous studies have shown that antigen stimulation of T cells in the absence of costimulation will leave T cells anergic, i.e. unresponsive to subsequent stimulation by the same antigen [166, 167, 191]. The most extensively studied costimulatory pathway required for the induction of full T cell immune responses is the interaction of CD80 and CD86 molecules expressed on DCs with the CD28 molecule expressed on T cells. Cytotoxic T lymphocyte antigen-4 (CTLA-4) is expressed by activated T cells and mediates a T cell inhibitory signal thus counteracting CD28-mediated signaling to limit excess T cell stimulation. CTLA-4 has a much higher binding affinity to CD80 and CD86 molecules than CD28. CTLA-4 immunoglobulin fusion proteins (CTLA-4Ig) have been pharmacologically engineered to block CD28-mediated costimulatory signaling to T cells and thus to induce tolerance [194]. CTLA-4Ig consists of the extracellular binding domain of CTLA-4 linked to an Fc domain of IgG and possesses a high binding affinity to CD80 and CD86, (reviewed in [126, 135]).

In 2002, Grohmann et al. [75] proposed a novel mechanistic concept for CTLA-4Ig mediating immunosuppression. In their model, CTLA-4Ig binding to CD80/CD86 molecules would provide a ‘reverse’ signal to DCs resulting in the production of IFN-γ and the induction of indoleamine 2,3 dioxygenase (IDO) [170]. IDO is the major and rate limiting enzyme of tryptophan metabolism in mammals initiating tryptophan metabolism along the kynurenine pathway [60]. The resulting tryptophan depletion and kynurenine accumulation in the intercellular microenvironment interfere with the T cell proliferative capacity and promote T cell apoptotic decline. Thus, IDO activity in DCs has been proposed to possess powerful immune regulatory and tolerance inducing capacity [171]. The concept of Grohmann et al. led to the assumption that binding of CTLA-4Ig to CD80/86 induces a regulatory DC phenotype

in an IDO dependent manner. This mechanism was proposed to critically underlie the tolerogenic capacity of CTLA-4Ig [130].

In the present study, our findings, unexpectedly, provide solid evidence that immunoregulation by CTLA-4Ig does neither require IDO activity nor the induction of a DC regulatory phenotype; CTLA-4Ig, instead, mediated regulation of allogeneic T cell proliferation by directly interfering with DC/T cell interaction and supported the emergence of $CD4^+$ T regulatory cells.

Materials and Methods

Mice and reagents

Female C57BL/6 (H-2^b), DBA/2J (H-2^d), and Balb/c (H-2^d) mice, aged six to 10 weeks (Charles River Laboratories, Sulzfeld, Germany) were maintained under specific pathogen-free conditions at the Biomedical Research Institute, Medical University of Vienna (Austria). All experiments were approved by the institutional review board and followed the national and international guidelines of laboratory animal care.

Human CTLA-4Ig fusion protein (IgG1) (abatacept) [129, 157] was generously provided by Bristol-Meyers Squibb Pharmaceuticals (Princeton, NJ, USA). Recombinant mouse cytolytic CTLA-4Ig (IgG2a), termed CTLA-4Ig (S), was purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA). Interferon-gamma (IFN-γ) cytokine secretion was examined using the BD OptEIA mouse IFN-γ ELISA set (BD Biosciences, San Diego, CA, USA).

Cell culture medium

All assays were performed in IMDM (Invitrogen, Carlsbad, USA) containing 2 mM L-glutamin and 25 mM Hepes supplemented with 10% FBS (PAA Laboratories, Austria), antibiotics and 50 μM 2-mercaptoethanol (Sigma), hereafter termed complete medium. Cell cultures were maintained in humidified air containing 5% CO_2 at 37°C.

Cell preparation

Splenic DCs were enriched from C57BL/6 mice following previously described protocols [74, 75] and personal recommendations (F. Fallarino, Perugia, Italy).

In brief, spleens were injected with collagenase (type IV, Sigma, 100U/ml) and placed in collagenase IV (400U/ml) solution for 30min at 37°C and made into single cells suspensions. Single cells were recovered in 50% isoosmotic Nycodenz solution (Sigma), and centrifuged at 450*g*. The low density fraction was adhered for 2h and non-adherent cells were removed. After further 18h, the detached cells were recovered. Alternatively, DCs were enriched by magnetic cell sorting (MACS) using CD11c selection columns (Miltenyi Biotec, Bergisch-Gladbach, Germany). The DC phenotype was examined by assessing the expression of CD11c, CD11b, MHC class I and II, CD80 and CD86. Either procedure yielded ~90% $CD11c^+$ cells which contained ~90-95% $CD8\alpha^-$ and 5-10% $CD8\alpha^+$ cells. $CD8\alpha^+$ subpopulations were enriched by ≥90% using the $CD8^+$ DC isolation kit (Miltenyi Biotec). BM derived DCs (BMDCs) were generated from mouse BM as previously described [144].

Immature BMDCs expressed CD11c and CD11b (> 80%) and low levels of MHC class I/II and CD80/CD86.

For DC activation, splenic DCs ($1x10^6$/mL per well) were plated in 24-well culture plates (IWAKI) and exposed to CTLA-4Ig (40µg/ml) [75] for 24h. BMDCs were activated by 100ng/mL LPS (*E. coli* O111:B4, Calbiochem, San Diego, CA) and IFN-γ (200U/ml, BD Biosciences) for 48h, which induced high levels of MHC class I/II and CD80/86 expression.

T cells were enriched from Balb/c spleens using the Pan T Cell Isolation Kit (MACS; Miltenyi Biotec, routinely yielding >95% $CD3^+$ cells.

T cell stimulation and mixed lymphocyte reaction (MLR)

$CD3^+$ T cells ($1x10^5$) were co-cultured with allogeneic DCs ($1x10^4$) for 3 to 6 d in 96-well round bottom plates (NUNC; Thermo Fisher) in triplicates in 200µL complete medium per well (MLR). For DC independent T cell proliferation assays, $CD3^+$ T cells ($1x10^5$) were stimulated with 3µg/mL immobilized anti-CD3 and 1µg/mL anti-CD28 (BD Biosciences) for 48h.

T cell proliferation was assessed by CFSE (Sigma) dilution as previously described [146]. Where indicated, the FlowJo Proliferation Platform (Tree Star, Ashland, OR, USA) was used to calculate the per cent divided cells. These are defined as the total per cent of cells of the starting population that divided (assuming that no cells died during the culture) irrespective of the number of cell divisions. Inhibition of proliferation was calculated as follows: Per cent inhibition = [1- (per cent $CFSE^-$ T cells in co-cultures with CTLA-4Ig/per cent $CFSE^-$ T cells in co-cultures without CTLA-4Ig)] x 100.

Flow cytometry

Flow cytometric examinations were performed using a FACSCalibur or a BD LSR II flow cytometer (BD Biosciences). List mode data were analyzed using either DivaCell (BD Biosciences) or FlowJo (Tree Star) software. The following Abs were used: unconjugated anti-CD16/32 (2.4G2), FITC-anti-H-2D[b] (KH95), PE-anti-I-A[b] (AF6-120.1), PE-Cy7-anti-CD11c (HL3), APC-Cy7-anti-CD11b (M1/70), PerCP-anti-CD8a (53-6.7), APC-anti-CD3 (145-2C11), PerCP-anti-CD4 (RM4-5), APC-Cy7-anti-CD8a (53-6.7), PE-Cy7-anti-CD25 (PC61) (all from BD Biosciences), PerCP/Cy5.5-anti-CD80 (16-10A1), Alexa Fluor 700-anti-CD86 (PO3), FITC-anti-CD62L (MEL14), APC-anti-CD90.2 (30-H12) (all from BioLegend). The Alexa Fluor 647 anti-mouse/rat/human FOXP3 Flow Kit (BioLegend, San Diego, CA, USA) was used for detection of FoxP3 expression in T cells,.

Immunoblotting

IDO protein expression in DCs was investigated using a rabbit anti-mouse IDO polyclonal Ab kindly provided by O. Takikawa (National Institute for Longevity Sciences, National Center for Geriatrics and Gerontology, Japan) [147]. Mouse monoclonal anti-mouse GapdH Ab (Ambion, Austin, TX, USA) was used as an internal control. Ab binding was visualized using the Odyssey Infrared Imaging System (Odyssey Classic; LI-COR Biosciences, Lincoln, NE, USA) and the respective fluorescent secondary Abs: goat anti-rabbit IgG, DyLight800 conjugated and goat anti-mouse IgG, DyLight680 conjugated (Pierce Biotechnology, Rockford, IL, USA).

IDO mRNA detection

Expression levels of IDO transcript in DCs were determined by semiquantitative RT-PCR. In brief, total RNA was isolated from cells with the use of trizol reagent (Invitrogen, Lofer, Austria). RNA was reversely transcribed with 200 Units Moloney-murine leukemia virus RT (Invitrogen) and 100pmol random hexamers (GE Healthcare, Vienna, Austria) at 42°C for 1h. RT-PCR was performed using Hot Start Taq polymerase (Qiagen, Vienna, Austria).

Oligonucleotides (MWG Biotech AG, Ebersberg, Germany) used for amplification of the murine IDO or of the murine GapdH were as follows: IDO, 5'-CGACATAGCTACCA GTCTGGAGAAAG-3' and 5'-GCGAGGTGGAACTTTCTCACAGAG-3'; GapdH, 5'-AC CACAGTCCATGCCATCAC-3' and 5'-TCCACCACCCTGTTGCTGTA-3'. Amplification products were size-fractionated by agarose gel electrophoresis on a 1% agarose gel, stained with ethidium bromide and quantified by scanning densitometry (Gel-Doc 1000, Molecular Analyst Software, Biorad, Hercules, CA).

Quantification of tryptophan and kynurenine

IDO enzymatic activity was determined by measuring the levels of tryptophan and kynurenine in the cell culture supernatants by HPLC as described [85].

Statistical analysis

All statistical analyses were performed with the Student *t* test (paired, 2-tailed). A *P*-value <0.05 was considered to indicate statistical significance.

Results

CTLA-4Ig primes DCs for IFN-γ production but fails to initiate IDO competence

To pin down the effect of CTLA-4Ig on the induction of a DC regulatory phenotype via IDO, we prepared C57BL/6 splenic $CD11c^+$ DCs in the same manner as described [75, 195] and stimulated these DCs using two isotypes of CTLA-4Ig. Splenic $CD11c^+$ DCs displayed a mature phenotype, indicated by MHC class I/II expression and by expression of high levels of the costimulatory molecules CD80 and CD86 [158, 159] (Figure 1A). CTLA-4Ig binding to CD80/CD86 reduced the detectability of these costimulatory molecules with the notion that abatacept affected particularly the CD80 molecule while leaving expression levels of CD86 largely unchanged [134]. Consistent with the specificity of CTLA-4Ig, no effect was observed on the expression levels of MHC class I/II molecules. (Figure 1A).

Next, C57BL/6 splenic DCs were analyzed for expression of IDO-specific transcript by RT-PCR. Consistent with previous reports [154], some basal level of expression of IDO mRNA was detected in DCs even in the absence of CTLA-4Ig (Figure 1B), compatible with a constitutive expression of IDO. However, this baseline level of IDO mRNA expression was not increased after exposing the DCs to either CTLA-4Ig isotype (Figure 1B). Likewise, the baseline levels of IDO protein expression detectable by immunoblotting remained the same upon exposing DCs to CTLA-4Ig (Figure 1C). Finally, CTLA-4Ig did not induce IDO activity. Indeed, kynurenine production in both untreated control and CTLA-4Ig stimulated DCs consistently remained <1 μmol/L (Figure 1D). Even excess doses of CTLA-4Ig up to 100μg/ml did not induce DC IDO activity (Supplementary Table 1). Thus, CTLA-4Ig failed to induce IDO expression or enzymatic activity in splenic C57BL/6 DCs.

We next sought to test whether CTLA-4Ig had an effect on DC IFN-γ production, since CTLA-4Ig binding to costimulatory molecules was reported to elicit IFN-γ production by DCs finally inducing IDO activity [76, 77]. The exposure of DCs to both CTLA-4Ig isotypes caused a slight increase of IFN-γ secretion by C57BL/6 splenic DCs (Figure 2A). However, this increase did not result in tryptophan consumption or kynurenine production (Figure 2B). Similar findings were obtained with splenic DCs obtained from DBA/2J mice [76] (Supplementary Figure 1). These findings finally excluded that the lack of IDO by CTLA-4Ig induction was limited to the C57BL/6 strain.

In a separate experiment, we specifically enriched C57BL/6 DCs for their minute (~5%) CD8α+ population and examined whether this particular DC subpopulation acquired IDO competence through CTLA-4Ig [154, 196]. However, like the total splenic DCs, the CD8α+

cells, while displaying baseline levels of IDO protein, did not acquire the capability to metabolize tryptophan and produce kynurenines by CTLA-4Ig (Supplementary Table 2)
In summary, the two isotypes of CTLA-4Ig tested caused DCs to produce IFN-γ but consistently failed to induce IDO expression and activity.

CTLA-4Ig does not confer a DC regulatory phenotype but directly affects the interaction of stimulator and responder cells

To explicitly address whether CTLA-4Ig induces a DC regulatory phenotype, we devised an experiment in which the DC population was pre-exposed to CTLA-4Ig before stimulating allogeneic T cells. C57BL/6 splenic DCs were exposed to CTLA-4Ig for 24 hours as above and thoroughly washed before being co-cultured with Balb/c T cells. Allogeneic T cell responses were quantified by CFSE dilution.
C57BL/6 derived splenic DCs were potent stimulators of allogeneic Balb/c T cells, resulting in ~70% (71 ± 1.9%, mean ± SEM, n=3) $CFSE^{-}$ T cells after a 6 day co-culture (Fig 3A). Strikingly, a pre-exposure of these DCs to CTLA-4Ig did not reduce the stimulatory capacity of the DCs. In fact, C57BL/6 DCs induced comparable allogeneic T cell proliferation irrespective of whether they were left untreated or pre-exposed to CTLA-4Ig (Fig 3A).
In contrast, when CTLA-4Ig was added at the initiation of the MLR and was present throughout the DC/T cell co-culture, we found, as expected, a significant inhibition of allogeneic T cell proliferative responses. CTLA-4Ig inhibited T cell proliferation in a dose dependent manner (not shown) with 100μg/ml resulting in an inhibition of proliferation of 68%± 0.5% (mean ±SEM) by day 3 of the MLR [145], in three consecutive experiments ($P<0.001$) (Fig 3B). Of note, similar effects of CTLA-4Ig were observed when BMDCs were used as stimulators of allogeneic T cells (see below).
To exclude that CTLA-4Ig reduces T cell responses independently of binding to costimulatory molecules on DCs, we performed DC independent T cell proliferation assays in the presence of increasing concentrations of CTLA-4Ig. In these experiments, CTLA-4Ig did not reduce T cell proliferation even at excess concentrations (Figure 3C) and did not have a toxic effect on T cells (not shown).
Together, these findings indicate that CTLA-4Ig neither promotes regulatory activity in DCs nor affects *per se* the proliferative capacity of T cells but directly interferes with the interaction of stimulator and responder cells in DC/T cell co-cultures.

CTLA-4Ig preferentially affects the $CD4^+$ T cell response and propagates T cells displaying a $CD4^+CD25^+FoxP3^+$ phenotype

Next, CFSE dilution was examined separately in $CD4^+$ and $CD8^+$ T cell populations. Consistent with previous results [136, 162], particularly the $CD4^+$ T cells were inhibited to proliferate when CTLA-4Ig was present throughout the MLR (79±0.5%, mean inhibition ±SEM), whereas the proliferation of $CD8^+$ T cells was largely spared form the effect (9±1%, mean inhibition ± SEM) (Fig 4).

Since $CD4^+CD25^+$ T cell populations may represent activated or regulatory cells [163], we examined T cells recovered from MLRs performed in the presence of CTLA-4Ig for expression of the key molecules associated with murine Treg cells, FoxP3 and CD62L [164]. In this set of experiments, C57BL/6 BMDCs were used as stimulators of MLRs which were performed in the presence (abatacept) or absence of CTLA-4Ig. In the experiment depicted in Fig 5, the allogeneic T cell proliferation was inhibited by 50% (not shown). Strikingly, the $CD4^+CD25^+$ T cell population emerging from MLRs performed in the presence of CTLA-4 Ig largely (83.0%) displayed a $FoxP3^+CD62L^+$ phenotype. In contrast, in $CD4^+CD25^+$ T cells recovered from MLRs in which CTLA-4Ig was absent the proportion of $FoxP3^+CD62L^+$ cells was low.

Thus in allogeneic stimulation, CTLA-4Ig, in addition to quantitatively down-regulating proliferation of $CD4^+$ T cells, supported the development of regulatory T cells.

The effect of CTLA-4Ig on allogeneic $CD4^+$ cells is preserved upon restimulation

To test whether the effect of CTLA-4Ig dampening allogeneic $CD4^+$ T cell responses was sustained, a second MLR was performed. Briefly, the $CD3^+$ T cells of the first MLR, performed in the presence or absence of CTLA-4Ig, were harvested, stained with CFSE and restimulated in a second MLR with the same stimulator cells but in the absence of CTLA-4Ig. Both, $CD4^+$ and $CD8^+$ cell populations having been stimulated by allogeneic DCs in the absence of CTLA-4Ig showed a rapid proliferative response upon restimulation (Fig 6A, upper panel). However, when the first MLR was performed in the presence of CTLA-4Ig, the $CD4^+$ cells remained significantly inhibited to mount a full proliferative response in a second MLR (Fig 6B). Particularly, the per cent divided cells as calculated by the FlowJo program was reduced by 50%. Like in the first MLR, the $CD8^+$ population was less affected by CTLA-4Ig, (24% inhibition, Fig 6B). Remarkably, in addition to the quantitative reduction of proliferation, the expression levels of CD25 and FoxP3 were retained in $CD4^+$ T cells upon restimulation when the first MLR was performed in the presence of CTLA-4Ig. This was in

contrast to $CD4^+$ T cells having undergone allogeneic stimulation in the absence of CTLA-4Ig (Fig 6B).

Together, we here demonstrate that the inhibitory effect of CTLA-4Ig on allogeneic stimulation is sustained in the $CD4^+$ T cell population and that a T cell regulatory phenotype is preserved upon restimulation.

Discussion

The present study advances the understanding of the cellular mechanisms of immune regulation by CTLA-4Ig as follows: CTLA-4Ig immune inhibition (i) does not rely on IDO activity, and not on the induction of a DC regulatory phenotype, but (ii) acts through directly impairing DC/T cell interaction and (iii) affects $CD4^+$ T cell responses not only quantitatively but also qualitatively by supporting the emergence of regulatory T cells.

CTLA-4Ig has been originally developed based on the concept that specific binding to CD80/CD86 molecules expressed on DCs interrupts CD28 mediated signalling to T cells (reviewed in [135, 167]) and thus can support the emergence of anergy [168]. This effect of CTLA-4Ig has been solidly evidenced in numerous in vitro and in vivo studies [96, 166, 169]. The recently reported new facet of the cellular mechanism of CTLA-4Ig claiming that CTLA-4Ig binding to CD80/CD86 transmits a signal back to DCs to ultimately result in activation of the immunomodulatory IDO pathway [75, 196] laid the basis for conceptualizing immunoregulatory CTLA-4Ig to involve a DC regulatory phenotype in an IDO dependent fashion. This understanding has stimulated a controversial debate. Pree et al showed that CTLA-4Ig, while being essential for the development of mixed chimerism in a murine model of non-myeloablative BMT, did not involve tryptophan metabolism and its effect was not reversed by inhibition of IDO [156]. Several further murine and human studies reported that the tolerogenic effect of CTLA-4 Ig occurred without the involvement of IDO [157, 197].

The present study comprehensively addressed the issue of IDO induction through CTLA-4Ig, including the use of different mouse strains, different CTLA-4Ig fusion protein preparations, and broad assessment of IDO expression on mRNA and on protein level and enzymatic activity, ultimately seeking for CTLA-4Ig mediated tolerance induction via IDO. However, the composite findings unequivocally demonstrated that CTLA-4Ig immunoregulatory activity occurred in an IDO independent fashion. In effect, while we confirmed previous findings of CTLA-4Ig inducing DCs to produce IFN-γ [76], CTLA-4Ig did not induce the IDO pathway. Moreover, CTLA-4Ig, when added to DC cultures and removed before using the DCs to stimulate allogeneic T cells, had no effect on subsequent allogeneic T cell responses. This finding might be related to the rapid dissociation rate of CTLA-4Ig from CD80 and CD86 [134]; yet, it clearly argues against the view that CTLA-4Ig induces a DC regulatory phenotype.

The problem of discrepant findings in the diverse studies addressing an association of CTLA-4Ig and IDO induction remains unsolved at this point. As previously debated, seemingly trivial differences in experimental procedures [85, 179, 180] or CTLA-4Ig

preparations (e.g., in the original study by Grohmann et al., CTLA-4 was fused to an IgG3 subclass [75]) may underlie the discrepancies. Irrespectively, the present findings strongly indicate that the immunomodulatory effect of CTLA-4Ig is not strictly dependent on the IDO pathway. In fact, CTLA-4Ig was, as expected from previous reports [145], able to potently inhibit allogeneic T cell proliferation when present at the time when DCs interacted with allogeneic T cells. This suggests that CTLA-4Ig directly interfered with costimulatory pathway signals, which is in keeping with the original concept of CTLA-4Ig.

In additio to the previously reported preferential effect on $CD4^+$ T cells [162, 181], we show that CTLA-4Ig promoted the $CD4^+CD25^+$ cell population to display a regulatory $FoxP3^+CD62L^+$ phenotype. FoxP3 expression in mice is viewed as the master transcription factor controlling development and function of Tregs [164, 182, 183]. In the murine system, T cells stimulated *in vitro* do not express FoxP3 (ibidem), contrary to the observations in the human system [85, 184, 185]. Thus, FoxP3 expression of the $CD4^+$ T cell population as propagated by CTLA-4Ig indicates the emergence of *bona fide* regulatory T cells. Notably, these Treg cells, as they are sustained upon a second stimulation even in the absence of CTLA-4Ig, appear to represent a stable population.

The role of Tregs cells in the context of CTLA-4Ig mediated costimulation blockade is puzzling. In principle, the homeostasis of Tregs has been proposed to be dependent on CD28 signalling. Therefore, CTLA-4Ig as it blocks CD28 mediated signal transduction has been suspected to negatively interfere with Treg survival [186]. Furthermore, blocking CD80/86 may also impair counterstimulatory signals to T cells, as provided by CTLA-4 [135]. Contrary to this hypothetical view, some reports suggested that CTLA-4Ig leads to an augmentation of Tregs [187], possibly by conversion of $CD4^+25^-$ T cells to $CD4^+CD25^+$ T cells [188], yet, in an IDO independent fashion [189]. In human studies, CTLA-4Ig was shown to fail to enlarge the $CD4^+CD25^+$FOXP3 Treg population [190]. In the long term, however, CTLA-4Ig did not negatively interfere with Treg survival [192, 198]. Finally, a specific role for $CD4^+$ Treg cells for tolerizing the $CD8^+$ population was proposed [167].

While published reports preclude a definitive conclusion, the findings of this study, i.e. CTLA-4Ig propagating Tregs, warrant further testing. Generating Treg populations through CTLA-4Ig ex vivo might be beneficial for adoptive transfer strategies after transplantation to ameliorate pathogenic alloreactivity in the host-versus-graft as well as in the graft-versus-host direction. Such an approach is currently extensively studied in our laboratory.

In summary, the findings of this study support the view that costimulation blockade by CTLA-4Ig achieves potent immunoregulatory activity in an IDO independent fashion. By

direct interference with the interaction of APCs and T cells, CTLA-4Ig affects predominantly the $CD4^+$ T cell population through two cellular mechanisms, inhibition of proliferation and allowing the emergence of a $CD4^+$ cell population harbouring a regulatory phenotype.

Figure Legends and supporting information

Figure 1. No induction of IDO and tryptophan metabolism by CTLA-4Ig in C57BL/6 splenic DCs. (A) Phenotype of splenic DCs after 24h of stimulation with CTLA-4Ig fusion proteins (40µg/ml); shaded histograms, isotype control; open histograms, DCs without (grey) or with (black) stimulation. One representative of 3 similar experiments is shown. **(B)** IDO mRNA expression as analyzed by RT-PCR. Total RNA of murine Baf3 cells was used as negative control and GapdH as internal standard; NTC, non template control. (**C)** IDO protein expression in the same DCs as in (A) analyzed by IB. Human DCs stimulated by LPS/IFN-γ as described (19) were used as positive and murine $CD3^+$ T cells were used as negative controls. Internal standard, Gapdh. **(D)** Concentrations of tryptophan (TRP; black bars, left scale) and kynurenine (KYN; grey bars, right scale) in cell culture supernatants of the same DCs as in (C) as determined by HPLC (n=5).

Figure 2. Induction of IFN-γ release in C57BL/6 splenic DCs by CTLA-4Ig. (A) Cell culture supernatants of DCs stimulated as in Fig 1 were examined for IFN-γ release (ELISA, left panel) and tryptophan metabolism (as described in Fig 1D, right panel) in parallel. Tryptophan (TRP; black bars, left scale) and kynurenine (KYN; grey bars, right scale). (n=3). *, p<0,05.

Figure 3. CTLA-4Ig does not induce a DC regulatory phenotype but impairs the interaction of stimulator and responder cells in MLRs. (A) C57BL/6 splenic DCs were stimulated with CTLA-4Ig fusion proteins for 24h. Thereafter, cells were thoroughly washed and co-cultured with Balb/c $CD3^+$ T cells. Proliferative responses were determined by CFSE dilution after 6 days and are expressed as per cent CFSE negative cells or as the per cent divided cells (see Material and Methods). Results of one experiment, representative of 3 experiments, are shown. **(B)** C57BL/6 splenic DCs were stimulated with CTLA-4Ig fusion proteins as above. Thereafter, DCs were thoroughly washed and co-cultured with Balb/c $CD3^+$ T cells in the presence or absence of CTLA-4Ig (abatacept, 100µg/ml). Proliferative responses were examined as in (A). Results of 1 experiment, representative of 3 experiments, are shown (P<0.01). **(C)** Purified Balb/c $CD3^+$ T cell were stimulated with plate bound anti-CD3/anti-CD28 for 48h in the presence or absence of increasing concentrations of CTLA-4Ig fusion proteins. T cell proliferation was assessed by CFSE dilution. Results of 1 experiment, representative of 2 experiments, are shown.

Figure 4. In an MLR, CTLA-4Ig preferentially affects the $CD4^+$ T cell population. An MLR was performed as in Fig 3 in the absence (black bars) or presence (grey bars) of CTLA-4Ig and the proliferative response (CFSE dilution) was assessed separately for CD4 and CD8 cells. **, $p<0.01$; n.s., not significant.

Figure 5. $CD4^+CD25^+$ T cells recovered from an MLR performed in the presence of CTLA-4Ig harbour a regulatory phenotype. C57BL/6 bone marrow derived DCs (BMDCs) were used as stimulators of Balb/c derived $CD3^+$ T cells in the absence or presence of CTLA-4Ig (abatacept). At the end of the co-culture, the $CD4^+CD25^+$ T cell population was examined for the expression of the key cellular markers indicating a regulatory phenotype, intracellular FoxP3 and cell surface CD62L. Dotted lines, isotype controls. Balb/c $CD4^+CD25^+$ cell isolated from lymph nodes and representing naturally occurring regulatory T cells (nTregs) were used as positive control. Results of one experiment, representative of 2 experiments are shown.

Figure 6. Sustained effect of CTLA-4Ig, preferentially in $CD4^+$ cells, in a second MLR. (A) BMDCs were used as stimulators of Balb/c derived $CD3^+$ T cells in the absence or presence of CTLA-4Ig as in Fig. 5. At the end of the MLR, T cells were recovered and stained with CFSE and restimulated with the same DCs but in the absence of CTLA-4Ig. Proliferative responses to 2^{nd} stimulation were examined by CFSE dilution, including the determination of the per cent divided cells (see inserted numbers) **(B)** $CD4^+$ T cells of the second round MLR were examined for expression of FoxP3 indicating their regulatory phenotype, as in Fig 6A.

Supplementary Table 1. Tryptophan (TRP) and kynurenine (KYN) concentrations were measured by HPLC in cell culture supernatants of $CD11c^+$ splenic DCs stimulated for 24h with increasing concentrations (0-100 μg/mL) of CTLA-4Ig (abatacept).

Supplementary Table 2. Tryptophan (TRP) and kynurenine (KYN) concentrations measured by HPLC in cell culture supernatants of $CD8\alpha^+$ splenic DCs stimulated for 24h with 40 μg/mL CTLA-4Ig (S) or abatacept.

Supplementary Figure 1. The effect of CTLA-4Ig(S) on splenic DCs obtained from DBA/2J mice. **(A)** Splenic DCs were exposed to CTLA-4Ig(S) (40μg/ml) and IDO protein expression

was examined by IB as described in Material and Methods. **(B)** Concentrations of tryptophan (TRP; black bars, left scale) and kynurenine (KYN; grey bars, right scale) were examined by HPLC in cell culture supernatants of the same DCs as in (A). (C) Cell culture supernatants of the same DCs as in (A) were examined for IFN-γ release.

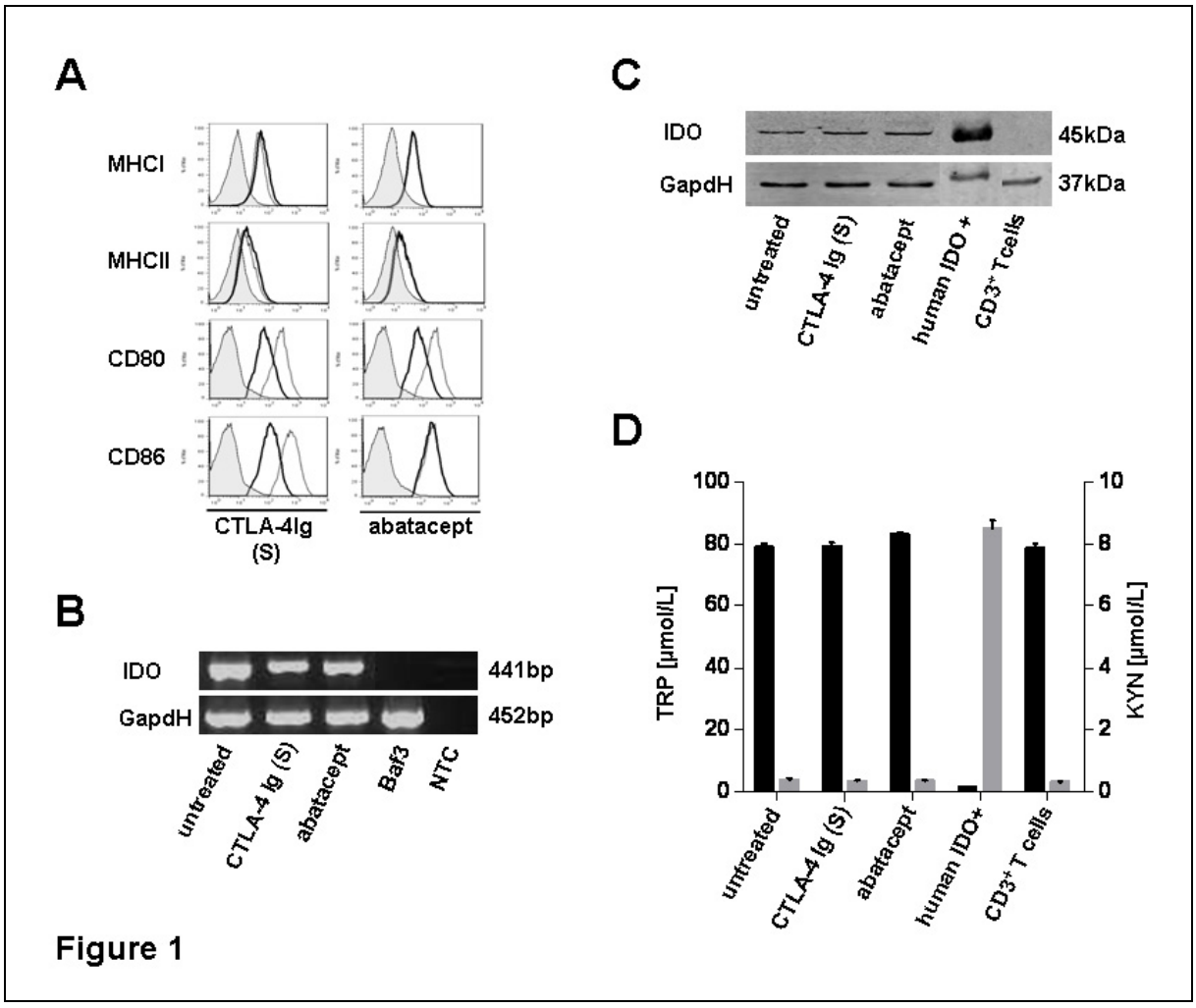


Figure 1

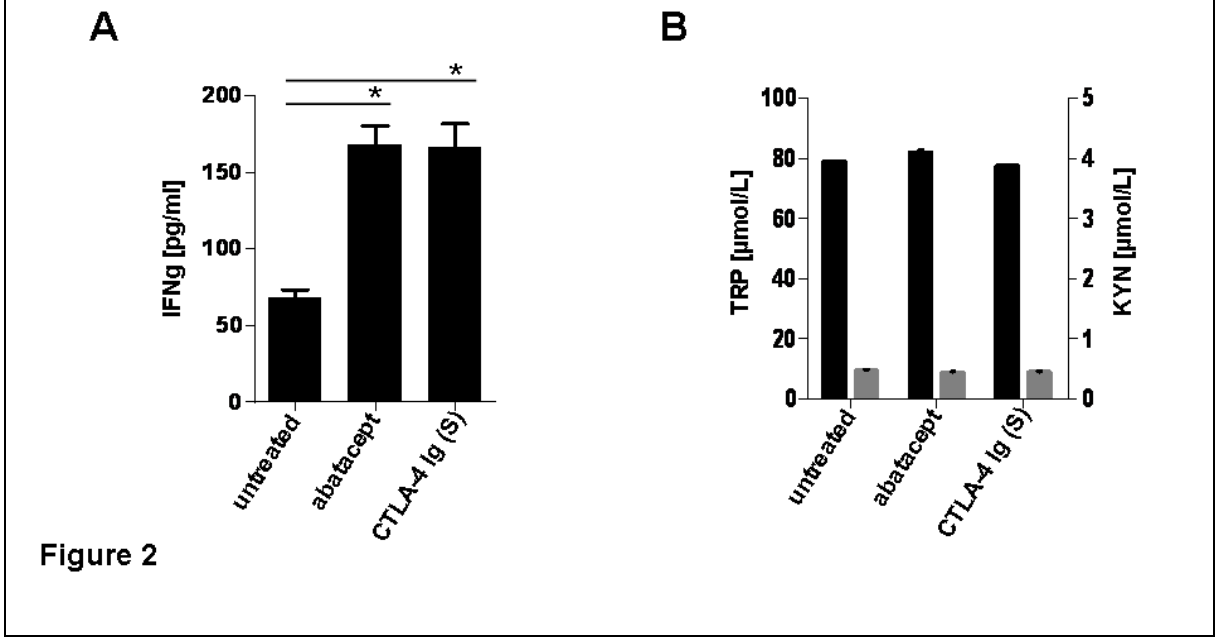


Figure 2

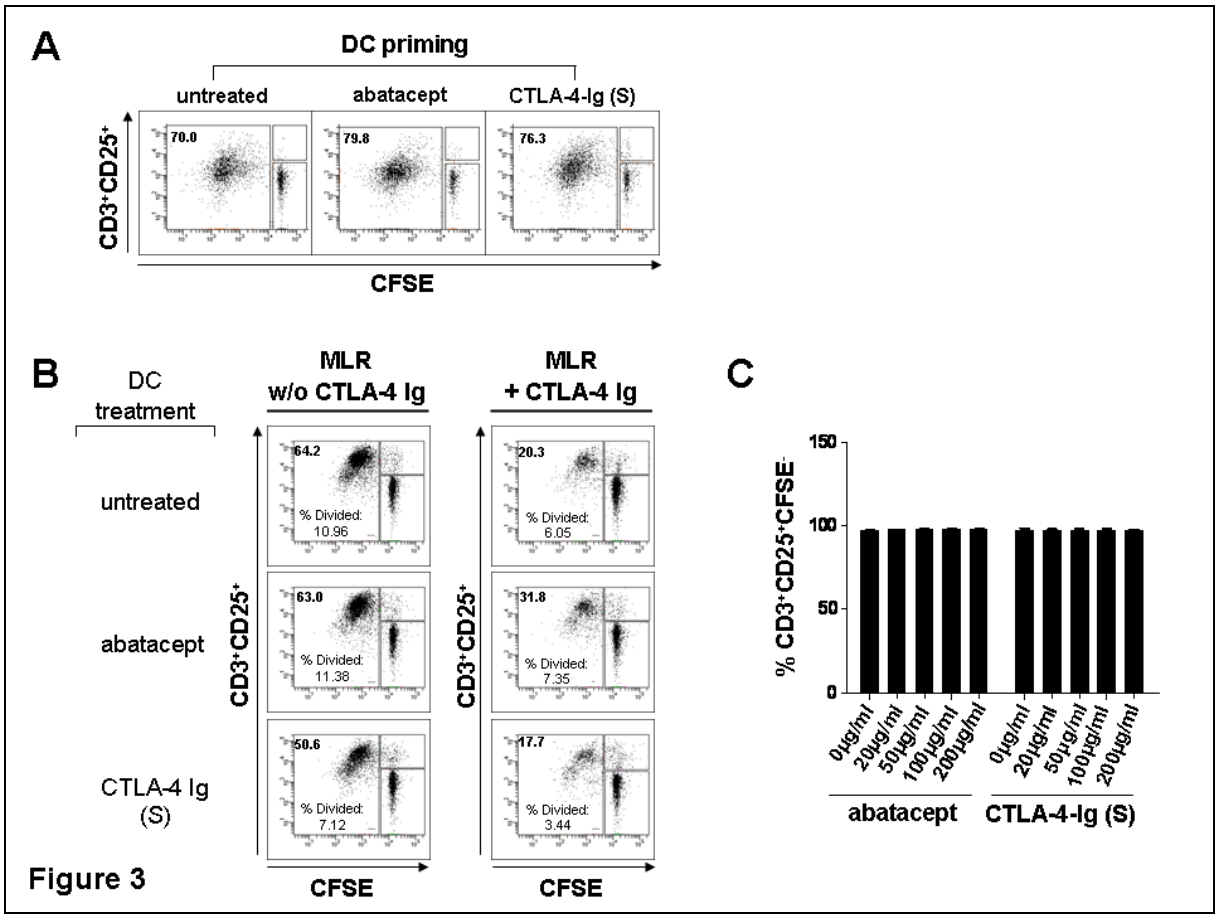


Figure 3

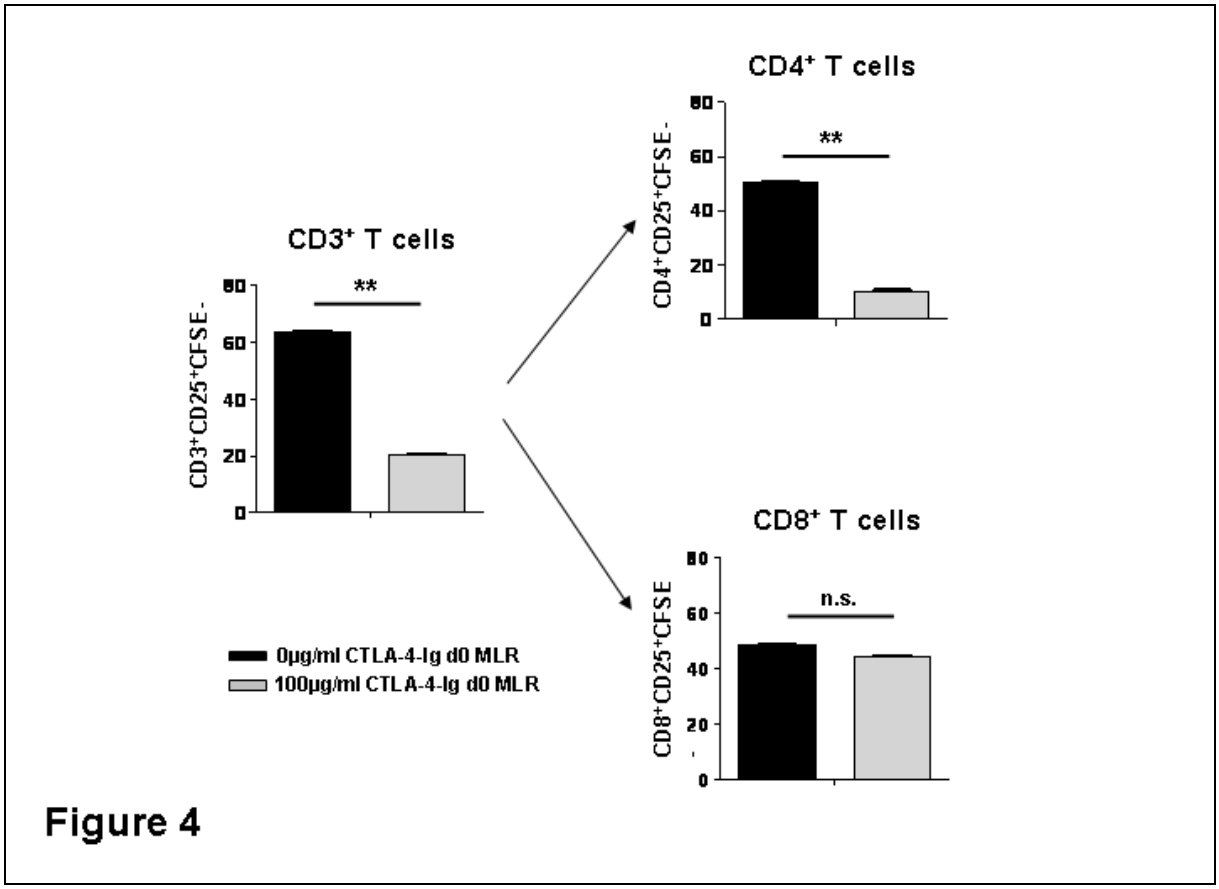


Figure 4

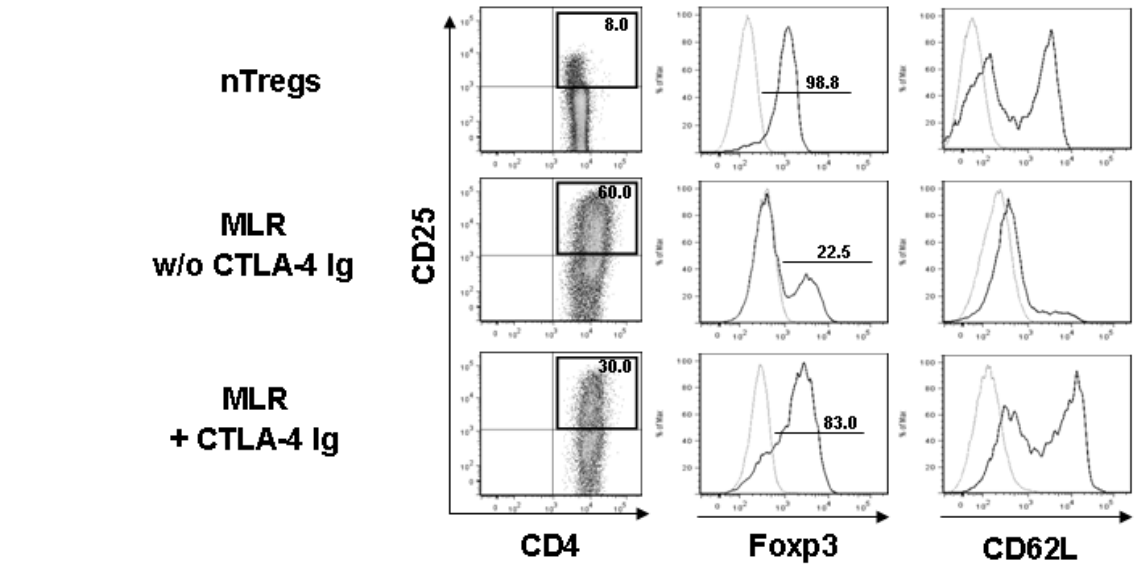


Figure 5

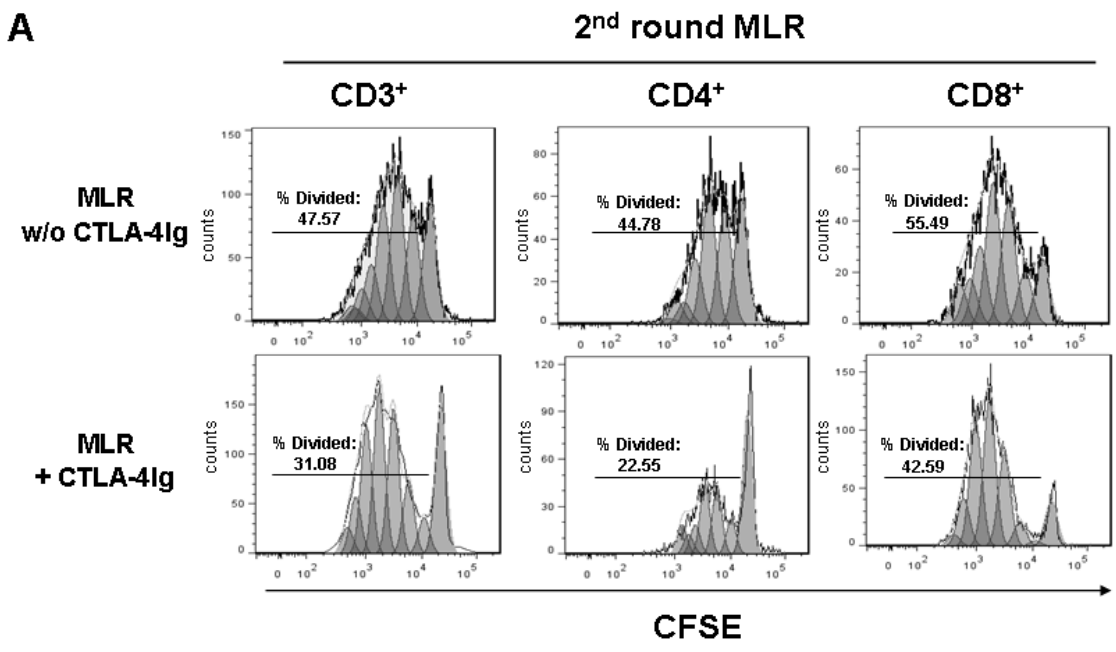


Figure 6A

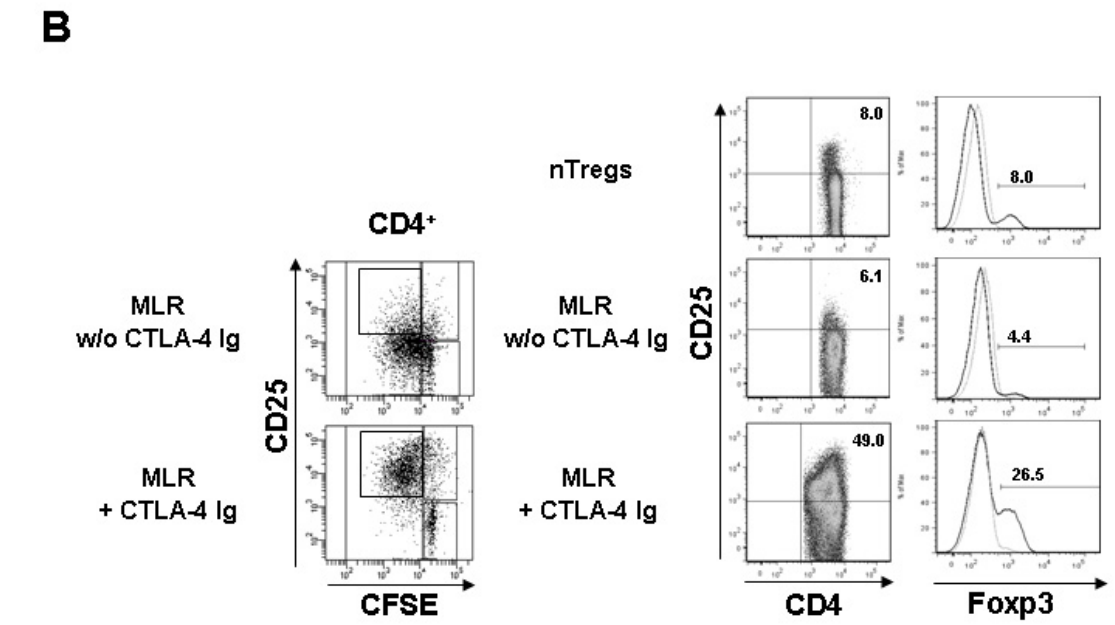
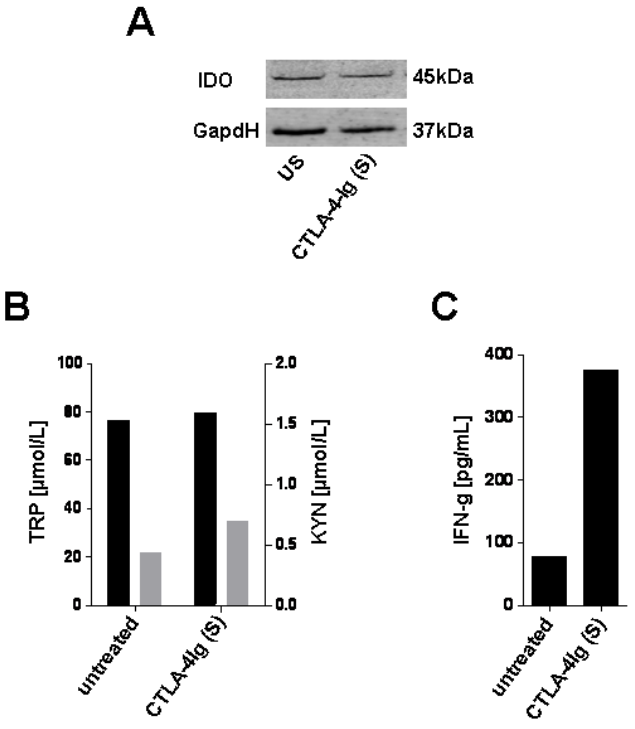


Figure 6B



Supplementary Figure 1

treatment	TRP [µmol/L]	KYN [µmol/L]
0 µg/mL CTLA-4Ig	80.2	0.32
20 µg/mL CTLA-4Ig	83.5	0.28
40 µg/mL CTLA-4Ig	77.8	0.30
100 µg/mL CTLA-4Ig	79.6	0.26

Table 4. Tryptophan (TRP) and kynurenine (KYN) levels measured by HPLC in cell culture supernatants of CD11c+ splenic DCs stimulated for 24h with increasing concentrations (0-100 µg/mL) CTLA-4Ig.

Supplementary Table 1

treatment	TRP [µmol/L]	KYN [µmol/L]
w/o stimulus	80.6	0.42
CTLA-4Ig (S)	80.5	0.28
abatacept	77.7	0.28

Table 1. Tryptophan (TRP) and kynurenine (KYN) levels measured by HPLC in cell culture supernatants of CD8a+ splenic DCs stimulated for 24h with 40 µg/mL CTLA-4Ig (S) or abatacept.

Supplementary Table 2

8 Abbreviations

APC	antigen-presenting cell
BMC	bone marrow cells
BMDC	bone marrow derived dendritic cells
BMT	bone marrow transplantation
CMV	cytomegalovirus
CNI	calcineurin inhibitor
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
CTLA-4Ig	CTLA-4-immunoglobulin
DC	dendritic cell
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
GapdH	glyceraldehyde-3-phosphate dehydrogenase
G-CSF	granulocyte colony-stimulating factor
GvHD	graft-versus-host disease
HLA	human leukocyte antigen
HPLC	high-pressure liquid chromatography
HSCT	hematopoietic stem cell transplantation
ICAM-1	intracellular adhesion molecule 1
iDC	immature dendritic cell
IDO	indoleamine 2,3 dioxygenase
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
LFA-1	lymphocyte function-associated antigen 1
LPS	lipopolysaccharide
MACS	magnetic cell sorting
MHC	major histocompatibility complex
NAD	nicotinamide-adenine-dinucleotide
NO	nitric oxide
NK	natural killer cells

PGE2	prostaglandin E2
p.t.	post transplantation
RT	room temperature
RT-PCR	real-time polymerase chain reaction
TCD	T cell depletion
TDO	tryptophan 2,3-dioxygenase
TNF	tumor-necrosis factor
TNFR	TNF receptor
TRAFs	tumor necrosis factor receptor-associated factors
Tregs	regulatory T cells
TRM	transplant-related morbidity and mortality
TRP	Tryptophan

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10 Curriculum vitae

PERSÖNLICHE DATEN

Name	Mag. rer. nat. Edda Veith
Datum und Ort der Geburt	1. Jänner 1981, Graz, Österreich
Staatsangehörigkeit	Österreich

BILDUNGSWEG

seit 08/ 2007	**Doktoratsstudium** unter der Betreuung von Univ.-Doz. Dr. Andreas Heitger (Abteilung für Transplantationsimmunologie, Children's Cancer Research Institute (CCRI) Vienna) an der Fakultät für Lebenswissenschaften der Universität Wien, Dissertationsgebiet Genetik und Mikrobiologie *Immune-regulation of allogeneic stimulation by CTLA-4Ig: Propagating $CD4^+CD25^+FoxP3^+$ T cells in an IDO independent fashion*
08/ 2006 – 07/ 2007	wissenschaftliche Mitarbeiterin im Rahmen des Projekts *"STAT1 as a tumor promoter for leukemia development"* im Labor von Univ.-Doz. Dr. Veronika Sexl, Zentrum für Physiologie und Pharmakologie, Medizinische Universität Wien
07/ 2006	Abschluss des Diplomstudiums Biologie (Studienzweig Genetik/ Mikrobiologie) mit Auszeichnung
02/ 2005	**Diplomarbeit** unter der Betreuung von A.o. Prof. Dr. Manuela Baccarini (Department für Mikrobiologie und Immunbiologie, Universität Wien) und A.o. Prof. Dr. Klaus Holzmann (Abteilung Institut für Krebsforschung, Klinik für Innere Medizin I der Medizinischen Universität Wien) *Gene expression studies of NMP265 in Coloncarcinogenesis*

	Die Arbeit wurde in der Research Group von A.o. Univ. Prof. Dr. Holzmann (Abteilung Institut für Krebsforschung, Medizinische Universität Wien) durchgeführt
seit 09/ 2003	Diplomstudium Biologie (Studienzweig Genetik/ Mikrobiologie), Universität Wien – Schwerpunkt Immunbiologie
08/ 2002	1. Diplomprüfung
1999 – 2002	Diplomstudium Biologie (Studienzweig Mikrobiologie), Karl-Franzens-Universität Graz
1991 – 1999	Akademisches Gymnasium, Graz (Matura 14.06.1999)
1987 - 1991	Volksschule Graz St.Peter

PUBLIKATIONEN

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PRÄSENTATIONEN AUF KONFERENZEN

Co-stimulation blockade to modulate allogeneic T cell responses. E. Veith, N. Pilat, T. Wekerle, A. Heitger. EBMT 2011, 03.-06.04.2011, Paris, France

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