**In vitro** treatment of dendritic cells with tacrolimus: impaired T-cell activation and IP-10 expression

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**Abstract**

**Background.** High doses (10⁻⁶–10⁻⁸ M) of tacrolimus (FK506) were reported to induce a type-2 T-helper cell (Th2)-promoting function in developing dendritic cells (DC). We used a therapeutic dose (2.4 × 10⁻⁹ M) of tacrolimus to investigate its effect on human monocyte-derived DC.

**Methods.** Using untreated and treated immature and mature DC we compared T cell-activating capacity, surface marker expression, T cell and DC cytokine profile and transcription of genes coding for a panel of DC function-related molecules.

**Results.** Tacrolimus-treated mature DC had reduced T-cell stimulatory capacity. Although interleukin (IL)-12 production of DC was impaired, they did not promote Th2 development as T cells activated by tacrolimus-treated DC produced less interferon-γ (IFN-γ), IL-4 and IL-10. The up-regulation of the T-cell activation marker CD69 and the production of IL-2 were impaired. In addition, tacrolimus-treated DC produced less IP-10 (CXCL10), which is known to be involved in allograft rejection. Other molecules related to DC function remained unchanged.

**Conclusions.** Tacrolimus treatment reduces the ability of DC to stimulate T cells and the impaired production of DC-derived IP-10 (CXCL10) and IL-12 might play a role in the immunosuppressive action of tacrolimus.

**Keywords:** chemokines; dendritic cells; immunosuppression; T lymphocytes; transplantation

**Introduction**

The major role of immature dendritic cells (DC) is to immunologically monitor the environment for invading pathogens. DC take up antigen, process it and load it onto their major histocompatibility complex (MHC) molecules for a later presentation to T cells [1,2]. In the steady state, these cells traffic through tissues and die in the lymphoid organs [3]. When an additional stimulus arising from the innate immune system is encountered, e.g. during inflammatory events, a maturation process is initiated, which induces DC to migrate via the lymphatic ducts or blood vessels to lymph nodes and spleen, respectively. These mature DC are now able to selectively activate naïve resting antigen-specific T cells [4–7]. It has been postulated that during transplantation, immature DC are constantly trafficking through tissues and phagocytosing apoptotic cells. If DC from the recipient pick up graft-derived cells or debris and also encounter a maturation stimulus, these DC will go to the lymph node and stimulate immunity to donor-derived peptides [8]. It seems, therefore, that modulating DC might be a desirable goal for immunosuppressive therapy following transplantation.

The second-generation calcineurin inhibitor, tacrolimus, is converted to active conformation by forming complexes with the cytoplasmic FK-binding protein [9]. These complexes bind to calcineurin, a phosphatase that dephosphorylates nuclear regulatory proteins, hence facilitating their passage through the nuclear membrane. Inhibition of calcineurin thereby impairs the expression of T-cell activation genes, including those for interleukin (IL)-2 and interferon (IFN)-γ as well as their receptors. In addition, tacrolimus enhances the expression of transforming growth factor-β, which inhibits IL-2-stimulated proliferation and cytotoxic T-lymphocyte generation. As opposed to the well-described effects of tacrolimus on T cells, it is largely unknown whether and how this drug affects DC. There
are few reports showing that tacrolimus inhibits DC function. Shimizu et al. [10] reported that CD34+-precursor-derived DC treated with high doses of tacrolimus skew T-cell responses from type-1 T-helper cell (Th1)- to Th2-type response. Szabo et al. [11] showed inhibition of the allostimulatory capacity and cytokine production of immature human myeloid DC grown in the presence of tacrolimus. Matsue et al. [12] reported that in a transgenic T-cell receptor mouse model, tacrolimus inhibits changes resulting from intercellular signaling in both DC to T cell and T cell to DC directions. Woltman et al. [13] reported little effect of high doses of tacrolimus on DC.

In this study we investigate the action of tacrolimus in therapeutic doses on immature and mature monocyte-derived DC and report impaired T-cell stimulatory capacity and changes in the mRNA expression profile of DC generated in the presence of tacrolimus.

**Subjects and methods**

**Generation of CD14+- monocyte-derived DC**

Human DC were prepared from peripheral blood CD14+-monocytes (isolated from whole blood or leukocyte removal filters), essentially as described previously [14,15]. Monocytes were obtained by isolating CD14+ cells by Ficoll gradient centrifugation and magnetic sorting (MACSTM; Miltenyi Biotec, Bergisch-Gladbach, Germany) or by depleting T-cells by E-rosetting. Briefly, 1.5 × 10^6–2 × 10^6 cells/well are plated in 6-well tissue culture plates in 3 ml complete culture medium (Strathmann Biotech, Hamburg, Germany), supplemented with 50 μg/ml gentamycin, 10% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel), supplemented with 1600 U/ml GM-CSF and 1000 U/ml IL-4 (Strathmann Biotech, Hamburg, Germany). Culture medium was renewed every other day by adding the removed amount at feeding times.

Tacrolimus concentration was 2.4 nmol/l (2 ng/ml) during the culture period of 2 days in the absence (immature DC) or presence (mature DC) of a maturation cocktail consisting of 10 ng/ml IL-1β (Preprotech EC, London, UK), 10^3 U/ml IL-6 (Strathmann Biotech, Hamburg, Germany), 10 ng/ml tumour necrosis factor (TNF)-α (specific activity 6 × 10^7 U/mg; generous gift of Dr G.R. Adolf, Bender, Vienna, Austria) and 1 μg/ml Prostin E2 (Pharmacia & Upjohn, Buurs, Belgium). Tacrolimus concentration was 2.4 mmol/l (2 ng/ml) during the complete culture period and kept at this concentration by adding the removed amount at feeding times.

**Isolation of T cells**

Bulk T cells were isolated from the rosettes that had formed with neuraminidase-treated sheep red blood cells during the monocyte isolation procedure by lysing the sheep red blood cells with ammonium chloride, as described previously [16]. Naïve T-cells were enriched from bulk T cells using negative selection by panning with antibodies against CD8, CD14, CD16, CD19, CD45RO and CD56. FACS staining of the enriched population with anti-CD45RA showed at least 80% positive cells.

**Flow cytometry**

Fluorescence-labelled primary antibodies were from BD-Pharmlingen, San Diego, CA (CD40, CD80, CD86, HLA-ABC, HLA-DR and CD69), DAKO A/S, Glostrup, Denmark (CD11c) or Immunotech/Coulter/Beckman, Fullerton, CA, USA (CD83). Specimens were analysed on a FACScalibur instrument using CellQuest software (BD Immunocytometry Systems, San Jose, CA, USA).

**Cytospin preparations**

Cytospin preparations were either used immediately or stored dessicated at –80°C and thawed at room temperature before use. The cells were fixed by incubating in acetone for 5 min followed by air drying and pre-incubation with phosphate-buffered saline containing 1% bovine serum albumin for 5 min. Immunocytochemistry was performed with the DC-LAMP (CD208) specific antibody (Beckman-Coulter, Fullerton, CA, USA). Antibody binding was visualized using biotinylated species-specific sheep anti-mouse immunoglobulin (Amersham International, Amersham, UK) followed by a streptavidin–Texas Red™ conjugate (Molecular Probes).

**Mixed leukocyte reaction**

Graded doses of DC derived from monocytes (1 × 10^5, 3 × 10^5, 1 × 10^6, 3 × 10^6 and 1 × 10^7) were co-cultured with 1.5 × 10^3–2 × 10^5 allogeneic bulk peripheral blood T cells [6 days, proliferation measured by 3H-thymidine incorporation at 4 μCi-148KBq/ml [3H]TdR (specific activity 247.9 GBq/mmol = 6.7 Ci/mmol; New England Nuclear, Boston, MA, USA) over the last 16 h] in flat bottom 96-well microtitre plates (no. 3072; Falcon Labware, Oxnard, CA, USA) in 200 μl culture medium.

**Cytokine analyses**

To measure cytokines produced by T cells, 2.5 × 10^5 allogeneic mature DC or tacrolimus-treated mature DC were co-cultured with 1 × 10^6 human CD45RA+ T cells. After 6 days of DC–T cell co-culture, supernatants were analysed for IFN-γ, IL-4 and IL-10.

To measure cytokines produced by DC, DC were co-cultured with murine myeloma cells transfected with the human CD154/CD40-ligand molecule (P3 × TBA7 cells) (1 × 10^6 DC and 0.5 × 10^6 allogeneic bulk peripheral blood T cells) in 12 well plates (no. 3072; Falcon Labware, Oxnard, CA, USA) in 1 ml culture medium.

**Intracellular cytokine staining analysis**

CD45RA+ naïve T-cells were co-cultured with DC for 5 days (see above). During the last 4 h, 25 ng/ml PMA (Sigma
Aldrich), 1.5 μg/ml ionomycin (Sigma I-0634) and 10 μg/ml Brefeldin A (Sigma B-7651) were added. Cells were lysed and stained using the FIX&PERM cell permeabilization kit (GAS-002; An der Grub, Kaumberg, Austria) and Fastimmune FITC/PE anti-human IFN-γ/IL-4 and γ2a/γ1 control antibodies and PE anti-human IL-2 antibody and γ1 control (BD Biosciences, San Jose, CA, USA). The percentage of positive cells was evaluated on a FACScalibur instrument using CellQuest software (BD Immunocytometry Systems, San Jose, CA, USA).

Quantification of RNA expression
Quantitative polymerase chain reaction (PCR) analysis was performed using real-time PCR (Abi Prism 7700 sequence detector; Applied Biosystems, Vienna, Austria). Sequences for probes (FAM/TAMRA label) and primers (synthesized by Microsynth) specific for individual mRNA molecules were selected using the Primer Express software (Applied Biosystems) and are listed in Table 1. For PCR, the TaqMan PCR master mix from Applied Biosystems was used. Random-primed cDNAs were prepared (Superscript II RNase H–reverse transcriptase; Life Technologies, Vienna, Austria) from total RNA isolated from immature and mature, treated and untreated DC.

Intracellular chemokine staining analysis
Cells were lysed and stained using the FIX&PERM cell permeabilization kit (GAS-002; An der Grub, Kaumberg, Austria) and anti-IP-10 PE (BD-PharMingen, San Diego, CA, USA) and γ2a PE (BD Biosciences, San Jose, CA, USA) control antibodies. Evaluation was with a FACScalibur instrument using CellQuest software (BD Immunocytometry Systems, San Jose, CA, USA).

Results
All experiments shown are representative of at least six experiments with similar results, unless otherwise reported.

Surface marker expression and maturation markers are not changed by tacrolimus
DC derived from CD14+ monocytes were harvested on days 8 or 9 after continuous treatment with tacrolimus and either stimulated to maturation or not during the last 48 h. Cell numbers did not vary between cultures grown without or with tacrolimus. The expression of surface markers indicating DC specificity (CD11c) and maturation (up-regulation of CD80, CD83, CD86, MHC-I and MHC-II) were not altered on tacrolimus-treated DC (Figure 1). This result was confirmed by staining of cytopsin preparations of untreated and tacrolimus-treated DC with the novel intracellular maturation marker DC-LAMP (CD208) (DC lysosome-associated membrane glycoprotein) (data not shown) [18].

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<tr>
<th>Table 1. Primer and probe sequences for real-time PCR analyses</th>
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<tr>
<td><strong>Forward primer</strong></td>
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<tr>
<td>TGGTGGCCATCCTTGTCC</td>
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<td>TGCGTCTTGCTCG GATTG CAAGGTCCCCAGCTCCCTAAGTCAGGA</td>
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T-cell stimulatory capacity of tacrolimus-treated DC is reduced

Figure 2 shows the result of a mixed leukocyte reaction (MLR) with graded doses of DC as stimulators and bulk T-cells as responders. Although the expression level of maturation markers and co-stimulatory molecules were unchanged on the tacrolimus-treated (2.4 nmol/l) mature DC, the T-cell stimulatory capacity was reduced and more so when DC were treated with 1 μM tacrolimus. This reduction was donor-dependent in magnitude; the MLR shown is an intermediate result. To exclude a possible carry over effect of tacrolimus into the MLR reaction, we suspended untreated mature DC in the supernatant of the last wash of DC derived from monocytes in the presence of tacrolimus and performed an MLR as above. No effect on T-cell proliferation was observed (data not shown).

IL-12 p70 production by mature DC is reduced

IL-12 present at the time of primary T-cell stimulation is responsible for the shift towards Th1 differentiation. DC were the primary source of IL-12 in our setting. CD40-stimulated DC treated with tacrolimus produced lower amounts of IL-12 p70 than untreated control DC (Figure 3). IL-10 levels produced by mature DC in the presence of CD40 ligand were very low and if measurable levels were obtained, they were diminished by tacrolimus treatment (data not shown).

IFN-γ, IL-4 and IL-10 production by T cells stimulated with tacrolimus-treated DC is reduced

When CD45RA+ naive T-cells are co-cultured with allogeneic DC, they are stimulated to become CD69+ responder T-cells, which are either of the IFN-γ-producing Th1-type or the IL-4-producing Th2-type. Under certain conditions, DC can also shape T cells to become IL-10-producing regulatory or suppressor
T-cells [19]. We collected the supernatants of such co-cultures and measured the cytokine contents. In Figure 4A, the IFN-γ, IL-4 and IL-10 levels are depicted. T cells stimulated by tacrolimus-treated DC produced lower levels of IFN-γ. Skewing towards the Th2-type should result both in a lower IFN-γ production and an elevated IL-4 production. Therefore, in the same supernatant the IL-4 content was measured. Interestingly, we detected that also IL-4 levels were diminished in the DC–T cell co-culture supernatants. The same results were obtained when IFN-γ and IL-4 were measured by intracellular FACS analysis (Figure 4B). To test whether the tacrolimus-treated DC induce regulatory T-cells, we measured the IL-10 content in the same supernatant (Figure 4A): both mature and immature DC treated with tacrolimus induced lower IL-10 secretion by T cells than untreated control cells. Taken together, these results imply that stimuli provided by tacrolimus-treated DC are not able to skew the T-cell response towards a defined type.

**IL-2 production and CD69 expression of T cells stimulated with tacrolimus-treated mature DC are reduced**

IL-2 is the autocrine growth factor for T cells and CD69 is an early activation marker. We measured IL-2 production and CD69 surface expression of T cells after co-culture with tacrolimus-treated mature DC. Both activation-indicating molecules were clearly reduced when compared with T cells stimulated with untreated mature DC (Figure 5).

**Tacrolimus down-regulates IP-10 (CXCL10) expression in immature DC**

To investigate in more detail the molecular basis of the immunosuppressive action of tacrolimus on DC, we analysed the mRNA expression levels of a panel of chemokines and other molecules known to have an impact on DC function. From all mRNAs analysed (Table 1), only mRNA coding for IP-10 (CXCL10) was influenced by tacrolimus treatment. IP-10 (CXCL10) mRNA was down-regulated during the maturation of untreated DC. With tacrolimus treatment, the expression of IP-10 (CXCL10) mRNA in immature DC was reduced to the level of mature DC, while mRNA expression of mature DC was further reduced (Figure 6A). Measuring IP-10 (CXCL10) protein by intracellular chemokine staining in DC confirmed the mRNA data: tacrolimus-treated immature DC contained less IP-10 than untreated immature DC (Figure 6B).

Less pronounced changes in mRNA expression induced by tacrolimus were found for mRNA coding for Dectin-1 and DEC205 (CD205). Dectin-1 mRNA was reduced to 50% by tacrolimus. DEC205 mRNA up-regulation during maturation was 70% of untreated levels (data not shown).

**Discussion**

The survival of allografts is largely dependent on the suppression of alloreactive immune responses. This is
partially accomplished by the administration of tacrolimus post-transplantation. It is known that tacrolimus inhibits the effector cells (i.e. T cells) of such alloresponses [9]. Recently, it has been shown that also the cells that initiate such alloresponses (i.e. DC) are severely affected by tacrolimus treatment. The effects described include an impaired immunostimulatory capacity of tacrolimus-treated DC and the outcome of T cells of Th2-type after co-culture with DC. However, these data were obtained with immature monocyte-derived DC [11], which are not the optimal immunostimulatory DC, and on DC derived from CD34+ haematopoietic progenitor cells [10]. Other authors used doses widely exceeding those used for therapeutic purposes [13].

We show here that the impaired T-cell stimulatory function and cytokine production of monocyte-derived DC can be achieved with therapeutic doses (2.4 nM) of tacrolimus (Figure 2) and is neither due to lack of MHC nor due to reduced expression of the co-stimulatory molecules CD40, CD80 or CD86. The Th type-1-inducing IL-12 production of CD40 ligand-stimulated mature DC is diminished by tacrolimus treatment (Figure 3). The Th type-2-promoting IL-10 production is very low in CD40-stimulated mature DC and, if detectable, is further diminished by tacrolimus treatment. These data indicate that the immunosuppressive effect of tacrolimus is not due to a shift from Th1-type towards Th2-type immune response.

The production of the autocrine growth factor IL-2 as well as the early activation marker CD69 on T cells co-cultured with tacrolimus-treated mature DC are reduced as compared with T cells activated by untreated mature DC (Figures 5A and 5B). Recently, regulatory T-cells, which do not proliferate and do not produce the autocrine growth factor IL-2, have been described to produce high levels of IL-10 but not IFN-γ or IL-4 [20]. Since IL-10 production of T cells after co-culture with tacrolimus-treated DC is not elevated, the impaired immunostimulatory function observed in the allogeneic MLR is not due to induction of such IL-10 producing regulatory T-cells.

In addition to the cytokines known to be involved in the control of T-cell stimulation, chemokines are emerging to be as important in directing immune responses. One chemokine reported to be involved in the allograft rejection is IP-10 (CXCL10). Hancock et al. [21] reported that when allografts from IP-10 (CXCL10)-deficient mice are transplanted to wild-type recipients, the allograft survives for >40 days. In real-time PCR analyses, we show that among a panel of chemokines and other molecules related to DC functions (Table 1), the level of IP-10 (CXCL10) expressed by DC is markedly reduced by tacrolimus treatment (Figure 6A) and confirmed this finding at the protein level by intracellular IP-10 (CXCL10) staining (Figure 6B). IP-10 (CXCL10) was reported to be selectively induced after transplantation and is also induced by mechanical injury and ischaemia/reperfusion injury to blood vessels [21]. The effects of IP-10 (CXCL10) are thought to involve IFN-γ production of natural killer cells [22], T-cell activation and cytokine production [23], T-cell homing and recirculation [24] and development of multiple effector cell pathways [25]. Although the influence of tacrolimus on other immunomodulatory molecules remains to be tested, the reduced IP-10 (CXCL10) production of immature DC could lead to the impairment of these mechanisms in patients treated with tacrolimus. This could account for parts of the immunosuppressive action of tacrolimus. It remains to be investigated, however, whether tacrolimus affects the IP-10 (CXCL10) production of other leukocytes as well.
Minor but constant \((n = 6)\) reduction in mRNA expression by tacrolimus treatment was also found for Dectin-1 and DEC205. Dectin-1 binds \(\beta\)-glucans, a conserved structure present in the cell walls of plants, fungi and bacteria, representing a classical pathogen-associated molecular pattern. However, Dectin-1 also binds to the surface of \(T\) cells in a \(\beta\)-glucan-independent fashion. Dectin-1 binding promotes \(T\)-cell proliferation in the presence of anti-CD3 antibodies, i.e. it has co-stimulatory activity [26].

DEC205 is a receptor for adsorptive endocytosis which is implied in a very efficient delivery of its ligands to the antigen-processing machinery of \(DC\), thereby facilitating the initiation of primary immune responses [27,28]. Recently, it has been shown that this very efficient delivery system is used to induce antigen-specific tolerance under steady-state conditions, so that during inflammation or infection, when \(DC\) present both self-antigens with non-self, the immune system can focus on the pathogen-specific induction of immune responses, thereby avoiding autoimmunity [29].

Taken together, our findings allow for a new consideration of the effects of tacrolimus on \(DC\), suggesting a more pronounced impairment of the \(T\)-cell stimulatory capacity rather than a redirection from \(Th1\)-type vs \(Th2\)-type immune response. These effects might contribute to the reduction in allograft rejection as seen in clinical studies with tacrolimus therapy [30,31].

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Conflict of interest statement. None declared.

References


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