Aus der Klinik für Angewandte Zelltherapie
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im Universitätsklinikum Schleswig-Holstein, Campus Kiel
an der Christian-Albrechts-Universität zu Kiel

A functional characterisation of the Transplant Acceptance-Inducing Cell

Inauguraldissertation
zur
Erlangung der Doktorwürde
der Medizinischen Fakultät
der Christian-Albrechts-Universität zu Kiel

vorgelegt von

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aus Ankum

Kiel 2010

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Tag der mündlichen Prüfung:	30.06.2011
Zum Druck genehmigt, Kiel, den	03.08.2010
gez.	Prof. Dr. Dr. I. Cascorbi

1. Introduction

1.1. The challenges of transplantation medicine

1.1.1. Transplantation tolerance

In the last years, solid organ transplantation has become the therapy of choice for end stage kidney, liver and heart failure, and now transplantation is an established procedure in most university clinics. This development has been closed related to the fact that pharmacological immunosuppressive strategies during the last decades were optimized and have greatly enhanced the long-term allograft survival. However, most of immunosuppressive drugs non-specifically inhibit T-cell activation, clonal expansion or differentiation into effector cells, whereas only a fraction of the lymphocyte population in humans is responsible for initiating graft destruction (Fändrich, 2006). Generalised immunosuppression leads to numerous adverse effects including increased susceptibility to infections and increased risk of tumor development. An increase in cardiovascular diseases may also be associated with some conventional immunosuppressive drugs. Furthermore immunosuppressive drugs do not prevent chronic allograft rejection so that the long-term graft survival is still limited (Golshayan et al., 2007).

The development of strategies that might allow the minimisation or eventual the cessation of conventional immunosuppression is clearly desirable. The induction of permanent specific tolerance to donor alloantigens is the only true solution to the several problems which are associated with allotransplantation (Chatenoud, 2008). Numerous approaches to tolerance induction in animals have shown promising results, but so far, no clinically practicable therapies have been established (Fändrich et al. 2002a; Knechtle et al., 1997). Given the favourable outcomes achieved with conventional immunosuppressive therapy, it is important that the inherent risks of trialling novel approaches to immunosuppression, including strategies to induce specific immunological tolerance, in transplant recipients are carefully balanced against the potential benefits.

1.1.2. The immunological basis of graft rejection

Transplantation of HLA-mismatched tissues induces many changes in the host immune system and in the graft itself. These changes are caused, on the one hand, by the inevitable proinflammatory stimulation associated with surgical and ischaemic injury to the transplanted organ and the surrounding recipient tissue, and on the other hand through recognition of antigenic differences between the donor and recipient. These circumstances result in a powerful rejection response which inevitably leads to the loss of transplant if the rejection remains untreated (Dallmann et al., 1999).

From an immunological standpoint, it is possible to distinguish two principal mechanisms of graft rejection, namely, cell-mediated and antibody-mediated immune responses. The latter is due to preformed antibodies which can cause a hyperacute rejection response resulting in an immediate failure of the transplanted organ following revascularization though the activation of the complement and platelets. Fortunately, hyperacute rejection is now a rare event because the use of pretransplant cross-match tests ensure that transplant recipients never receive an organ from a donor against whom they are sensitized (Dragun et al., 2007).

The mechanisms of acute cellular graft rejection are very complex. We differentiate between a direct antigen response and an indirect antigen response according to the two different modes of antigen presentation. Rapidly after transplantation graft-resident donor antigen-presenting cells migrate to secondary lymphoid tissues and encounter recipient T cells. A high proportion of recipient T cells bear a TCR that binds donor MHC-peptide complexes with high affinity. The allogenic MHC-peptide complexes provide a strong stimulus for the recipient T cells and leads to a high frequency of T cells which become activated to engender a direct, allospecific acute rejection response. Furthermore, recipient dendritic cells are capable of presenting graft-derived antigens in the context of self-MHC molecules which, in the surgical environment, provoke an indirect graft-specific T cell response. The majority of antigenic determinants which are recognised by responding T cells are derived from the variable domains of allogenic MHC Class II molecules, with fewer determinants derived from MHC Class I molecules and minor histocompatibility antigens (Golshayan et al., 2007). As many as 1/200 of recipient T cells respond to donor

alloantigen through the pathway of direct recognition, whereas a lesser proportion of recipient T cells, estimated to be $1/10^6$ of the naive T cell pool, are activated by indirect recognition of donor allopeptides (Powell, 2006). Both T-cell mediated processes lead to acute cellular rejection.

Chronic rejection is still an unresolved problem in transplantation medicine and the main reason for transplant failure. Chronic rejection is histologically characterized by inflammatory vascular injury and tissue fibrosis in the graft. These pathological changes are caused by T-cell mediated alloreactive responses, which trigger the migration of further T cells and macrophages into the graft resulting in an intermittent or persistent damage. Over years, a chronic transplant dysfunction develops, characterized by a slow loss of function (Joosten et al., 2004). So far the immunological basis of chronic graft rejection is still relatively unknown and, therefore, hardly any clinical preventive strategies exist.

1.2. The mononuclear phagocyte system

1.2.1. General introduction

The mononuclear phagocyte system is a commonly used term which describes a family of cells which have their origin from bone marrow progenitors and further differentiate into blood monocytes and accordingly into different tissue macrophages and dendritic cells (Hume, 2006). Monocytes are extraordinarily versatile cell lineages which have the property to differentiate into diverse cell types, including inflammatory macrophages, various types of dentritic cells and specialised tissue macrophages. Macrophages exhibit great morphological variation and have diverse functional characteristics, directed by particular tissues and immunological microenvironment (Stout et al., 2004). They represent a constitutive cell population in most of the tissues in the human body and they play an important role in both innate and adaptive immunity. Diverse, often antagonistic processes are performed by macrophages: they participate in both tissue-destructive and reparative processes, and macrophages exert immunogenic and immunosuppressive functions (Gordon et al., 2005).

1.2.2. The role of the mononuclear phagocyte system in innate and adaptive immunity

Due to their wide distribution in the body and their prevalence in tissues like the submucosa of the bowel and the airways, macrophages represent the first line of defence against many pathogens. Macrophages have the ability to eliminate pathogens by phagocytosis or by initiating the recruitment of effector cells of the immune system; thus, macrophages are a cornerstone of both the innate and adaptive immune system. In addition, macrophages prevent the systemic dissemination of numerous pathogens by blood circulation contributing to the formation of abscesses and granulomata (Gordon, 1995; Hume, 2006).

Macrophages possess several different cell-surface receptors which binding specifically “molecular patterns” on pathogens (Janeway et al., 2002a; Taylor et al., 2005). These specific interactions guarantee that the “molecular patterns” of the host remained unaffected. The macrophage mannose receptor recognises sugar molecules on bacterial and viral surfaces, whereas dectin-1 binds bacterial and fungal β-glucan. The scavenger receptor particularly recognises negatively charged ligands like lipoteichoic acids, and further detects structures that are normally shielded by sialic acids on normal host cells. This attribute is important for the degradation of senescent cells. Fc-receptors and complement receptors allow macrophages to internalise opsonized pathogens. Finally, several receptors which belong to the Toll-like receptor (TLR) family are located on macrophage cell surface. Each receptor recognizes characteristic components of pathogenic microorganisms: TLR-2 recognises a wide variety of different ligands, including zymosan, peptidoglycan and lipoteichoic acid; TLR-4 detects mainly lipopolysaccharide (LPS); flagellin is recognized by TLR-5 receptor; TLR-3, TLR-8 and TLR-9 are stimulated by bacterial and viral nucleic acid (Janeway et al., 2002b).

Binding of specific “molecular patterns” of pathogens may induce the process of phagocytosis by which pathogens are eliminated in phagolysosomes. . Additionally many other bacterial toxic agents like nitric oxide, superoxide anion and hydrogen peroxide, which are produced by macrophages, contribute to this process. These mechanisms can be sufficient to prevent an infection before it becomes established.

Furthermore macrophages produce toxic substances such as perforin, granzyme and TNF-α to kill parasites, antibody-coated cells, and neoplastic cells (Aderem et al., 1999).

The production of cytokines, chemokines and other inflammatory mediators such as prostaglandins, leukotrienes and platelet-activating factor (PAF) by macrophages is a result of the interaction between macrophages and pathogens or immunological cells. These substances play a role initiating the inflammation at the site of infection reached by complex effects like recruiting other cells, increasing vascular permeability and expression of adhesion molecules in endothelial cells (Janeway et al., 2002b).

Besides their central function in the innate immunity, macrophages occupy an important role as professional antigen-presenting cells (APC) in the adaptive immune system. Antigen fragments of phagocytized pathogens are presented on the cell surface via MHC Class II proteins. Several co-stimulatory molecules on the macrophage cell surface, including CD80 and CD86, are further necessary for the proper activation of T cells. The binding between the T-cell receptor and the MHC-II protein leads to IL-1 secretion by macrophages. IL-1 binds to its receptor on T cells and initiates an autocrine stimulation process via IL-2 in the T cell. The result is a rise of a clone by proliferation of the T cell (Janeway et al., 2002b).

Not only the induction of inflammation within the innate and adaptive immunity, but also the resolution of inflammation is among other things performed by macrophages. It is partly based on anti-inflammatory activities of macrophages and further on the clearance of apoptotic cells from the site of inflammation (Roos et al., 2004; Serhan et al., 2007). The fact that macrophages produce several tissue-trophic factors and promote new blood vessel formations makes them become central cells in tissue repair processes (Gordon, 1995; Martin et al., 2005; Tsirogianni et al., 2006).

1.2.3. The heterogeneity of monocytes and macrophages

The monocyte ultimately derives from the haematopoietic stem cell in bone marrow, which is the precursor of the common myeloid progenitor (CMP). From these progenitor lineage arises the granulocyte-monocyte /monocyte- colony forming units (GM-CFU and M-CFU) which represent the precursor population for monoblasts. Monoblasts are the earliest form committed to becoming monocytes which then released from the bone marrow into the peripheral blood, where they circulate for a number of days before migrating several tissues (Hume et al., 2002; Volkman et al., 1965).

Peripheral blood monocytes are quite heterogeneous; in particular the morphology shows substantial variances such as differences in size, different degrees of granularity and varied nuclear forms (Gordon et al., 2005). In addition monocyte heterogeneity based on differential expression of CD14 and CD16 (FcγRIII) and even considerable differences in the expression of chemokine receptors exist. Accordingly, monocytes can be divided into two subsets: the $CD14^{high}$ $CD16^{-}$ cells, described as classic inflammatory monocytes, and the $CD14^{+}$ $CD16^{+}$ cells, which are the precursors of tissue macrophages and are more likely to differentiate into dendritic cells (Passlick et al., 1989; Randolph et al., 2002; Ziegler-Heitbrock et al., 1993). A further monocyte subset was detected by the expression of $CD14^{+}$, $CD16^{+}$ and $CD64^{+}$ (FcγRI). It is assumed that these cells combine characteristics of monocytes and dendritic cells and that they command immunoregulatory properties (Grage-Griebenow et al., 2001).

When monocytes are not recruited to inflammatory lesions to become activated macrophages, they undergo a progression of phenotypic alterations, through which they can maturate into different classes of tissue-resident macrophages. The following macrophages belong to this subset: osteoclasts, gut mucosal macrophages, splenic macrophages, thymic macrophages, alveolar macrophages, Kupffer cells and microglia. Probably monocytes also contribute to the pool of lymph node-resident dendritic cells (Gordon et al., 2005).

Macrophages are phenotypically polarized in response to extrinsic stimuli and their ambient microenvironment, which leads to extremely heterogeneous populations of cells (Mantovani et al. 2005). For this reason a near-unlimited number of alternative macrophage phenotypes exist and, therefore, it is difficult to categorize them systematically.

Mantovani has proposed a classification scheme in which macrophages are divided into M1-polarised macrophages, which preferentially drive Th1-type T cell responses, and M2-polarised macrophages, which generally participate in Th2-type T cell responses (Mantovani et al., 2007). M1-polarised macrophages can be produced by treating resting macrophages with a number of pro-inflammatory agents, including IFN-γ, TNFα and LPS. Depending on the exposure to different agents M1-polarised macrophages represent a phenotypically and functionally distinct cell population, but these cells bear greater similarity to one another than to M2-polarised macrophages. The M2-polarised macrophages can be further subclassified into M2a, M2b and M2c macrophages (Mantovani et al., 2004). M2a macrophages, also known as alternatively-activated macrophages, emerge from resting macrophages stimulated with IL-4 or IL-13, or the combination of both (Goerdt et al., 1999; Gordon, 2003). M2b macrophages are generated by stimulation with immune-complexes (Mosser, 2003; Sironi et al., 2006; Sutterwala et al. 1998). Many alternative ways producing M2c macrophages exist, including treatment with vitamin D derivatives, IL-10, dexamethasone or other corticosteroids (Barclay et al., 2002; Bhavsar et al., 2008; Bogdan et al., 1991; Copland et al., 2007; Joyce et al., 1997; Lang et al., 2002; Ogawa et al., 2006). Classifying macrophages in this way illustrates a somewhat artificial concept which not really reflects the extreme heterogeneity and plasticity of macrophages. Therefore, the polarised macrophage subsets should be considered as steady-state conditions within a wide spectrum of possible macrophage phenotypes.

1.3. Generalities of cell-based therapies

Exploiting the potential tolerance-inducing effects of cells like regulatory T cells and antigen presenting cells with T cell-suppressive properties, by transferring those cells

from donor to recipient is becoming an interesting possibility in transplant medicine (Becker et al., 2006; Fändrich, 2006).

It is quite evident that cell-based therapies like these are associated with a number of technical and clinical problems. The respective complications obviously depends on the exact nature of the cells used, their way of administration and the clinical condition of the patient; but also possible risks like malignancy, atypical infections, embolism of cellular aggregates, anaphylactic reactions, sensitisation, consequences of massive cell lysis and graft-versus-host type disease must be considered (Hutchinson et al., 2008b).

Since the identification and characterisation of cells with immunosuppressive or even tolerogenic features, great interest has developed in the possibility of inducing and expanding these cells types in culture before giving them to patients (Bluestone et al., 2007; Lechler et al., 2005; St Clair et al., 2007; Thomson et al., 2006). These methods might exploit cellular mechanisms which underlie other tolerance-inducing strategies, but ex vivo manipulation of cells opens the possibility of purification, expansion and quality control testing which might lead to more consistent therapeutic effects. If such ex vivo expansion of tolerance-inducing cells should become a clinically practicable strategy for immunosuppression, it is obligatory that the substrate cells are easily preservable from donor or recipient, that these cells should be subjected to the least possible manipulation to minimise the rate of technical failure and cost and further that the therapeutic cells should be readily and effectively transferred into patients.

1.4. History of the Transplant Acceptance-Inducing Cell

Interest in Transplant Acceptance-Inducing Cells originated from studies in which rat embryonic stem-cell (ESC) lines were shown to indefinitely prolong the survival of ectopic cardiac allografts in rats (Fändrich et al., 2002a; Inoue et al., 2006). The ESCs had their origin from WKY strain animals and were transferred pre-operatively to recipient DA rats [see figure 1.1.]. This tolerogenic effect was alloantigen-specific and depended on engraftment of the progeny of the transferred ESCs in the recipient

animal. Further investigations showed that the infused ESCs gave rise to a rather limited, but stable, state of chimerism and that their principal derivatives were of the myelomonocytic lineage (Fändrich et al., 2002a+b).

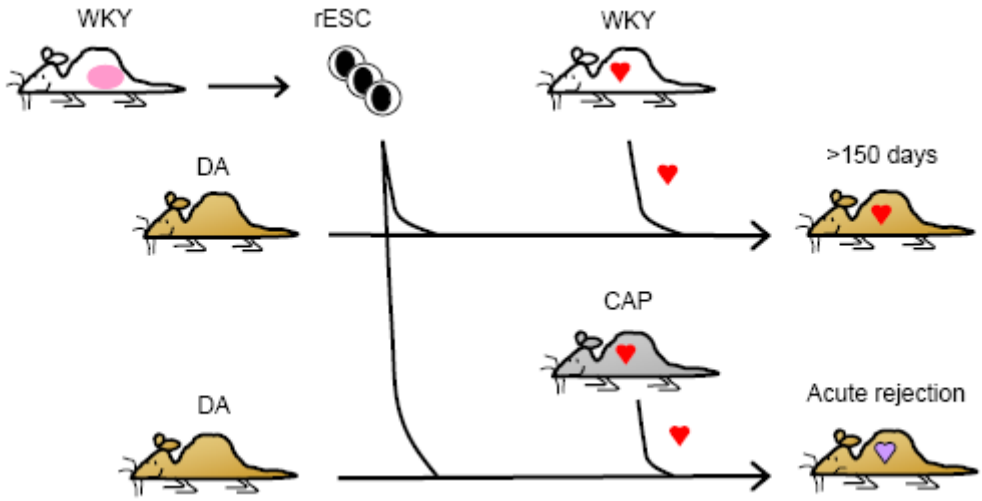

Figure 1.1. Rat embryonic stem-cells prolong the survival of cardiac allografts. Original experiments of Fändrich et al., (2002a) demonstrated that infusion of donor embryonic stem-cells prior to heart transplantation improve graft survival. Figure adapted from Riquelme (2008).

It was shown in these studies that the administration of rat ESCs did not sensitize the recipient animals and additionally a long-lived population of donor-derived cells established in the recipients providing a permanent antigenic stimulus. Brent et al. proposed that exactly such conditions are necessary for the induction and maintenance of peripheral transplantation tolerance (Brent et al., 1976). The fact that treatment with ESCs is not an accepted clinical practicable therapy so far; further studies focused their interest to identify and characterize the developed long-lived population of the ESCs. The phenotype of the ESC derived- cells determined them as a subtype of macrophages. Subsequently it was shown that equivalent cell types could produced from mononuclear splenocytes and blood and bone marrow mononuclear cells by ex vivo manipulation (Brem-Exner et al., 2008; Heumann et al., 2005; Hutchinson et al. 2008a+b). Several animal experiments demonstrated the tolerogenic potential of these cells.

Similar methods as used in animal experiments lead to the generation of TAIC cells from human monocytes; a characterisation of human TAIC cells was recently described by Hutchinson et al. (Hutchinson et al., 2008b). Both in humans and in animals, TAIC cells constitute a heterogeneous mixture of quite different macrophage phenotypes which are contaminated with lymphocytes when they are prepared by conventional isolation methods. Special isolation techniques were established in order to achieve a more homogenous cell suspension and hence to isolate the effector macrophage or macrophages. Therefore the purest possible starting monocyte population was isolated by magnetic bead sorting and then cultured under TAIC-generating conditions until they achieved a steady-state phenotype. This procedure leads to the isolation of a relatively homogenous macrophage population with a characteristic phenotype. Given that these macrophages are able to promote regulatory T cell responses and according to Mantovani`s convention, these cells were named regulatory macrophages (M regs) (Hutchinson et al., 2008b).

1.5. Characterisation of human M regs

The regulatory macrophage represents the ultimate effector cell in the heterogeneous mixture of TAIC cells. The morphology is strikingly different to that of other macrophage subtypes and even the constellation of cell-surface markers shows differences (Hutchinson J.A., unpublished data).

Plated at an appropriate density of 1-2x10^6 monocytes per cm^2 in cell culture plastic, M regs adopt a distinctive phenotype, which is characterised by a tessalating, epithelioid morphology with a prominent central body [see figure 1.2.]. Although binucleate forms can be seen occasionally, usually a single clear nucleus is visible surrounded by a thin skirt of cytoplasm. Further remarkable is the extreme granularity in M regs which probably is associated with their ability for phagocytosis of cellular debris. The size of M regs, which ranges to 50µm in diameter, constitute the most conspicuously morphological feature and is close related to the local cell density (Brem-Exner et al., 2008; Hutchinson et al., 2008b). M reg-like forms are sometimes detectable in cultures of other macrophages; it is assumed that the starting population of human peripheral blood monocytes already contained a subset of

monocytes that had been appropriately stimulated to become M regs. In contrast, pure M reg cultures contain almost exclusively cells which exhibit the typical morphological features described above (Hutchinson J.A., unpublished data).

The cell surface phenotype overlaps in some features with known macrophage types, nevertheless there exist clear differences. M regs express the myeloid markers CD13 and CD33, further the expression of CD205 identified them as cells of monocytic origin. During cell cultures M regs down-regulate CD14 expression which distinguish them from nearly all other macrophage subtypes. Further the absence of TLR2 and TLR4 and alike the lack of CD16 and CD163 differentiates M regs from other macrophages (Hutchinson et al., 2008b).

M regs are predominantly CD80 negative and they appear to express lower levels of CD86 and HLA-DR than classically-activated macrophages, but higher levels than resting macrophages. That fact identifies M regs as partially-matured and relatively inactivated macrophage subtype which is a hallmark of tolerogenic DC populations (Brem-Exner et al., 2008; Reis e Sousa, 2006).

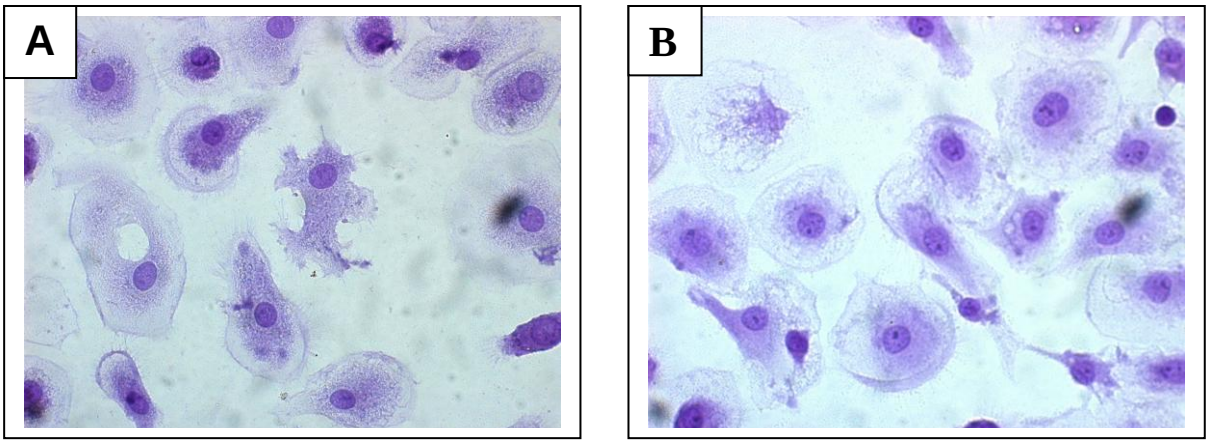

Figure 1.2. Morphology of human M regs derived from peripheral blood monocytes after CD14 purification. Isolated monocytes were cultured in 10% HABS macrophage medium (A) supplemented with ATRA (all-trans-retinoic acid) (B) for seven days. On day 6 IFN-γ was given to the culture medium. Original magnification 500x.

1.6. Characterisation of the mouse TAIC

Brem Exner et al. recently described the phenotypic and functional characteristics of IFN-γ stimulated monocyte-derived cells (IFN-γ-MdCs) in mice. Except for little differences in the culture medium the generation of those cells is similar to the generation of human TAIC cells. On the basis of their cell surface phenotype which mainly accords to that of TAIC cells, it is assumed that IFN-γ MdCs exist in a state of partial maturation (Brem-Exner et al., 2008).

Numerous experiments were undertaken to demonstrate their potential tolerogenic effect. In a first trial IFN-γ MdCs were co-cultured with lymphocytes from mesenteric lymphocytes of mice suffering from DSS-induced colitis. It was observed that the number of lymphocytes clearly decreased over the time in the co-culture. This effect was also seen in lymphocytes which derived from healthy mice. Further, it was demonstrated that the addition of the T cell activation inhibitor cyclosporine to the co-culture resulted in unchanged lymphocyte numbers. In contrast, lymphocytes stimulated with the lectine concavalin A were considerable more readily deleted by IFN-γ-MdCs. Therefore it was assumed that IFN-γ-MdCs preferentially eliminate activated T cells without consideration of their antigenic specificity (Brem-Exner et al., 2008).

The killing effect of IFN-γ-MdCs to T cells was specified in more detail. It was shown that the elimination of T cells is a clear cell-contact and caspase dependent process. Unfortunately the detailed mechanism for IFN-γ-MdCs-induced T cell death was not ascertained. Interestingly the IDO-mediated tryptophan depletion, which was responsible for induced T cell apoptosis in experiments with IFN-γ and sCD40L-induced macrophages performed by Munn and colleagues, was not relevant for the induction of T cell death (Brem-Exner et al., 2008; Munn et al., 1999; Munn et al., 1996).

In the course of the co-culture experiments a proportion of residual T cells survived. These cells were characterized as $CD4^{+}CD25^{high}$ T cells which also shown a relatively high expression of Foxp3 and IL-10 mRNA. The functional features of the $CD4^{+}CD25^{high}$ cell subset also confirmed the assumption that they represent a

population of regulatory T cells. During the co-culture process a true enrichment of regulatory cells was observed indicating that IFN-γ-MdCs are capable to expand T cells with regulatory phenotype and properties. This effect was strictly depended to the presence of $CD4^+$ cells during IFN-γ-MdCs generation. In comparison, an expansion of $CD4^+CD25^{high}$ cells was not observed in resting or M1 macrophage co-cultures (Brem-Exner et al., 2008).

A mouse colitis model was established in order to analyze the therapeutic potential of IFN-γ-MdCs. Initially, it was demonstrated with fluorescently-labelled IFN-γ-MdCs, that these cells are able to migrate to the inflamed intestinal tissue and accordingly get in contact with disease-causing T cells. On the basis of the promising in vitro experiments mice suffering from DSS-induced colitis received an administration of $5x10^6$ IFN-γ-MdCs. This treatment led to a significant reduction of colonic inflammation and according to this to a gain of body weight compared with an untreated control group. In a further experiment, healthy BALB/c mice were treated with DSS to induce chronic colitis. Directly after the last treatment with DSS, mice received either $5x10^6$ control cells, $5x10^6$ IFN-γ-MdCs or left untreated. Three weeks later the colon tissue was histologically analyzed with the result that the majority of the IFN-γ-MdCs treated animals showed low-to-intermediate grades of colitis and even almost histological resolution of colitis was observed in some mice. In contrast, the control groups showed predominately severe grades of chronic colitis (Brem-Exner et al., 2008).

1.7. Animal studies with TAIC cells

Several animal experiments with rats in the past demonstrated the tolerogenic potential of the TAIC cell, including a renal transplant model in which Lewis rats received grafts from DA strain animals. During this study it was revealed that non-immunosuppressed graft recipients succumbed to renal failure after 9.0±3.0 days. Rats which received an intravenous infusion of donor-specific TAIC cells seven days before transplantation survived for more than 100 days [see figure 1.3. A]. Both clinical data, in term of creatinine, potassium and urea levels, and the histological results did not significantly differ from a control group which received syngenic

organs. The graft biopsies did not shown any signs of acute or chronic rejections (Fändrich F., unpublished data).

The majority of transplants in Germany derive from deceased donors, so immune-conditioning strategies that rely on the pre-operative transfer of donor-derived cells to the recipients are obviously clinical impractical (Oosterlee et al., 2008). To identify whether post-operative administration of TAIC cells also result in prolonged graft function, Inoue et al. performed experiments in which BALB /c mice achieved TAIC cells five days after a heterotopic heart transplantation from C3H mice. It was shown that the untreated control group lost their transplant because of acute rejection after 8.5±0.8 days [see figure 1.3. B]. BALB/c mice which received $5x10^6$ TAIC cells of donor origin five days after transplantation showed a significantly prolonged graft survival to 21.4±8.3 days ($p<0.05$). The administration of recipient TAIC cells (BALB/c) demonstrated also a marginally lengthened graft survival to 12.4±3.4 days ($p<0.05$). The treatment with MACS-sorted monocytes from both C3H and BALB/c mice did not result in a significantly elongation of graft survival. This observation proves that the graft-protective effect was TAIC cell-dependent. TAIC cells of third-party origin (C57BL/6) did not attain an effect concerning the survival of heart transplants in BALB/c mice (8.8±0.8 days) (Inoue et al., 2006).

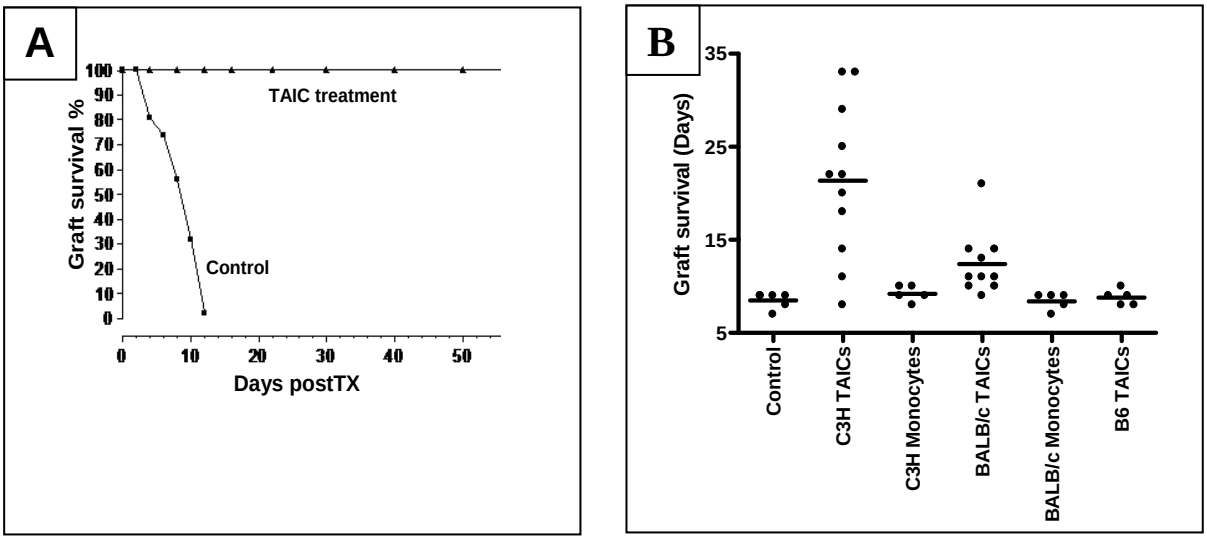

Figure 1.3. Pre-operative (figure A) and post-operative administration (figure B) of TAIC cells results in transplant survival prolongation in non-immunosuppressed animal models. Original experiments of Fändrich et al. (figure A) showed that an infusion of donor-specific TAIC cells prior to kidney

transplantations in rats considerably prolongs graft survival. Experiments performed by Inoue et al. (2006) (figure B) demonstrated that postoperatively given TAIC cells are capable of prolonging heterotopic heart transplants between C3H and BALB/c mice.

1.8. Clinical studies with human TAIC cells

The promising results in animal trials with TAIC cells and the detection of equivalent cell types in humans lead to the decision to trial TAIC cells as an immune-conditioning therapy in patients receiving kidney transplants from deceased donors. A phase 1 clinical trail, called the TAIC-1 study, commenced in 2003 (Hutchinson et al., 2008b). In this study 12 recipients of renal transplants from deceased donors received donor TAIC cells on the fifth day postoperatively. Four weeks after transplantation patients were gradually weaned from conventional immunosuppression over a time period of eight weeks.

During the first month, all participants of the TAIC-1 study were treated with conventional immunosuppressive triple therapy, including tacrolimus, sirolimus and steroids in the form of prednisolone and methylprednisolone. Initially patients were weaned from steroids over 14 days in week 5 and 6 postoperatively. If graft function remained stable at the end of the sixth week, sirolimus was also reduced namely in week 7 and 8. Then transplant recipients were slowly weaned from tacrolimus over a time-period of four weeks (weeks 9-12) up to plasma levels of 8-10 ng/ml, provided that the Cl_{cr} did not show a significant reduction (>25%) in relation to the value on day 28 after transplantation. Further reduction was undertaken in three patients who presented no signs of rejection or graft dysfunction.

For the TAIC-1 study, TAIC cells were generated from donor splenic mononuclear cells under GMP conditions. To minimize the risk of sensitising the patients against donor antigens, and for practical reasons, TAIC cells were administered five days after transplantation by central venous infusion. A number of $>1x10^6$ viable TAIC cells per kg bodyweight were infused.

The TAIC-1 study demonstrated that clinical-grade TAIC preparations could be reliably produced under GMP conditions and administered to patients postoperatively without complications. Notably, of the twelve patients enrolled in the trial, three were successfully weaned to tacrolimus monotherapy with trough serum tacrolimus levels of less than 4 ng/ml. However, four patients developed acute rejection episodes during the withdrawal of immunosuppression. Four further patients showed an acute increase in creatinine levels without any histological signs of rejection. In all eight cases conventional immunosuppressive regime was reintroduced and normal graft function was reconstituted.

It is obvious that because of the limited number of study participants and the lack of a control arm, it is not possible to obtain clear information about a potential beneficial effect of TAIC cells. Though it was demonstrated that the infusion of TAIC cells did not lead to a sensitization of the trial recipients to donor alloantigens and also evidence of potential acute adverse effects like embolism, transfusion reaction and graft versus host disease were not observed (Hutchinson et al., 2008b).

In a second clinical trial, the TAIC-2 study, TAIC cells were used as an immune-conditioning therapy in living-donor kidney transplantation (Hutchinson et al., 2008a). A number of five patients received TAIC cells five days before transplantation appointment. This was enabled by the fact that TAIC cells were generated from a leucapherisis product taken from the donor.

The administration of TAIC cells occurred without immunosuppression by a central venous catheter. After transplantation patients received anti-thymocyte globulin for the first three days. Additionally graft recipients were immunosuppressed with steroids and tacrolimus for the first eight weeks. During the eighth and tenth week postoperatively steroids were continuously reduced under strict monitoring for indices of rejection. Directly after cessation of steroids, tacrolimus was slowly reduced and even completely withdrawn, assuming that no signs for rejections were detectable. Through the RISET (Reprogramming the Immune System for the Induction of Tolerance) collaborative network, it was possible to monitor the TAIC-2 participants for various special indices of tolerance induction among other things through mixed lymphocyte cultures with multiple cytokine assays.

Under these conditions three patients tolerated low-dose tacrolimus monotherapy (< 4ng/ml) and another patient was completely withdrawn from immunosuppressive therapy for over eight months. However, one of these patient developed an acute rejection episode after reduction of tacrolimus trough serum levels to < 4ng/ml. Further two patients underwent acute rejection after complete cessation of immunosuppressive therapy. In all cases reintroduction of immunosuppression drugs lead to restoring of normal graft function.

Based on these results it is possible to say that TAIC treatment, as it was used in this study, is not capable of inducing complete operational tolerance in renal allografts. Anyhow it is amazing that the reduction of immunosuppressive therapy to such small doses was mostly well tolerated and that in three cases the maintenance of tacrolimus monotherapy was sufficient to prevent allograft rejection. It is assumed that TAIC cells might induce a state of donor-specific hypo-responsiveness in the recipient. While a complete cessation of immunosuppression is not tolerated, small doses are sufficient to maintain adequate immunosuppression. In addition this suggestion was confirmed in various mixed lymphocyte cultures in which an anti-donor T cell response was not detectable (Hutchinson et al., 2008a).

1.9. Objectives

There is still missing the knowledge about the concrete immunological effect of human TAIC cells and even long-term adverse effects so far are not excluded. The general objective of this thesis is the analysis of the influence of human TAIC cells to allogenic lymphocytes in co-culture. The main focus is fixed on a potential suppressive effect induced by TAIC cells. A case study, within an almost five year time period after the clinical application of TAIC cells as an adjunct immunosuppressive therapy in renal transplantation is described. Additionally a source of high quality leucocytes is presented which is very useful for the generation of experimental grade TAIC cells.

The specific objectives of the thesis are:

1. For experimental purposes, are leucocytes obtained from leucocyte reduction systems of Baxter Gambro^{TM} leucapheresis machines an equivalent or even better alternative for leucocytes from buffy coats for use in Immunological experiments?
2. Do human TAIC cells suppress T cell activation and proliferation, or even kill allogeneic lymphocytes?
3. Is the administration of TAIC cells a clinical feasible immunosuppressive therapy?

2. Materials and methods

2.1. Cell culture

2.1.1. Blood donors

Cells of human origin for experimental cell cultures were isolated from buffy coats or from cells obtained from the leukoreduction system chambers of apheresis instruments after platelet collection. Human cells were used as early as possible after their production, mainly between 6-12 hours after isolation, to keep the cell-quality high.

Both buffy coats and cells from leukoreduction system chambers of apheresis instruments derived from the Department of Transfusion Medicine of the University Clinic Schleswig-Holstein, so it was guaranteed that the cells stemmed from healthy donors. All blood donors gave their written informed consent to the use of their cells for research purposes. Blood type, age and gender of the donors were established.

2.1.2. Cell isolation

Peripheral blood monocytes were isolated from buffy coats or leukoreduction system chambers by density gradient centrifugation. Therefore cells from both sources were transferred into three 50 ml centrifugation tubes (Becton Dickinson, Germany) and each tube was filled up with DPBS without Ca^{2+} and Mg^{2+} (Dulbecco´s phosphate buffered saline, Cambrex Bio Science, Belgium) to a volume of 50 ml. Shortly after that six centrifugation tubes were filled with 20 ml of Ficoll (Biochrom AG, Germany). Each Ficoll-tube was overlayed with 25 ml of cell-suspension. After that the resulting density gradients were centrifuged at 2000 rpm for 20 min at 20°C without brake. After centrifugation the leucocyte interfaces were collected by pipetting and were transferred into two new 50 ml centrifugation tubes. Then DPBS were added to the tubes up to a volume of 50 ml and the resulting suspension was centrifuged at 1600 rpm for 10 min at 20°C with brake. The supernatant was carefully collected, in order to lose as few cells as possible, and discarded. The cell pellet on the bottom of the

tube was resuspended in 50 ml of DPBS and thereupon the cells were washed twice, centrifuging at 1000 rpm for 10 min at 20°C to remove platelets. Subsequently the pellet was resuspended in DPBS or medium and the number of cells was defined in an automatic cell counter.

2.1.3. Cell medium

TAIC culture medium: RPMI 1640 (BE12-918F, Cambrex Bio Science), 25mM HEPES (R&D Systems GmbH; Wiesbaden), 10% human AB serum (Cambrex Bio Science), 2mM glutamine (Gibco-Invitrogen GmbH; Karlsruhe), 100U/ml penicillin - 100µg/ml streptomycin (Gibco-Invitrogen), 5ng/ml human M-CSF (R&D Systems).

10% HABS macrophage medium: RPMI 1640 with phenol red (Cambrex Bio Science), 10% human AB serum (Cambrex Bio Science), 2mM glutamax (Gibco-Invitrogen), 100U/ml penicillin - 100µg/ml streptomycin (Gibco-Invitrogen), 5ng/ml human M-CSF (R&D Systems).

AB-Medium: RPMI 1640 with phenol red (Cambrex Bio Science), 10% human AB serum (Cambrex Bio Science), 2mM glutamine (Gibco-Invitrogen), 100U/ml penicillin - 100µg/ml streptomycin (Gibco-Invitrogen).

2.1.4. Generation of TAIC cells for experimental purposes

Peripheral blood monocytes were suspended in TAIC medium and plated at a density of $5x10^7$ cells/cm^2 in six-well plates or in T175 flasks (Sarstedt, Germany). The cell cultures went into the incubator overnight at 37°C and 5% CO_2. On the following day the non-adherent cells in the supernatant were discarded and fresh medium was added to the adherent cell fraction in the original flasks. The flasks were incubated for further two days before they were treated with IFN-γ (R&D Systems) on day 4 at a concentration of 25 ng/ml for 24 hours. After the stimulation with IFN-γ the cells were ready to harvest.

2.1.5. Generation of M regs for experimental purposes

To produce M regs, positive selected CD14+ monocytes were isolated from prepared buffy coats or leukoreduction system chambers with the MACS magnetic bead selection system (Miltenyi Biotec; Bergisch Gladbach, Germany) according to the manufacture's instruction. The purified monocytes were seeded at 10^5 cells/cm^2 in 10% HABS macrophage medium and incubated at 37°C and 5% CO_2. On day 6 the cells were stimulated with IFN-γ (R&D Systems) at a concentration of 25 ng/ml for 24 hours.

2.1.6. Cell Harvest

Because of the fact that monocytes and macrophages adhere strongly to tissue culture plastic, TAIC cells were harvested by scraping with a standard rubber cell scraper (Sarstedt). Before scraping process, the medium of the TAIC cells were removed and the adherent cells were gently washed with DPBS without Ca^{2+} and Mg^{2+} (Cambrex Bioscience). Then chilled TAIC-medium was added to the flask and cells rested in the medium for 10 minutes. TAIC cells were lifted with small, gentle strokes by a cell scraper. The resulting cell suspension was transferred into centrifuge tubes (Becton Dickinson), which were put into the centrifuge for five minutes at 1600 rpm.

2.2. Flow cytometry

2.2.1. CFSE staining of responder lymphocytes

For the flow cytometry-based T-cell proliferation assays the responder lymphocytes were labelled with the long term fluorescence CFSE (Gibco-Invitrogen). The use of CFSE labelled lymphocytes allows it to discriminate between responder lymphocytes, which were co-cultured with TAIC cells, and contaminating lymphocytes from the TAIC generation phase.

Shortly after the isolation of lymphocytes from buffy coats or leukoreduction system chambers a number of $250x10^6$ leucocytes was suspended in 10ml of DPBS without Ca^{2+} and Mg^{2+} (Cambrex Bioscience) in a 50ml centrifugation tube (Becton Dickinson). In parallel 10 µl of CFSE was diluted in 10ml of DPBS in another tube. Subsequently both volumes were pooled and stirred extensively before the suspension went for 7 minutes in a 37°C warm water bath. Then the labelling-process was interrupted with 10ml of FCS (Biochrom AG). Further the labelled lymphocytes were centrifuged for 10 minutes by 1600 rpm at room temperature with brake. Two more washes in DPBS followed, both for 6 minutes by 1000rpm. Finally the cells were suspended in AB-medium and incubated overnight in T175 flasks (Sarstedt).

On the following day the supernatant in the flasks was carefully collected, after agitating the cell cultures to lift the sedimented non-adherent cells, and transferred into 50ml centrifugation tubes. Afterwards the cells were centrifuged for 8 minutes at 1600 rpm. The resulting lymphocyte pellet was resuspended in 10 ml of TAIC culture medium and a cell-count was performed. CFSE labelled lymphocytes were co-cultured with TAIC cells in six-well plates (Sarstedt) in a 2:1 ratio.

2.2.2. Immunostaining for flow cytometry

To analyse the lymphocyte subsets after TAIC co-culture the responder CFSE labelled lymphocytes were stained with directly-conjugated antibodies. Initially the co-cultured cells were gently harvested with standard rubber cell scrapers (Sarstedt). The resulting cell solution in the six-well plates often contained aggregates of clustered cells. For this reason the solution was agitated with a pipette for several times. Then a volume of 300µl cell suspension was removed and transferred into cytometer tubes (Sarstedt) which were placed on ice. 30µl of 10% FcR blocking reagent (Miltenyi Biotec GmbH) was added to the tubes in order to reduce the unspecific antibody binding. After 30 minutes of incubation by room temperature, 10µl of the respective antibodies (see table 2.1.) were added to the samples. The tubes received a strong vortex and a half hour of incubation in the dark at 4°C in the fridge followed. Before an absolute quantification of responder lymphocytes was

performed, the cells in the tubes were spiked with $5x10^6$ CountBright beads (Invitrogen) and stained with 20µl of 7-aminoactinomycin (7-AAD; BD Biosciences, Heidelberg, Germany).

Antibodies	**Cat. No**
CD4	BD, 555349
CD8	BD, 555367
CD3	BD, 555339
CD14	BD, 555399
CD19	BD, 555413

Table 2.1. List of used conjugated antibodies. Manufacture: BD Biosciences

2.2.3. Flow cytometry analysis

The samples were measured with a FACS Calibur flow cytometer (BD Biosciences) and the data analysis was performed with CellQuest software (BD Biosciences). The use of CFSE and several different antibodies allowed the measurement of the lymphocyte subsets of interest.

2.3. Histology

2.3.1. M reg cytology

One day after the stimulation with IFN-γ the M-regs adopt their typical phenotype. In order to conserve the M reg cell structure and to fix them on microscope slides, the cells in the six well plates were gently washed three times with DPBS without Ca^{2+} and Mg^{2+} (Cambrex Bioscience). Further, 1ml of HyQ®Tase™ Cell Detachment Solution (Perbio Science GmbH, Bonn, Germany) was added to every well. In the following the cells were incubated by room temperature until the M regs apparently detached from cell culture plastic. The detached cells were transferred into 50ml tubes and centrifuged at 1000rpm for 8 minutes by room temperature. The resulting cell pellet was suspended into 8ml of 10% HABS macrophage medium and plated on microscope slide chambers (Lab-Tek II, Nunc, Germany).

2.4. Case study

2.4.1. Ethics

The administration of TAIC cells to a single patient in the reported case study was undertaken in accordance with all the relevant German laws. An independent local ethics committee approved the experimental set up for the described renal transplant recipient. The patient was informed in detail about possible risks and the procedure of the healing attempt before he gave his full, informed, written consent to it. The donor organ derived from the Eurotransplant pool and was transferred to the patient in conformity with the Eurotransplant and Bundesärztekammer allocation procedures.

2.4.2. Generation of clinical grade TAIC cells

TAIC cells for patients which received renal allografts from deceased donors were generated from splenic mononuclear cells. For this reason the donor spleen was recovered together with the transplanted kidney at the same time. Then the splenic capsule and the perisplenic adipose tissue were recovered before the spleen was subdivided into pieces of an approximately size of 1 cm^3. These pieces were washed with DPBS without Ca^{2+} or Mg^{2+} (Cambrex Bioscience); subsequently a filtration of this suspension was performed to receive a relatively homogenous cell mixture. Afterwards 20ml of this cell suspension was layered onto 15ml of Ficoll (Biochrom AG) in 50ml centrifuge tubes (Becton Dickinson). The tubes went into the centrifuge at 400g for 20 minutes without brake by room temperature. The resulting leukocyte interface was removed carefully and transferred into a new 50ml centrifuge tube. A threefold wash in DPBS without Ca^{2+} and Mg^{2+} followed.

The washed cells were suspended into normal TAIC culture medium at a density of $5x10^7$ cells/ml. A volume of 30 ml was added into T175 flaks (Sarstedt) which went directly into the incubator overnight at 37° C and 5% CO_2.

On the following day the supernatant cell fraction in the flasks were carefully extracted and transferred into new flasks. The adherent cells in the original flasks

obtained fresh TAIC medium, so that both the new and the original flasks were charged with a volume of 30 ml of TAIC culture medium. Subsequently all culture flasks returned back into the incubator.

24 hours later a second transfer of the supernatant cells from those cultures which had been replated one day before was performed. This procedure guaranteed that as many as possible monocytes sedimented and adhered to culture plastic with the result that the best possible number of clinical feasible TAIC cells was produced. After the transfer the flasks were reincubated.

On day 4 freshly prepared TAIC medium was supplemented with IFN-γ (R&D Systems) in a concentration of 25 ng/ml. Then the culture flasks were gently agitated and the old medium with all non-adherent cells was removed and discarded. Afterwards the resting cells in all flasks were put in culture with the IFN-γ supplemented TAIC medium. The cells went back into the incubator overnight.

The harvest of the TAIC cells, which was performed on day 5, was done with great care by cell scraping. Initially the culture flasks were swung carefully before the medium was removed by pipetting. Following the adherent cells were washed with DPBS without Ca^{2+} and Mg^{2+}; the cells were left for 10 minutes in DPBS before they were detached from the culture plastic with standard rubber cell scraper. To receive high cell viability TAIC cells were lifted with small gentle strokes which were performed very carefully. In the past it was shown in several trials that the use of trypsin as an alternative for detaching TAIC cells from the plastic bottom results in a reduced viability. In the end all TAIC cells were pooled together and suspended in an isotonic human albumin solution for central venous infusion.

3. Results

3.1. Obtaining PBMC cells from LRS chambers

3.1.1. General comments on leucocytes from LRS chambers

Due to the fact that during our lab work we had several problems with the consignment of buffy coats, and in addition the cell quality of the isolated leucocytes was not satisfactorily, we decided to search for an alternative. It was discovered that during clinical platelet collection a spin-off product emerges that contains a high number of leucocytes. These leucocytes are collected in leucoreduction system chambers of apharesis instruments. In comparison to leucocytes from buffy coats these cells do not undergo numerous preparation steps which might have negative effects on cell morphology and function; instead the leucocytes from LRS chambers can be used directly after the separation process. In addition, the LRS chamber leucocytes derive from examined healthy patients because the criteria for admission are stricter than for normal blood donors. Since LRS chambers are conical in shape, we refer to then as “cones” for convenience.

3.1.2. Significant numbers of PBMC cells with high viability can be isolated from LRS chambers

To analyze if leucocytes from cones compare well in quality to those from buffy coats, six samples of cone leucocytes were verified in terms of cell quantity and viability. For this reason leucoreduction system chambers were drained and the resulting cell suspension was diluted into a final volume of 45ml DPBS without Ca^{2+} and Mg^{2+}. The cells were then enumerated with an automated cell counter.
The results illustrated in figure 3.1. are similar to those recovered from a good quality buffy coat. Further it was shown that lymphocyte and $CD14^{+}$ monocyte viability was greater than 95% in donors 1-3.

	WBC	RBC	PLT	LYM	MON	GRA
Donor 1	$7.32x10^8$	$4.76x10^7$	$1.33x10^{10}$	$5.32x10^8$	$8.40x10^7$	$1.16x10^8$
Donor 2	$7.52x10^8$	$5.56x10^7$	$1.43x10^{10}$	$5.52x10^8$	$1.00x10^8$	$1.00x10^8$
Donor 3	$5.92x10^8$	$5.80x10^7$	$1.05x10^{10}$	$3.88x10^8$	$7.60x10^7$	$1.28x10^8$
Donor 4	$1.23x10^9$	$5.24x10^7$	$1.22x10^{10}$	$8.52x10^8$	$1.08x10^8$	$2.72x10^8$
Donor 5	$6.16x10^8$	$4.68x10^7$	$1.03x10^{10}$	$4.44x10^8$	$6.40x10^7$	$1.08x10^8$
Donor 6	$7.96x10^8$	$6.00x10^7$	$7.84x10^9$	$5.00x10^8$	$1.00x10^8$	$1.96x10^8$

Figure 3.1. Cell numbers of cone leucocytes derived from six different donors. Cell counts were performed with an automated cell counter. Cell numbers were expressed per ml.

In order to prove the influence from leukocyte isolation process on cell viability and cell quantity, the number of cells after Ficoll density gradient centrifugation was analyzed with an automated cell counter.

The leukocyte isolation process resulted in a considerable decrease of red blood cells and blood platelets. Likewise the cell numbers of leucocytes were reduced but not such extensive than those of erythrocytes and platelets [see figure 3.2]. This outcome was also observed in leukocyte isolation of buffy coats. The leukocyte separation did not influence the cell viability in a significant way.

	WBC	RBC	PLT	LYM	MON	GRA
Donor 1	$4.64x10^8$	$<8.00x10^4$	$6.00x10^8$	$3.32x10^8$	$4.00x10^7$	$9.20x10^7$
Donor 2	$4.60x10^8$	$<8.00x10^4$	$4.40x10^8$	$3.48x10^8$	$4.00x10^7$	$7.20x10^7$
Donor 3	$3.56x10^8$	$<8.00x10^4$	$4.40x10^8$	$2.40x10^8$	$4.00x10^7$	$7.60x10^7$
Donor 4	$6.60x10^8$	$1.2x10^5$	$1.08x10^9$	$4.84x10^8$	$6.00x10^7$	$1.16x10^8$
Donor 5	$3.12x10^8$	$1.2x10^5$	$7.60x10^8$	$2.36x10^6$	$2.80x10^7$	$4.80x10^7$
Donor 6	$4.68x10^8$	$<8.00x10^4$	$4.40x10^8$	$3.08x10^6$	$6.40x10^7$	$9.60x10^7$

Figure 3.2. Cell numbers of cone leucocytes derived from six different donors after leucocyte isolation process. Cell counts were performed with an automated cell counter. Cell numbers were expressed per ml.

3.1.3. Mitogen stimulation assay

Ascertaining in which intensity lymphocytes from cone origin react to PHA stimulation and in order to find out the mitogen concentration which leads to the strongest proliferation, lymphocytes were treated with different concentrations of PHA. The outcome was from interest because in several experiments before it was observed that at certain concentrations of PHA a cytotoxic effect occurred.

Lymphocytes from three different donors were isolated and labelled with CFSE. CFSE staining is suited to demonstrate very clearly the several lymphocyte subpopulations which emerge as a consequence of the mitogen stimulation: With every cell division the CFSE signal halves its intensity and therefore it is possible to estimate the division index of various T cell subpopulations by flow cytometry. After the staining a number of $0{,}5 \times 10^6$ lymphocytes per well was seeded into 24-well plates. Further, the lymphocytes were divided into six trial groups; each group was stimulated with a different concentration of PHA (0µg/ml, 0,5µg/ml, 1µg/ml, 2µg/ml, 4µg/ml and 8µg/ml).

In both $CD4^+$ and $CD8^+$ lymphocytes from all donors were responsive to the administration of PHA, whereas the $CD8^+$ fraction was more sensitive to the mitogen stimulation [see figure 3.3.]. The T cells responded to PHA stimulation in a dose-dependent fashion with a near-maximal response at 2 µg/ml. Therefore and because of the assumption that higher concentrations of PHA might generate cytotoxic effects to some cell populations, the decision was made to use PHA in a concentration of 2 µg/ml in the following mitogen experiments.

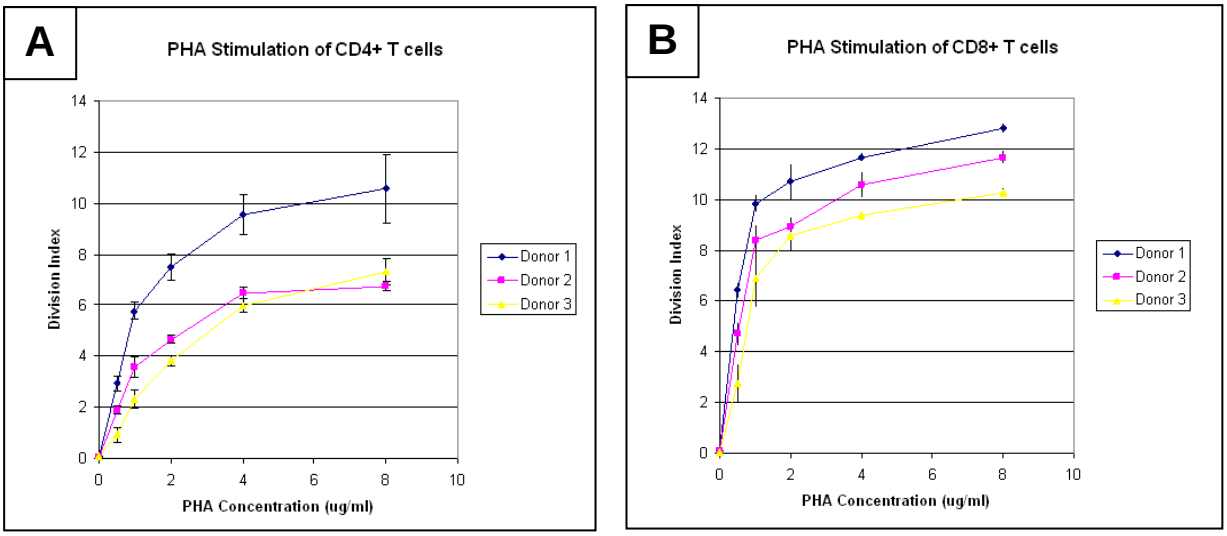

Figure 3.3. Mitogen stimulated proliferation within the $CD4^+$ (A) and $CD8^+$ (B) subset of cone-lymphocytes. Lymphocytes from three different donors were treated each with six different concentrations (0µg/ml, 0,5µg/ml, 1µg/ml, 2µg/ml, 4µg/ml and 8µg/ml) of PHA. Division index was measured by flow cytometry.

3.2. A description of the behaviour of allogeneic lymphocytes in TAIC co-culture

3.2.1. Establishing a technique for quantifying lymphocytes by flow cytometry

As described earlier, allogeneic mouse lymphocytes added into co-culture with IFN-γ treated MdCs disappear from the culture over time, which is thought to reflect an IFN-γ-MdC-mediated cellular cytotoxicity (Brem-Exner et al., 2008). In order to analyse whether human TAIC cells feature similar T-cell eliminating abilities, and to develop an experimental system in which the molecular mechanism of any such effect could be studied, a flow cytometry-based technique for counting lymphocytes was established. Figure 3.4. illustrates the experimental model: TAIC cells were generated according to standard protocols, and then a determined number of allogeneic lymphocytes were added into the culture; at different time-points, the absolute number of remaining lymphocytes was counted.

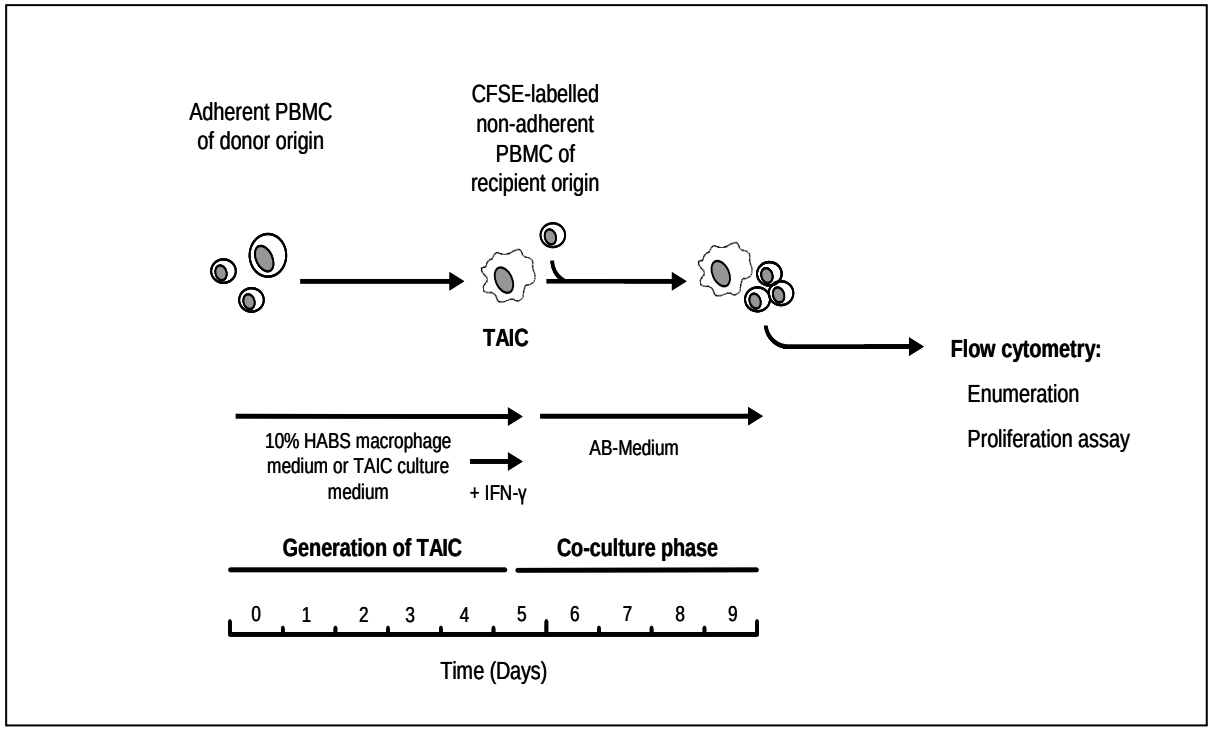

Figure 3.4. An overview of the experimental design. Human TAIC cells were co-cultured with CFSE-labelled allogeneic responder cells for 5 days and were then analysed by flow cytometry.

It is not trivial to make an absolute quantification of the loss of lymphocytes from TAIC co-cultures. The absolute number of lymphocytes in the co-culture at any fixed time-point is determined by the initial number of lymphocytes, less those lost spontaneously, less those eliminated possibly by TAIC cells, plus any lymphocytes that arose by division in culture. Additionally this dynamic situation is complicated by the fact that the lymphocytes in co-culture are of two origins: those lymphocytes which are freshly added and those that remain as contaminants of the first phase of TAIC culture. Discriminating the residual and added lymphocytes is possible by flow cytometry using CFSE to label one or other population.

Flow cytometry is a technique which has numerous advantages over alternatives methods for quantifying the number of lymphocytes in TAIC co-culture. Conventional cytotoxicity assays based on the release of radio label, particularly Chromium-51, from lysed responder cells are technically difficult and inherently hazardous. Cytotoxicity assays based on the release of cytosolic enzymes for instance G6PD do not differentiate between the death of responder cells, contaminating lymphocytes or

even TAIC cells. The measurement by flow cytometry is made separately on each cell and does not present only a mean value for a whole population.

Establishing the optimal labelling of responder lymphocytes for cell tracking and proliferation is a nontrivial procedure. The fact that the lymphocytes spent some time in co-culture before they are counted by flow cytometry makes it necessary to label them with a long term fluorescence like CFSE. But even CFSE staining loses intensity with the time and additionally by cell proliferation. Furthermore the intensity of the CFSE signal must be adapted to flow cytometry and even to the cells to be labelled because high doses of CFSE lead to cytotoxic effects. In order to find the accurate concentration several trials were undertaken. A concentration of 0,85 µg/ml incubated with $250x10^6$ cells over 7 minutes at 37°C was found to be optimal.

The ability of the flow cytometry to measure several cell-parameters at the same time allows it to determine the different lymphocyte subsets in the TAIC co-culture by adding monoclonal antibodies shortly before counting process. This procedure enables to analyze accurately the effect of TAIC cells to the lymphocyte subsets. In experiments unspecific binding was reduced by adding FcR Blocking reagent before the administration of antibodies occurred. Further antibodies for CD4 and CD8 afford a separation into three discretely identifiable groups of cells, namely $CD4^+$, $CD8^+$ and $CD4^-$ $CD8^-$. Unfortunately both CD4 and CD8 are not completely cell-type specific so that for detailed characterizations for several cell populations accessorily CD3, CD14 and CD19 antibodies were used.

Due to the assumption that TAIC cells have the property to eliminate allogenic lymphocytes it was necessary to discriminate between living cells and dead cells. For this reason we worked with 7- aminoactinomycin (7-AAD), a DNA-binding dye which permeates apoptotic cells and which can be easily combined with fluorescent surface markers.

Having statistical relevant means, samples were measured in triplicate and the standard deviation was defined, but there are still several sources of variation which have to be considered. To keep both TAIC and lymphocyte numbers exactly in same concentrations in every samples is unfeasible, impreciseness by pipetting and

counting are a remaining problem. Experiments were designed to minimize these effects by using flow cytometry as a highly specific and sensitive technique to determine the lymphocyte cell number at time-point zero which served as a relatively accurate reference. However, the absolute lymphocyte and TAIC number which was added per well in co-culture was calculated with a Coulter counter which possesses a considerable lesser accuracy of measurement than a flow cytometer. Accessorily the cell harvest is a source of variation because every harvest technique leads to a lost of cells which can diversify. In order to reduce the variation of cell lost, it was tried to harvest the cells in a standardized way.

3.2.2. Co-culture of allogeneic lymphocytes with TAIC cells promotes lymphocyte survival

In a first experiment, TAIC cells from three donors were prepared in six well plates according to the standard protocol; except that instead of TAIC medium, 10% HABS macrophage medium was used. On the final day of culture the TAIC cells were put into co-culture with freshly prepared CFSE labelled lymphocytes of two donors at 1:2 ratio. Subsequently the number of allogeneic lymphocytes per well was analyzed and again 24 hours and 96 hours later. The data after 24 hours co-culture (data not shown) showed nearly no difference, the lymphocyte numbers both in TAIC co-culture and control remained nearly stable. After 96 hours a considerable reduction of about 20% was observed. However in the lymphocyte control even a more intensively cell lost was noticed [see figure 3.5. A].

In a second experiment, the design remained the same as in the first experiment with the difference that the lymphocyte numbers were evaluated at 0 hours, 24 hours and 210 hours. The absolute number of lymphocytes in co-culture was nearly unchanged after 24 hours (data not shown) as well as after 210 hours. By contrast the control lymphocytes were reduced by approximately the half [see figure 3.5. B]. These data illustrate exactly the opposite of what Brem-Exner and colleagues described in same experiments in mice. In these experiments it was demonstrated that mice TAIC cells effectively kill lymphocytes in co-culture (Brem-Exner et al., 2008).

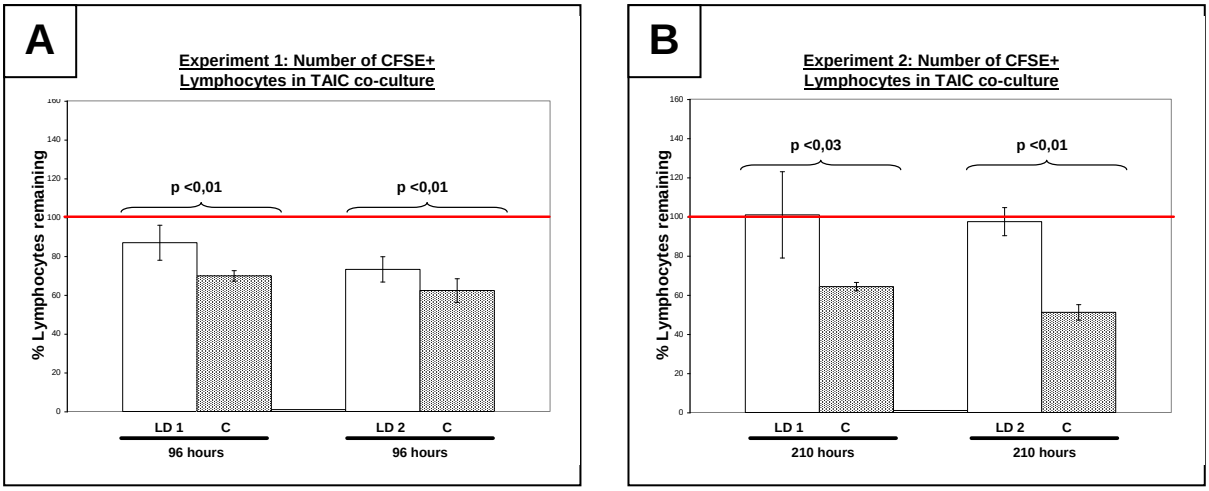

Figure 3.5. Absolute quantification of CFSE$^+$-Lymphocytes after co-culture with human TAIC cells of three different donors. Lymphocyte cell numbers are expressed as a percentage of the number of cells initially added to the cultures (red line). Cell counts were performed in triplicate. Values are shown are mean± standard error of the mean. LD= lymphocyte donor; C= control.

3.2.3. An analysis of allogeneic lymphocytes in IFN-γ treated TAIC co-culture

Further the effect of IFN-γ to the co-culture in the first and second experiment was studied. At the same day when TAIC cells and allogeneic lymphocytes were put in co-culture, 25 µg/ml IFN-γ was added to the cell suspension. In both experiments the treatment with IFN-γ did not show any relevant difference to the outcome we have seen in the IFN-γ untreated samples [see figure 3.6. A+B].

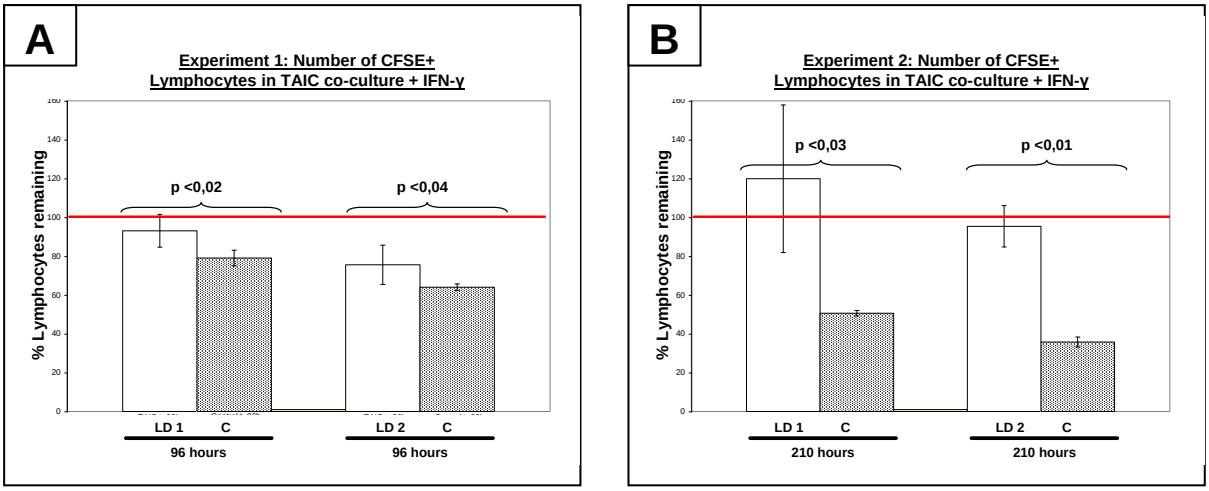

Figure 3.6. Absolute quantification of CFSE$^+$-Lymphocytes after co-culture with human TAIC cells of three different donors and supplementation of IFN-γ. Lymphocyte cell numbers are expressed as a percentage of the number of cells initially added to the cultures (red line). Cell counts were performed in triplicate. Values are shown are mean± standard error of the mean. LD= lymphocyte donor; C= control.

3.2.4. Co-culture of PHA stimulated allogeneic lymphocytes with TAIC cells dampens lymphocyte proliferation

To characterize in which way activated lymphocytes are affected by TAIC cells, the co-cultures were treated with PHA. The outcome of the first experiment revealed a considerable decrease both in co-culture and in the control, even the control lymphocytes declined more intensively [see figure 3.7. A]. This effect was already seen in experiments before (data not shown). An extensive augmentation of PHA-treated lymphocytes was revealed in the second experiment both in the control and co-culture; notably the control-cells presented a massive proliferation whereas the lymphocyte-increase in co-culture comparatively was damped [see figure 3.7. B].

This suppressive effect also was detected in flow cytometry dot plots. 96 hours after PHA treatment the control lymphocytes in the first experiment presented a wide expanded population in which divers clearly separated lymphocyte clones were identifiable. However, in the PHA treated co-cultures as well a broadened population was observed in comparison to the PHA untreated samples, but the cluster was not

such wide as the control cluster and even it was difficult to differentiate several lymphocyte clons.

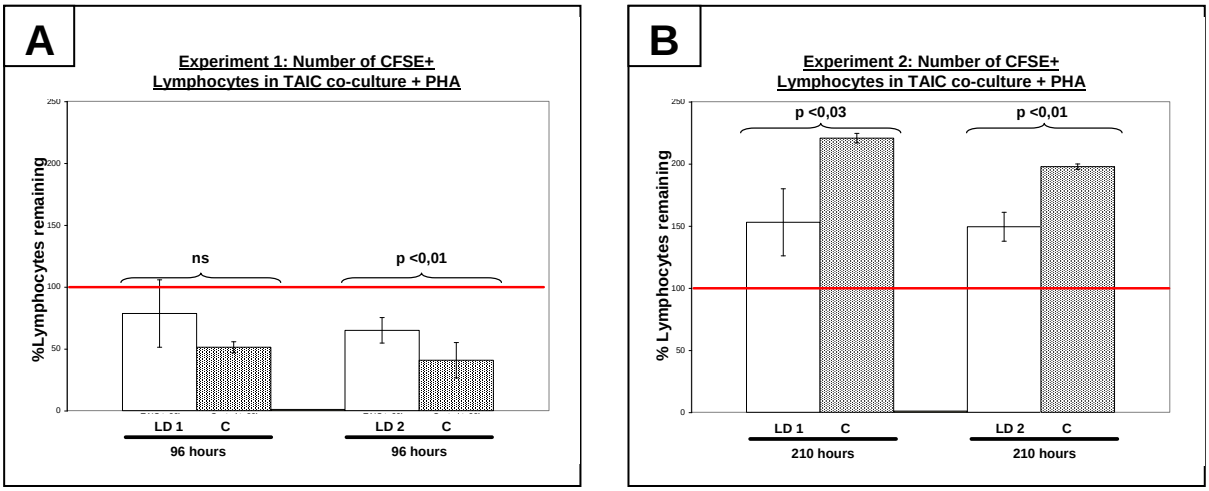

Figure 3.7. Absolute quantification of CFSE$^+$-Lymphocytes after co-culture with human TAIC cells of three different donors and supplementation of PHA. Lymphocyte cell numbers are expressed as a percentage of the number of cells initially added to the cultures (red line). Cell counts were performed in triplicate. Values are shown are mean± standard error of the mean. LD= lymphocyte donor; C= control.

3.2.5. An analysis of the CD4$^+$, CD8$^+$ and CD4$^-$ CD8$^-$ subsets in PHA treated TAIC co-cultures

In order to further dissect the suppressive effect on the T cell subsets in experiment two, the lymphocytes were labelled with monoclonal antibodies for CD4 and CD8 shortly before flow cytometry measurement. The number of the CD4$^+$ lymphocytes in co-culture and control did not shown significant differences [see figure 3.8. A] except that the CD4 fraction in the PHA stimulated control decreased compared to the co-culture [see figure 3.9. A]. In comparison, there was a considerable increase of CD8$^+$ lymphocytes in the control; up to five times more cells were detected than at time-point 0 h. In contrast, the CD8$^+$ fraction in co-culture did not expand and remained relatively stable [see figure 3.8. B +3.9. B]. The CD4$^-$ CD8$^-$ lymphocyte population did not show significantly changes between co-culture and control [see figure 3.8. C].

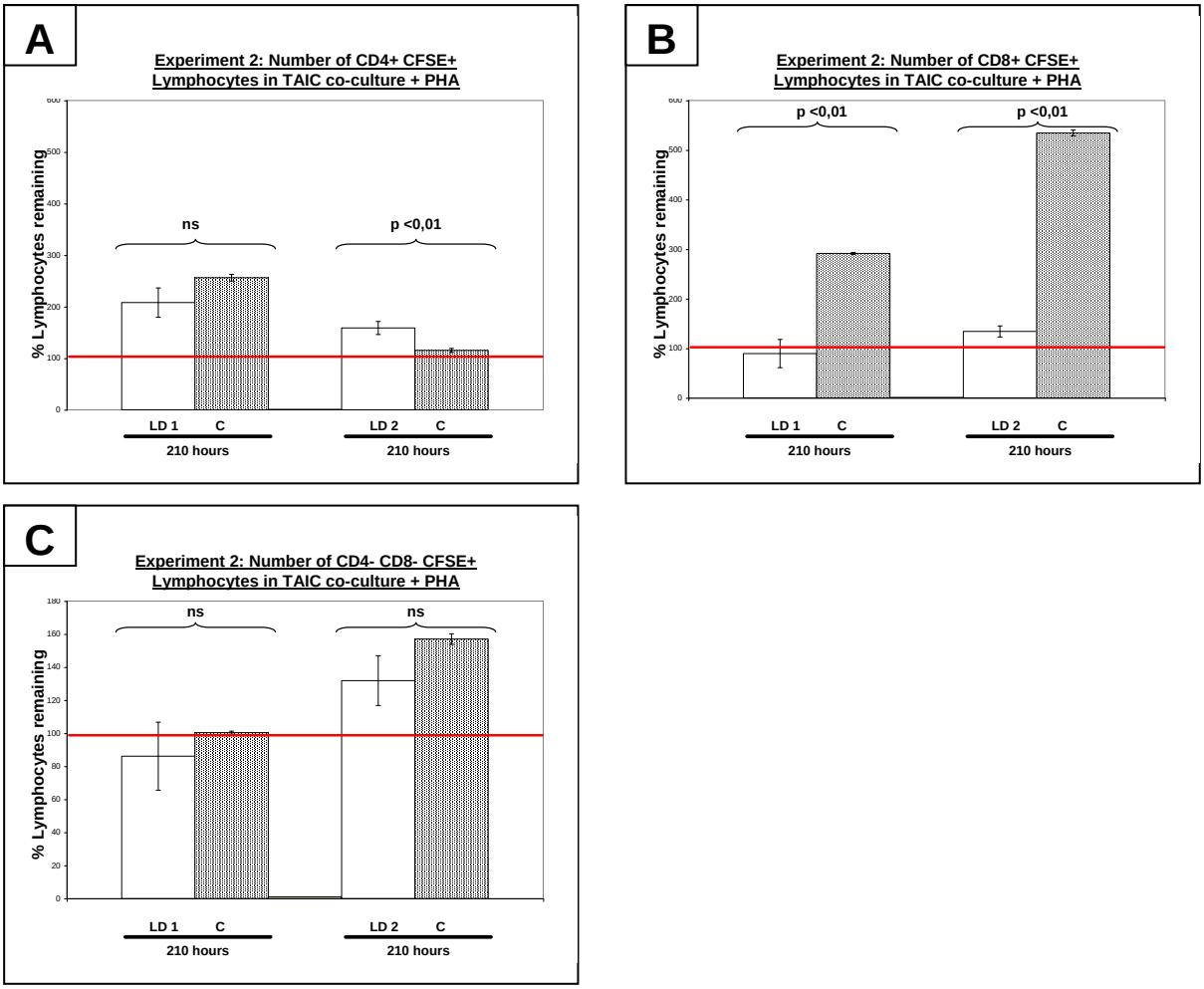

Figure 3.8. Absolute quantification of different CFSE$^+$-Lymphocyte subsets after co-culture with human TAIC cells and supplementation of PHA. CD4$^+$ (A), CD8$^+$ (B) and CD4$^-$CD8$^-$ (C) Lymphocyte cell numbers are expressed as a percentage of the number of CD4$^+$ (A), CD8$^+$ (B) and CD4$^-$CD8$^-$ (C) cells initially added to the cultures (red line). Cell counts were performed in triplicate. Values are shown are mean± standard error of the mean. LD= lymphocyte donor; C= control.

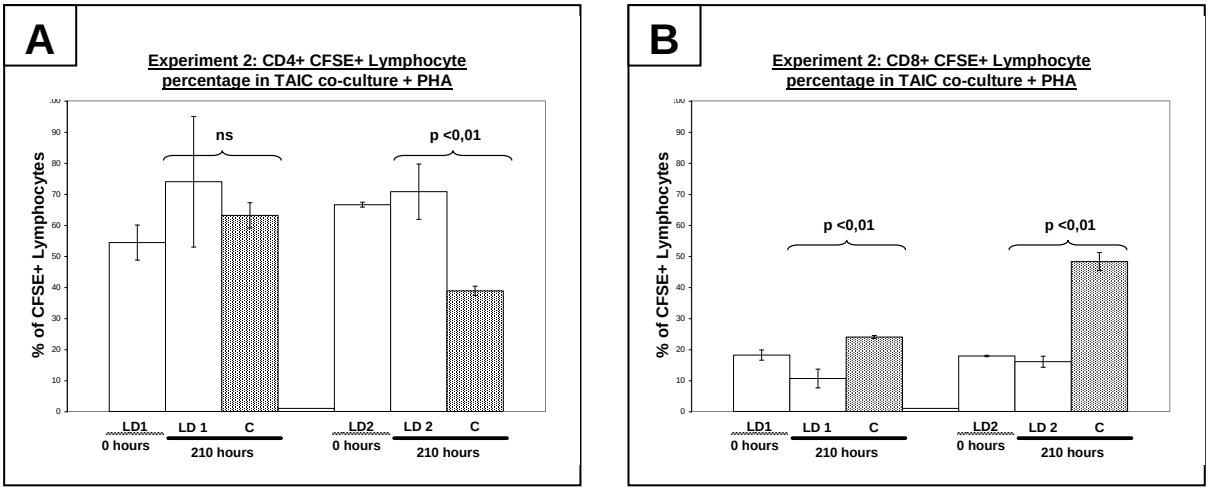

Figure 3.9. Percentage values of CD4+ (A) and CD8+ (B) CFSE+ Lymphocytes after co-culture with human TAIC cells and supplementation of PHA. CD4+ (A) and CD8+ (B) Lymphocyte numbers are expressed as a percentage of the total number of CFSE+ Lymphocytes. Cell counts were performed in triplicate. Values are shown are mean± standard error of the mean. LD= lymphocyte donor; C= control.

3.2.6. Establishing an experiment with similar conditions as in the TAIC trials

It is obvious that it is not possible to achieve same conditions in an in-vitro experiment as in a complex human organism. However in-vitro experiments depict a central role in clinical investigation and they are able to give us important information about potential modes of action and possible adverse effects.

As allude in the introduction TAIC cells already were given to patients in the course of the TAIC-1 and TAIC-2 clinical studies as an adjunct immunosuppressive therapy (Hutchinson et al., 2008 a+b). The fact that a part of the trial participants were successfully weaned from an immunosuppressive triple therapy to a low doses monotherapy or even to a drug free period let us assume that TAIC cells have a significant immunosuppressive or even a potential tolerogenic effect. The results we have seen in the first experiments partly confirmed the assumption of a possible immunosuppressive effect. In order to specify this effect in more detail and to investigate the impacts of the cell preparation steps, which are inescapable for the generation of clinical grade TAIC cells, it was tried to set up an experiment which reflects similar conditions as in the TAIC 1 and 2 studies so far as possible [see

Figure 3.10.]. The main differences to the previous experiments were that different medium was used and that the TAIC cells were harvested before they were put in co-culture with allogeneic lymphocytes. These two aspects might influence the outcome of an experiment considerably. In the past it was already shown that even a careful harvest with a cell scraper leads to immense cell destruction, so that a lot of dead cell debris is generated. It is to be assumed that such dead cell particles affect complex immunological processes with allogeneic lymphocytes. In addition the modification of the medium changes the microenvironment to which the TAIC cells are exposed and that might lead to phenotypically and functionally changes.

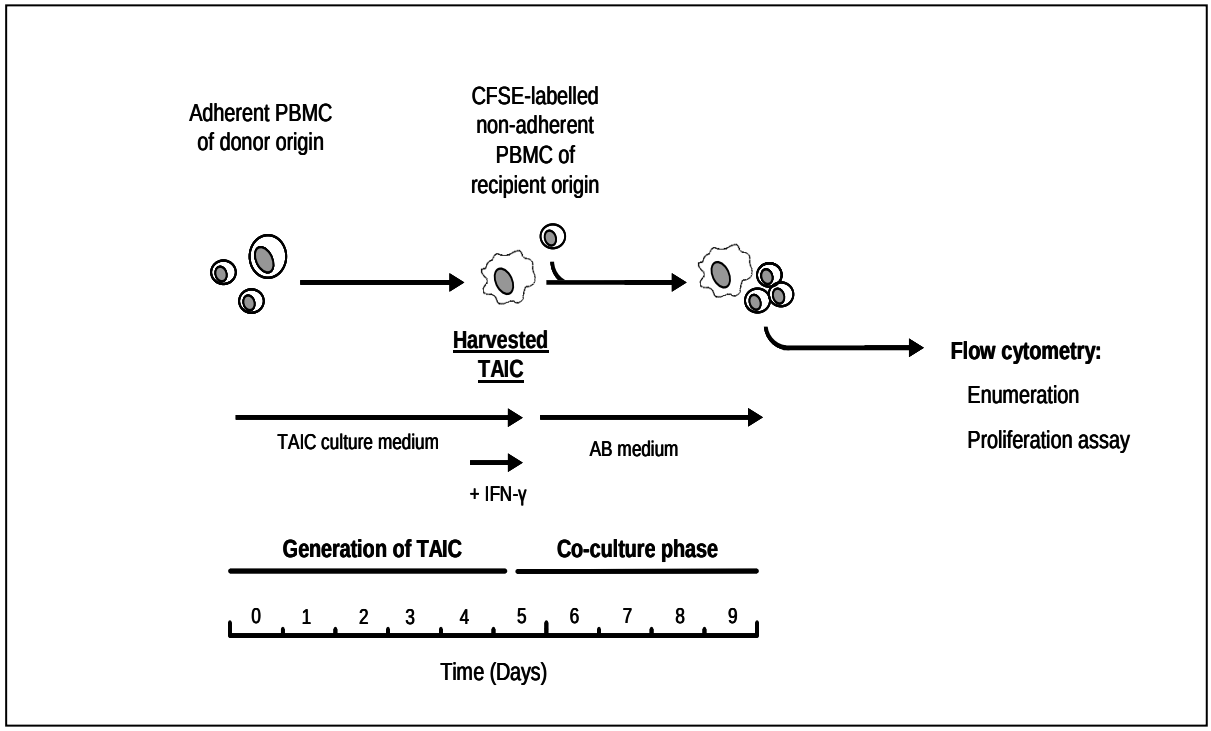

Figure 3.10. An overview of the experimental design. Human TAIC cells were harvested before they were co-cultured with CFSE-labelled allogeneic responder cells for 5 days and were then analysed by flow cytometry.

In a first experiment three different TAIC donors were combined with two different lymphocyte donors and in a second one two TAIC donors were put in co-culture with three lymphocyte donors; so that twelve different donor-recipient constellations were generated.

TAIC cells were produced like in the reported experiments except that the medium was adjusted to the medium which was used in the TAIC studies. Furthermore the source of cells was changed; instead of leucocytes from buffy coats, cone leucocytes were used. TAIC cells were harvested with a cell scraper, gently washed and then transferred into 6 well plates, in each well $2x10^6$ cells. In parallel lymphocytes from donor different cones were isolated and further they were labelled with CFSE. Both TAIC cells and labelled lymphocytes were incubated overnight. On the following day $2x10^6$ CFSE+ lymphocytes were put in co-culture with the TAIC cells.

Due to the fact that we have seen in the previous experiments a damped lymphocyte proliferation in PHA treated co-cultures and a survival effect in the untreated fraction, it was decided to divide the co-cultures into an untreated group and a PHA treated group, so that we can compare the outcome with the previous experiments. The cells were counted directly after the adding and once again 5 days later by FACS. The set up for the FACS-count and the harvest process remained the same as described in the previous experiments.

3.2.7. Co-culture of allogeneic lymphocytes with “harvested” TAIC cells does not promote lymphocyte survival

After five days of co-culture a cell lost of nearly 60% was revealed [see figure 3.11.]. Compared with the first experiments the cell death increased considerably but as also seen before the lymphocytes in monoculture showed a more extensive reduction. Nevertheless the difference between the co-culture data and the control data are nowhere near impressive then in the undertaken experiments before.

3.2.8. Co-culture of PHA treated allogeneic lymphocytes with “harvested” TAIC cells affects lymphocyte proliferation

In this experiment the addition of the mitogen PHA to the co-culture lead to a strong reduction of lymphocytes. Approximately 20% lymphocytes of recipient origin were detectable after the fifth day in the PHA treated co-cultures. The control showed approximately four times more lymphocytes after mitogen-stimulation [see figure

3.11.]. Such intensive effect was not seen in the experiments before. However in comparison to the previous undertaken long term experiment the proliferation effect induced by PHA was considerably weaker both in co-culture and in the control which might be caused by the different sources of lymphocytes and the shorter experimental time of five days.

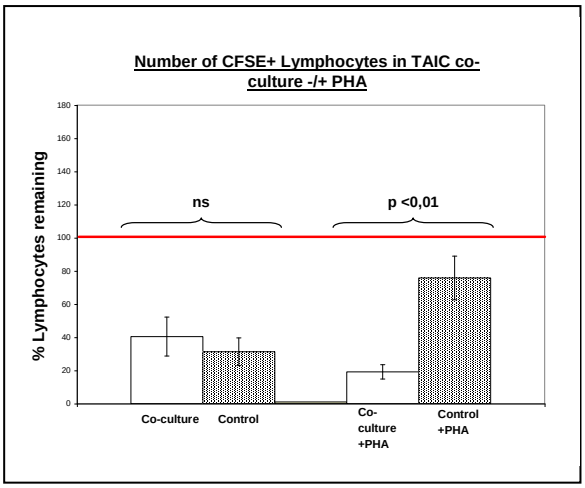

Figure 3.11. Absolute quantification of CFSE$^+$-Lymphocytes after co-culture with human TAIC cells. The right two columns represent the PHA stimulated lymphocyte fractions whereas the lymphocytes represented in the left columns remained unstimulated. Lymphocyte cell numbers are expressed as a percentage of the number of cells initially added to the cultures (red line). Cell counts were performed in triplicate. Values are shown are mean± standard error of the mean.

3.2.9. An analysis of the CD4$^+$ and the CD8$^+$ lymphocyte subsets in PHA treated "harvested" TAIC co-cultures

Both the CD4$^+$ and the CD8$^+$ fraction in PHA treated TAIC co-culture were clearly hindered in their proliferation and reduced in their number. After five days of co-culture only 20% of CD4$^+$ and CD8$^+$ lymphocytes were verifiable whereas in the controls approximately 100% CD4$^+$ lymphocytes and about 120% CD8$^+$ lymphocytes were detectable [see figure 3.12. A+B]. This TAIC mediated suppression effect of mitogen stimulated proliferation within the CD4$^+$ and CD8$^+$ lymphocyte populations is also mirrored nicely in the CFSE signal plots [see figure 3.13.]. The PHA treated control-lymphocytes represent clearly five till six subpopulations with different strong

CFSE signals as a consequence of cell division both in the $CD4^+$ and the $CD8^+$ fraction, whereas in TAIC co-culture the CFSE signal remained nearly unchanged. A damped lymphocyte proliferation in the previous experiments was only seen for the $CD8^+$ lymphocyte fraction whereas the $CD4^+$ subset remained unaffected.

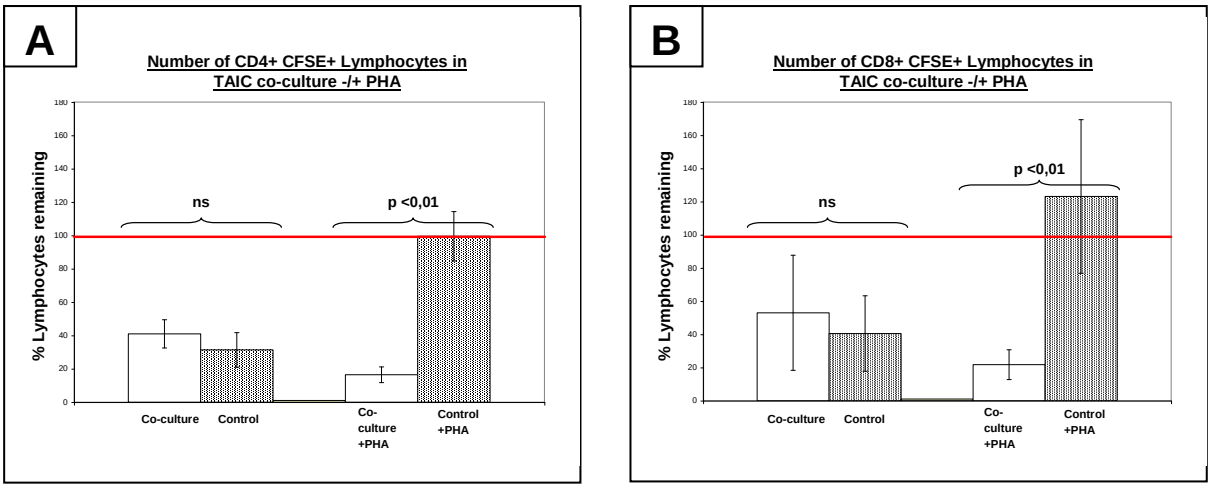

Figure 3.12. Absolute quantification of $CD4^+$ (A) and $CD8^+$ (B) $CFSE^+$-Lymphocytes after co-culture with human TAIC cells. The right columns represent the PHA stimulated lymphocyte fractions whereas the lymphocyte numbers represented in the left columns remained unstimulated. Lymphocyte cell numbers are expressed as a percentage of the number of CD4+ (A) and CD8+ (B) cells initially added to the cultures (red line). Cell counts were performed in triplicate. Values are shown are mean± standard error of the mean.

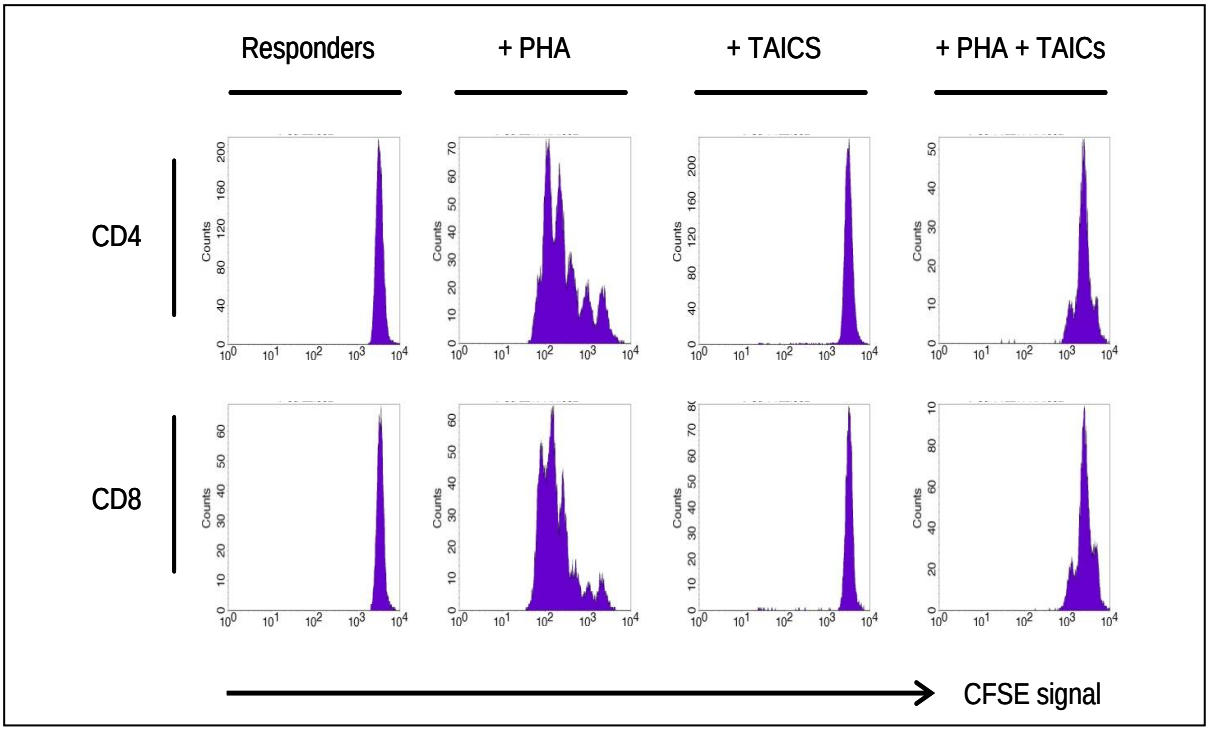

Figure 3.13. TAIC-mediated suppression of mitogen-stimulated proliferation within the CD4$^+$ and CD8$^+$ T cell subsets. The CFSE signal of lymphocytes was measured after the cells were cultured for five days in four different culture conditions by flow cytometry.

3.3. TAIC cells of Donor Origin as a Novel Adjunct Immunosuppressive Therapy in Renal Transplantation – A Case Report

Patient KW was a 36-year-old man who had an IgA Nephropathy, which was first diagnosed in year 1996. In March 2003, after he had been on haemodialysis for nearly three years, he received a deceased-donor kidney transplant. The donor kidney derived from a 57 year-old female with whom patient KW shared 5/6 HLA matches [see figure 3.15.]. The transplantation went without complications and the kidney was successfully engrafted with a warm ischaemia time of 30 min and a cold ischaemia time of 23 hours. After an initial dysfunction of the transplant during the first days after transplantation, the patient returned to theatre for a surgical treatment of a haematoma at the graft site.

At first the patient was treated with a conventional immunosuppressive therapy comprising Tacrolimus (6mg p.o.), Rapamycin (3mg p.o.) and Prednisone (50mg

p.o.) which was then slowly reduced with the time [see figure 3.14. A]. TAIC cells were procured from the donor's spleen, which was recovered at the same time as the donor kidney. A cell number of $1,07x10^6$/kg bodyweight were prepared and was given to the patient by central venous infusion on the fifth day post-transplantation. The patient did not show any kind of acute complications because of the TAIC infusion.

Due to the fact that the blood parameters only decreased slowly after the revision, with serum creatinine levels > 2,6 mg/dl, a transplant kidney biopsy was performed [see figure 3.14. C]. The biopsy showed a minimal acute tubular necrosis, a tubular microcalzification and a minor edema, leading to the diagnosis of tacrolimus toxicity. Thereupon the tacrolimus-dosis was reduced and a relevant decrease of his creatinine and his urea was detectable, such that, in the fifth week the patient's creatinine-level was 1,56 mg/dl and the urea-level was 48 mg/dl. The levels remained stable over the following ten days, so that the decision was made to wean the patient from steroids over the following two weeks. A subsequent biopsy, 3 days after the steroid withdrawal, did not show any evidence for a rejection [see figure 3.14. D].

In the following two weeks, the graft function remained stable, so Rapamycin was also gradually phased out. In parallel, Tacrolimus was reduced with the time, such that serum levels were in the range of 5,0 to 7,0 ng/ml. The blood parameters were not affected by this reduction of immunosuppression. Two weeks later the weaning of tacrolimus was continued, the tacrolimus doses was changed from 0,5 mg/d BD to a 0,5 mg/d OD, with the result that, at the end of the tenth week, the serum levels were below the detection threshold of clinical assay (< 4 ng/ml).

At the end of the eleventh week, the patient's creatinine level increased up to 2.76 mg/dl and a maximal urea level of 89 mg/dl was measured. A biopsy taken at this time showed a medium-grade tubulitis with focal tubular atrophy and a high-grade interstitial lymphocyte infiltration [see figure 3.14. E]. This pathology conformed to a moderate focal rejection. The rejection episode was treated with a high dose of Decortin (100 mg/d) and 4 mg/d Tacrolimus. Under these conditions the creatinine and the urea-levels were reduced quickly and the graft function was restored at the end of the twelfth week. In the following weeks the graft function remained stable under prednisolone and tacrolimus.

The abrupt rise of creatinine and urea five weeks later were due to a uric-acid stone which resolved without surgical intervention. A few weeks later, a further biopsy was taken with the diagnosis of a low-grade rejection [see figure 3.14. F]. The biopsy showed a non-invasive, focal mononuclear cell infiltration and a minor focal fibrosis of the intertsitium; the glomeruli und the tubules appeared normal. Due to the fact that the time after the brief episode of renal failure caused by the uric-acid stone, the graft function was recovered and the creatinine and urea levels did not show any evidence of rejection, the decision was made not to increase the immunosuppressive therapy. In the past, such focal infiltrations of leucocytes were seen in several spontaneously tolerant kidney transplant recipients (Burlingham et al., 1999).

During the 21st week, and up to the end of the first year posttransplantation, the patient received a tacrolimus monotherapy, so that his tacrolimus serum levels were kept in the range of 5.5 to 10 ng/ml. The following nearly four years the tacrolimus levels ranged between 3.1 to 8 ng/ml. Under these low-level immunosuppressive conditions the patient did not shown any evidence of rejection or other pathological processes. During this time-period his creatinine levels were consistenly between 1.7 to 2.2 ng/ml. Presently, the patient is in a good condition and not impaired because of his transplant.

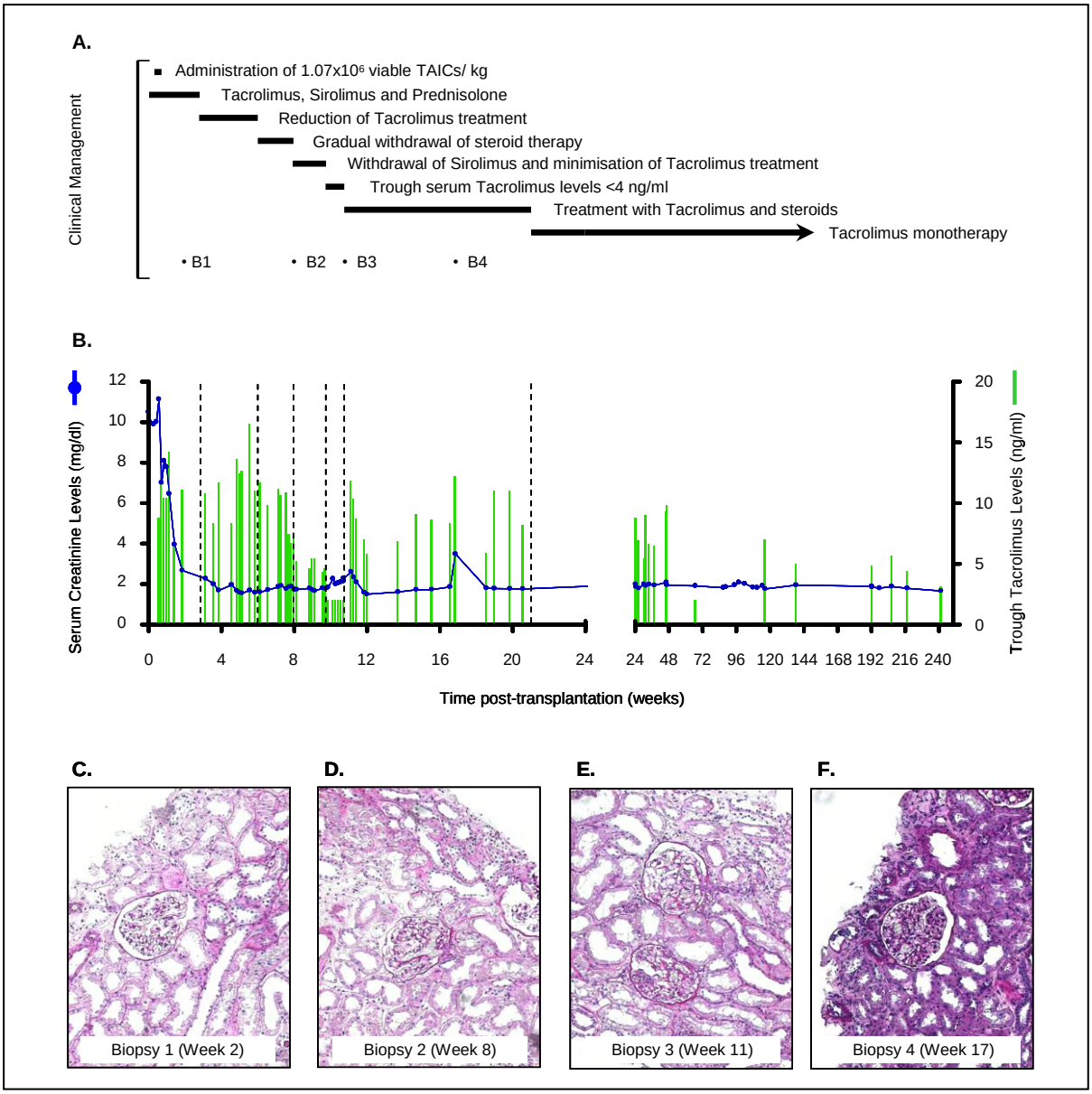

Figure 3.14. Summary of the clinical course of patient KW. *(*A) Patient KW received immunosuppressive therapy as illustrated. Four graft biopsies (B1-4) were taken at the time-points indicated. (B) Serum creatinine levels and trough serum tacrolimus levels. (C-F) PAS-stained sections from graft biopsies taken at week 2 (C), week 8 (D), week 11 (E) and week 17 (F): original magnification 200x.

	Age	Sex	Race	HLA Type	CMV
Recipient KW	36	male	White caucasian	A1 A3 B7 B8 BW6 BW6 CW7 DR1 DR2 DR15 DR51 DQ1 DQ5 DQ3	negative
Donor	57	female	White caucasian	A1 A3 B7 BW6 DR1 DR2 DR15 DR51 DQ1 DQ5 DQ6	negative

Figure 3.15. Patient characteristics of donor and recipient KW.

4. Discussion

4.1. PBMC cells from LRS chambers

To receive significant and meaningful results in experimental assays it is of particular importance to work with reliably substances, this applies especially to experimental trials with viable human cells. The discovery of leucocytes which are a by-product of clinical platelet collection, provides an excellent source for cell experimental purposes. Their quick availability directly after donation and the minimal preparation steps for their isolation are the main advantages compared to conventional cell sources like buffy coats. We demonstrated that these conditions result in high cell quality and viability and therefore present ideal properties for cell culture experiments. Further, it was shown that $CD4^+$ and $CD8^+$ T cells from LRS chambers react intensively to mitogen stimulation in a dose-dependent manner, with a maximal response at approximately 8 µg/ml PHA. Due to the fact that we have seen cytotoxic effects of PHA to some cell subsets in previous experiments, we concluded that a concentration of 2 µg PHA per ml cell culture medium present an optimal lymphocyte proliferation stimulus. Nevertheless, the work with lymphocytes from cones also exhibit disadvantages in comparison to buffy coats: The usage of those cells is still limited because platelet collection with apharesis instruments is normally restricted to clinics with an associated institute for transfusion medicine and further the number of donors is considerably smaller than those for blood donors. In conclusion, PBMC from cones represent a very useful and good quality source for experimental purposes and therefore we preferred in our experiments lymphocytes from cone origin to those from buffy coats.

4.2. TAIC co-culture with allogeneic lymphocytes

Developing TAIC treatment as a clinical option for immunosuppressive therapy in transplantation medicine requires that the function of TAIC cells be better understood and the potential advantage of TAIC cells over conventional immunosuppressive therapies has to be demonstrated.

For more than twenty years, a wealth of very potent chemical and biological immunosuppressants was successfully used in order to prevent acute rejection episodes leading to about a 95% graft-survival one-year posttransplant. Though these immunosuppressive drugs became more and more selective for the defined subsets of immune cells, particularly for T cells, their non-specific way of immunosuppression and the involved consequences like higher risks for tumors and infections are still an unresolved problem. Furthermore these approaches have no bearing on the chronic rejection process and the assertion that acute rejection is a main factor leading to chronic rejection graft dysfunction, an often used argument for high doses immunosuppressive protocols, is now obsolete (Chatenoud, 2008). Therefore, it is obvious that alternatives are badly needed and that over the long-term the induction of tolerance is the goal which is to be achieved. Brem-Exner and colleagues engaged in this topic and detected immunosuppressive and anti-inflammatory properties of TAIC cells in mice (Brem-Exner et al., 2008). Based on these results the effects of human TAIC cells to allogeneic lymphocytes were analyzed in this work.

In the first experiments TAIC cells neither provoked a T cell depleting effect nor a strong proliferative response in co-cultured allogeneic T cells, but did promote allogeneic lymphocyte survival effect under the given conditions. This ability of TAIC cells was not seen in TAIC experiments before and is obviously caused by interaction between TAIC cells and allogeneic lymphocytes. In experiments in the past it was observed that single cultured cell subsets rely on stimuli from other cells to be rescued from death-by-neglect. Otherwise isolated cell subsets lose cell viability constantly over the time how it was observed in control lymphocytes which were not co-cultured with TAIC cells. In conclusion, an immunosuppressive effect of TAIC cells co-cultured with unstimulated allogeneic lymphocytes was not detectable.

Munn et al. already described macrophages with T-cell suppressive properties among others the IDO-expressing subset of macrophages. For the induction of their immunosuppressive features CD40 ligation and IFN-y stimulation were essential (Munn et al., 1996; Munn et al., 1999). It is also assumed that IFN-γ plays a crucial role in the generation of human TAIC cells and further we speculated that the addition of IFN-γ to the co-culture might enhance the immunosuppressive properties

of TAIC cells. Contrary to expectation, the additional administration of IFN-γ did not influence the co-culture and the outcome was the same as we have seen it in co-cultures without IFN-γ.

Based on the previous results and the observation that mouse TAIC cells delete mitogen stimulated T cells more intensively (Brem-Exner et al., 2008) the idea has been evolved that human TAIC cells only exert suppressive properties to allogeneic lymphocytes when those are in an activated state. In order to demonstrate this, co-cultures were treated with PHA. The depletion of lymphocytes after four days in both co-cultures and control cultures is presumably ascribed to the fact that PHA might affect cytotoxic to some lymphocytes. After a certain time, the addition of PHA resulted in a considerable suppression of stimulated allogeneic lymphocytes in TAIC co-culture. Interestingly, this damping effect initially was only seen in the $CD8^+$ lymphocyte fraction. The recognized suppression could be caused by different mechanisms. One possibility might be that TAIC cells are capable to inhibit selectively the proliferation of activated $CD8^+$ lymphocytes. This hypothesis is supported by the fact that nearly same cell numbers of $CD8^+$ lymphocytes remained in co-culture with TAIC cells after PHA stimulation compared to the same lymphocytes co-cultured with TAIC cells alone. A further supporting point is that the flow cytometry dot plots depict clearly an impaired cell proliferation in the $CD8^+$ lymphocyte fraction. Beyond the resulting CFSE- cell fraction in the co-cultures arose evidentially from CFSE+ lymphocytes as a consequence of mitogen stimulation. The fact that these cells are continuously positive stained for CD4 but not for CD8 is yet another argument for the assumption that under these specific conditions, given in the first experiments, TAIC cells selectively suppress the division of activated $CD8^+$ lymphocytes. Nevertheless it is also possible that the observed suppressive effect might be caused by selective elimination of activated $CD8^+$ lymphocytes or even might be a consequence of shortage of culture medium. These possibilities must be considered and therefore it is necessary that further studies dissect the detailed mechanism of the suppressive effect under these conditions.

The preparation of the TAIC cells in the first two experiments did not mirror the circumstances under which clinical grade TAIC cells for the both TAIC-studies were prepared (Hutchinson et al., 2008 a+b). For this reason the experimental used

medium was adapted and most important the TAIC cells were exposed to the harvest procedure. These are two important points which might influence the outcome of an experiment considerably. Macrophages are sensitive responsive to their ambient microenvironment, therefore even little differences in the culture medium might result in phenotypically and functionally modifications (Stout et al., 2004). The cell harvest which was performed with conventional rubber cell scrapers leads demonstrable to an extensive loss of viable and functional TAIC cells. Hence, it is assumable that the immunosuppressive effect would be conspicuously mitigated in comparison to the results seen in the experiments before. We also have to consider a potential influence of the cell debris resulting during the TAIC cell harvest procedure. Probably such an influence is negligible in simplified in-vitro experiments but the administration of a large quantity of allogeneic cell debris into patients obviously represents a strong stimulus for the immune system. Therefore the treatment might accompany by sensitization of the recipient for donor alloantigens which can trigger an acute rejection episode or even a hyper acute rejection provided that TAIC cells are given prior to transplantation. Although so far no case of sensitization or pre-sensitization was observed, it must be aspired to develop a way of harvest without producing such high cell-damage.

A further point which might affect the outcome in comparison to the preceding experiments is the usage of cone lymphocytes. As we demonstrated before the cell quality and viability are considerably higher compared to standard buffy coats which were used in the first experiments. The fact that we worked with the cone lymphocytes directly after their isolation from patients guaranteed that the cells were absolutely fresh. Hence, the work with such cells represents better the conditions how they would be due in vivo.

The observation in the first experiments that TAIC cells lead to an allogeneic lymphocyte survival in co-culture compared to mono-cultured lymphocytes was not confirmed in the ensuing experiments. Though a reduced cell death in co-culture than in control cells was demonstrated, it was shown that TAIC cells were not able to rescue co-cultured allogeneic T cells from death-by-neglect. This finding might be related to the fact that the number of functional TAIC cells was reduced by cell-scrapping. Also the usage of cone lymphocytes may contribute to that observation;

this has to be assumed because even in monoculture the cell death clearly increased compared to the first experiments. However, the most interesting outcome of these experiments is the strong suppressive effect in mitogen stimulated co-cultures. Not only the intensity of suppression was extensively higher in comparison to previous results, but also TAIC cells suppress proliferation of both allogeneic $CD4^+$ and $CD8^+$ T cells in almost the same manner. Further it is concluded that the observed suppressive effect is caused by a selective elimination of activated T cells as fewer lymphocytes were detectable in PHA treated co-cultures than in untreated TAIC co-cultures. As aforementioned T cell-suppressive features were already seen in IDO-expressing subsets of macrophages and dexamethasone-treated macrophages and therefore these properties are not unique to TAIC cells (Munn et al., 1996; Munn et al. 1999). Anyhow, in connection with the quite remarkable results in the TAIC-2 study (Hutchinson et al., 2008 a), the outcome of these experiments demonstrates a potential way of function of TAIC cells, namely that they specifically suppress activated T lymphocytes.

4.3. Case report

This case report describes the first attempt to treat a renal transplant patient with TAIC cells of donor origin. The application of TAIC cells via central venous infusion went without acute complications, including embolism, transfusion reactions, graft-versus-host disease or infection. After a time period of almost five years, no signs of long-term adverse effects like malignancies arising from the transfused cells were detectable. Furthermore there was no evidence that the administration of TAIC cells leads to a sensitization to donor antigens or otherwise accelerates graft rejection. It is quite obvious that is not possible to draw relevant conclusions concerning possible adverse effects from a single case report but such single attempts are the basis for Phase-1 safety trials with a larger number of transplant recipients. Therefore the TAIC-1 and TAIC-2 study was performed, in which a number of 17 patients were treated with TAIC cells and in no case any evidence of acute side effects due to the cell application were registered (Hutchinson et al., 2008 a+b).

In the TAIC-2 study it was shown that the pre-transplantation exposure to donor alloantigen in terms of TAIC cells allows an early withdrawal of conventional immunosuppression. Though, a complete reduction of immunosuppression was not tolerated. It is adopt that TAIC therapy with a suitable immunosuppressant weaning protocol might allow renal transplant patients to accept low-dose tacrolimus monotherapy, but without necessarily tolerating a complete withdrawal of immunosuppressive therapy. Hence, it is concluded that TAIC treatment is responsible for this state of donor-specific hyporesponsiveness (Hutchinson et al., 2008a). Several previous attempts were undertaken to establish immunosuppressive protocols with and without adjunct tolerance-promoting therapies to maintain renal transplant patients on low-dose tacrolimus monotherapy (Bäckman et al.,2006; Coupes et al.,2002; Krämer et al.,2005; Shapiro et al., 2003; Starzl et al., 2003; Tan et al., 2005; Tan et al., 2006; Thomas et al, 2007). From these studies, it is clear that a substantial proportion of renal transplant recipients tolerated a low-dose monotherapy, although there is no way of predicting which patients might tolerate such a regime.

Of course, this single case report can give us no information about the potential efficacy of TAIC treatment in the transplantation setting; particularly not when in previous studies transplant recipients successfully maintained on tacrolimus monotherapy without adjunct immune-conditioning therapy strategies. For this reason further clinical studies with larger numbers of participants will be necessary in order to asses the clinical benefit of TAIC treatment as an adjunct immunosuppressive therapy in renal transplantation. Beyond the lack of adequate control groups, because of ethical reasons in such trials, is a remaining problem and makes it difficult to draw firm conclusions. However, this case contains some intriguing features which deserve comment. Firstly, the graft biopsy taken at week 17 showed an unusual pattern of focal lymphocytic infiltration, which is nearly consistent with the histological appearance in two spontaneously tolerant renal transplant recipients with good graft function described by Burlingham and colleagues (Burlingham et al., 1999). In both cases biopsies shown small focal mononuclear cell infiltrates mainly consisting of $CD4^+$ T cells. Further analysis demonstrated in the case of patient JB that the majority of the focal cells were from recipient origin surrounding a donor-derived cell. The $CD4^+$ T-cell infiltrate contained a high proportion of anti-donor specific T-cell

clones. Therefore it was assumed that the presence of the donor cell might have immune regulatory properties. This adoption was additionally confirmed by studies of focal infiltrates which were seen in long-surviving rhesus monkeys with well-functioning kidney allografts (Kirk et al., 1997; Knechtle et al., 1997). The focal interstitial infiltrates were characterized by aggregates of $CD4^+$ T-cells arranged around a central antigen-presenting cell. For these reasons the described mononuclear cell infiltrates of the presented patient has to be analyzed in more detail with special focus on the origin of the cells and further detection for regulatory T cells would be from great interest.

5. Abstract

Clinical organ transplantation became the therapy of choice for endstage organ diseases. This development was closed related to the history of potent immunosuppressive drugs. Although major progress in the improvement of immunosuppressive therapy regimes was made, long-term graft survival is still limited and recipients are affected due to several adverse effects. For this reason numerous attempts were undertaken to induce operational tolerance. This thesis engaged in an attempt in which a subset of monocytes, named Transplant Acceptance Inducing Cells (TAIC), is given to patients in order to substitute conventional immunosuppressive therapy. The focus of the thesis was the detection of potential immunosuppressive effects to allogeneic lymphocytes induced by TAIC cells in in-vitro co-cultures. Further a case study was performed to demonstrate an interesting outcome of a renal transplant patient who received TAIC cells and to show the clinical feasibility of this treatment.

First experiments indicated that TAIC cells are able to suppress the proliferation of allogeneic $CD8^+$ T cells under co-culture conditions, provided that the T cells are existent in a mitogen activated state. Without the addition of mitogen to co-cultures, TAIC cells promoted the survival of allogeneic lymphocytes. Under changed conditions, which were closer related to previous undertaken clinical TAIC trials, TAIC cells suppressed both $CD4^+$ and $CD8^+$ mitogen stimulated allogeneic lymphocytes. In absence of mitogen stimulation TAIC cells were not able to rescue

allogeneic lymphocytes from death by neglect. Although these results apparently demonstrate immunosuppressive properties of TAIC cells adverse activated allogeneic lymphocytes, it is obligatory to identify the exact mode of function of these cells in order to distinguish them clearly from other macrophage subsets with immunoregulatory features.

The described renal transplant patient in the case study received TAIC cells afterwards the transplantation as an adjunct immunosuppressive therapy within an alternative healing attempt. The infusion of TAIC cells proceeded without any complications and the subsequent reduction of immunosuppressants up to a low grade tacrolimus monotherapy was well tolerated by the patient for the almost last five years. A biopsy, which was taken in the 17th week after transplantation, showed an unusual pattern of focal lymphocytic infiltration how it was already seen in spontaneously tolerant kidney transplant patients.

Both the undertaken experiments and the described case study in this thesis demonstrate that TAIC cells have immunosuppressive characteristics which might have the potential to become a feasible immunosuppressive therapy approach in the clinic. But therefore it is necessary that further studies depict the specific mode of function of those cells and that meaningful clinical trials definitely prove the beneficial effect of TAIC cells in transplant patient. Furthermore it is needed that long-term experiments exclude potential adverse effects.

6. Appendices

6.1. Bibliography

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6.2. Abbreviation list

APC, antigen presenting cell
ATRA, all-trans-retinoic acid
BD, blood dose
°C, degree Celsius
CD, cluster of differentiation
CFSE, carboxyfluorescein succinimidyl ester
CFU, colony forming unit
Cl_{CR}, creatinine clearance
cm^2, square centimetre
CMP, common myeloid progenitor
CMV, cytomegalovirus
CSF, colony stimulating factor
DC, dentritic cell
dl, decilitre
DPBS, Dubelcco`s phosphate-buffered saline
DSS, dextran sulphate sodium
ESC, embryonic stem cell
FACS, fluorescence activated cell sorting
FcR, Fc receptor (e.g. FcγRI)
FITC, fluorescein isothiocyanate
FOXP3, forkhead box P3
g, gram
GM-CFU, granulocyte-monocyte CFU
GMP, good manufacturing practice
GRA, granulocytes
G6PD, glucose-6-phosphate dehydrogenase
HABS, human AB serum
HEPES, N-2-hydroxyethylpiperazine N´-2 ethanesulfonic acid
HLA, human histocompatibility leukocyte antigen
IDO, indoleamine 2,3-dioxygenase
IFN-γ, interferon gamma
Ig, immunoglobulin
IL-, interleukin
LPS, lipopolysaccharide
LRS, leucocyte reduction system
LYM, lymphocytes
MACS, magnetic cell sorting
M-CFU, monocyte CFU
M-CSF, macrophage CSF
MdCs, monocyte-derived cells
mg, milligram
MHC, major histocompatibilty complex
ml, mililiter
mM, milimolar
MON, monocytes
M reg, regulatory macrophage
µg, microgram
µl, microliter
µm, micrometer
n, sample size
ng, nanogram
ns, non-significant
OD, oral dose
PAF, platelet-activating factor
PBMC, peripheral blood mononuclear cell
PE, phycoerythrin
PHA, phytohemagglutinin

PLT, platelets

p.o., per os

RBC, red blood cells

rpm, revolutions per minute

TAIC, Transplant Acceptance Inducing Cell

TCR, T cell receptor

TLR, Toll-like receptor

TNF, tumor necrosis factor

T reg, regulatory T cell

U, unit

WBC, white blood cells

7-AAD, 7-Amino-actinomycin D

6.3. Acknowledgement

I would like to take this opportunity to express my gratitude to all those people who contributed, directly or indirectly, to this work.

My very sincere thanks to Prof. Dr. Fred Fändrich for giving me the possibility to work in project under supervision of Dr. Dr. James Hutchinson. I am really indebted to Dr. Dr. James Hutchinson for his excellent mentoring, teaching and support throughout the entire thesis.

Furthermore I would like to thank Prof. Dr. Hendrik Ungefroren, Dr. Paloma Riquelme, Martina Matthäi and Ingrid Berg who always offered me advice and support.

Special thanks also to my family for their strong support.

6.4. Curriculum vitae

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Education

1991-1995	Grundschule Damme
1995-1997	Orientierungsstufe Damme
1997-2004	Gymnasium Damme

Medical School

2004-2006	Preclinical part at the Christian-Albrechts-Universität, Kiel
2006-2010	Clinical part at the Universitätsklinikum Schleswig-Holstein,Kiel
2007-2010	Scientific collaboration in the Transplantation research group at the Universitätsklinikum Schleswig-Holstein,Kiel
2008	Participation at the ITN-RISET Symposium on “Biomarkers in Transplantation”, Guy’s Hospital, London
2010	Participation at the International Research Conference, Medical School Sechenov, Moscow

Honors

2008	Grant for the participation at the ITN-RISET Symposium on “Biomarkers in Transplantation”, Guy’s Hospital, London
2009	Finalist for the MLP Medical Excellence Grant - Category: Science Excellence
2010	Grant for the participation at the International Research Conference, Medical School Sechenov, Moscow
	Recipient of the Pirogov Medal for the best dissertation, Medical School Sechenov, Moscow

6.5. Publications

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