Modulation of immune tolerance: the role of tolerogenic dendritic cells and TNF
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CHAPTER

An optimized CFSE-based T-cell suppression assay to evaluate the suppressive capacity of regulatory T-cells induced by human tolerogenic dendritic cells

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Abstract

In autoimmune diseases or transplant graft rejection a therapy that will prevent or reduce the present immune activation is highly desired. *Ex vivo* generated tolerogenic dendritic cells (DCs) are considered to have a strong potential as cellular therapy for these diseases. One of the mechanisms of immune suppression mediated by tolerogenic DCs is the induction of regulatory T cells (Tregs). Consequently, the efficacy of such DCs to induce Tregs will reflect their tolerogenic capacity. Because no specific markers have been described for human induced (i)Tregs yet, the Tregs can only be appreciated by functionality. Therefore, we have optimized an *in vitro* suppression assay to screen for human DC-induced-Treg activity. IL-10-generated tolerogenic DCs were used to induce Tregs that were previously shown to effectively suppress the proliferation of responder T cells stimulated with allogeneic mature DCs (mDCs). Our results show that the suppressive capacity of IL-10 DC-induced Tregs measured in the suppression assay increases with the iTreg dose and decreases with higher numbers of antigen presenting cells (APCs) as T cell stimulation. Lowering the ratio between responder T cells and stimulator mDCs present in the coculture clearly improved the read-out of the suppression assay. Furthermore, mDC-primed T cells in the suppression assay were shown to be an essential control condition. In conclusion, we recommend titrations of both APCs and iTregs in the suppression assay and to include a negative control condition with T cells primed by mDCs, to distinguish specific and functional suppression by iTregs from possible generalised suppressive activity.
Introduction

In many diseases, like in autoimmune diseases or transplant graft rejection, the immune system has shifted towards an undesired status of immune activation. Therapies that prevent induction of immune activation or reduce immune activation are explored to combat disease or graft rejection and to increase the patient's wellbeing. DCs are highly specialized professional antigen presenting cells. In recent years it has become clear that DCs not only induce immunogenic responses, but can also initiate tolerogenic responses.\(^1\)-\(^3\) In this respect, DCs play an important role for the maintenance of immune homeostasis. The tolerogenic properties of DCs can be mediated via soluble factors, like cytokines, via cell contact-dependent mechanisms, like inhibitory receptor-ligand interactions, or via impaired costimulation. Tolerogenic DCs induce anergy or apoptosis of effector T cells and/or expand or activate regulatory T cells.\(^2\),\(^4\),\(^5\) For these reasons, ex vivo generated tolerogenic DCs are considered as potential cellular vaccines to treat autoimmune disorders or to prevent undesired immune responses against allogeneic transplants.

Human tolerogenic DCs can be cultured in vitro from DC precursors using different compounds. Often used for this purpose are the anti-inflammatory cytokine IL-10, the active form of vitamin D\(_3\) or analogues of vitamin D\(_3\), and anti-inflammatory/immunosuppressive drugs like corticosteroids, rapamycin, calcineurin inhibitors and aspirin.\(^6\)-\(^8\)

For DCs that are generated for tolerance inducing therapy, their capacity to induce Tregs is preferred more than induction of anergy or apoptosis of effector T cells. The reason for this is that DC-induced Tregs can in turn induce a more widespread tolerance via secretion of IL-10, TGF\(\beta\) and/or granzymes and perforin, via expression of inhibitory molecules and via competition for growth factors.\(^9\)-\(^11\) In addition, Tregs may influence the memory response of T cells.\(^12\) A read-out system for the reliable measurement of Treg induction by tolerogenic DCs is therefore of great importance. In the murine system, Tregs can be identified by a combination of markers, e.g. CD4, CD25 and FOXP3. In the human system several markers have been described for naturally occurring Tregs, e.g. CD25\(^{hi}\), CD127\(^{lo}\), FOXP3, cytotoxic T lymphocyte antigen (CTLA)-4 and GITR.\(^9\),\(^10\),\(^13\) CD4\(^{+}\)CD25\(^{hi}\) iTregs are characterized by the same markers and resemble the naturally occurring Tregs, but are generated in the periphery in an antigen-specific manner.\(^14\) None of these markers however, are specific for human Tregs, as they are also expressed by activated T cells. Different types of iTregs have been described of which T regulatory type 1 cells (Tr1) and T helper 3 cells (Th3) are the best studied. These iTregs arise after antigen-specific stimulation, in the presence of IL-10 or TGF\(\beta\), respectively. They are characterized by production of IL-10 and/or TGF\(\beta\) and regulate immune responses through the secretion of these cytokines.\(^9\),\(^15\)-\(^18\) A unique phenotype that defines all the different types of iTregs has not yet been identified. Therefore, definitive characterization of Treg induction by tolerogenic DCs still requires an in vitro suppression assay as a functional read-out.
Various *in vitro* suppression assays have been described but the setup of these assays is often variable and poorly defined. Therefore, we set out to develop a robust and clearly defined suppression assay. Often the assay for analysis of Treg suppressive capacity consists of a coculture setup of a constant number of responder T cells, which are antigen-specific or polyclonal stimulated, and a titration of Tregs. Responder T cell proliferation is measured by \(^{3}H\)-thymidine incorporation or CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester) dilution of proliferated cells.\(^{19,21}\) The information obtained from \(^{3}H\)-thymidine assays, however, is limited as the proliferation of cells is only determined during the final 16 hours of culture. CFSE dilution as a read-out mechanism allows longer monitoring, while the expression of surface molecules or cytokine production of proliferated cells can be simultaneously determined.\(^{20,21}\) Therefore, in this study, we have optimized an assay using the fluorescent dye CFSE. Furthermore, we have chosen to use APCs in stead of a polyclonal stimulation in our setup to mimic the *in vivo* situation more closely as Tregs can suppress T cell responses both in a direct way or an indirect way via APCs.\(^{10,11}\) This APC-mediated indirect way would be missed in a suppression assay with a polyclonal stimulus. As tolerogenic DCs to induce Tregs we used IL-10-treated DCs.

During optimizing the suppression assay for human tolerogenic DC-induced Tregs, we found that the number of APCs for T cell stimulation in a suppression assay is important for optimal read-out of suppression by DC-induced Tregs. Furthermore, we show the importance of including T cells primed by mDCs in suppression assays as a negative control condition, because it is essential to discriminate specific from aspecific suppression by DC-primed T cells. Longer T cell priming results in less specificity of the assay due to aspecific suppression in the negative control condition.

**Materials and methods**

**Reagents and antibodies**

CellGro DC medium, IL-4, IL-1β and TNFα were all obtained from CellGenix (Freiburg, Germany). GM-CSF was either obtained from CellGenix or from Berlex (Leukine) (Seattle, USA). PGE\(_2\) was obtained from Sigma-Aldrich (Steinheim, Germany). IL-10 was obtained from PeproTech (Rock Hill, USA). For read-out assays Iscove’s Modified Dulbecco’s Medium (IMDM, Bio Whittaker, Verviers, Belgium) was used supplemented with 10% FCS (Bodinco, Alkmaar, The Netherlands), 20 μg/ml human transferrin, 50 μM 2-mercaptoethanol (Sigma-Aldrich), 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco, Merelbeke, Belgium). All monoclonal antibodies (mAbs) used for flow cytometry were obtained from Becton Dickinson (BD Biosciences, San Jose, USA).

**Isolation of monocytes from healthy donors**

Peripheral blood mononuclear cells (PBMCs) were isolated via separation over a Lymphoprep gradient (\(d = 1.077\) kg/L, Axis-Shield PoC AS, Oslo, Sweden) from fresh aphaeresis material (Sanquin Blood Bank North West, Amsterdam, The Netherlands).
Netherlands), obtained from healthy volunteers upon informed consent. Monocytes were isolated from the PBMC fraction by positive selection using CD14 microbeads and a magnetic cell separator (MACS, Miltenyi Biotec, Bergisch Gladbach, Germany). Alternatively, monocytes were isolated from fresh aphaeresis material by separation using the Elutra™ cell separation system (Gambro, Lakewood, USA).

**Culture of monocyte-derived dendritic cells**

Monocytes were cultured at a concentration of 0.5 x 10^6 cells/ml in a 6 well plate (Nunc, Roskilde, Denmark) in CellGro serum-free medium supplemented with IL-4 (800 IU/ml) and GM-CSF (1000 IU/ml). At day 6, the immature DCs (imDCs) were either left untreated, or matured with IL-1β (10 ng/ml), TNFα (10 ng/ml) and PGE₂ (1 µg/ml) (mature (m)DCs), or alternatively activated by a 1 hour pre-incubation of IL-10 (40 ng/ml) followed by the maturation cocktail (IL-1β, TNFα and PGE₂) (IL-10 DCs). After 2 days of maturation, the cells were harvested, extensively washed and analysed.

**Flow cytometric analysis**

For phenotyping, the DCs were washed with PBS containing 0.5% bovine serum albumin (PBA) and incubated with 50 µl mAb or appropriate isotype controls diluted in PBA with 3 mg/ml human gamma globulin for 30 minutes in the dark at 4°C. Cells were washed and resuspended in PBA. DAPI (Sigma-Aldrich) was added to the cells before analysis to assess cell viability and exclude dead cells from analysis. Cells were analysed on an LSRII flow cytometer (BD Biosciences) and analysed with FACS Diva software (BD Biosciences).

**Cytokine production of dendritic cells**

DCs were harvested, washed and cultured in 96 well flat bottom plates (Nunc) at a concentration of 1 x 10^4 cells/ well in culture medium. To mimic the interaction with CD40 ligand (CD40L)-expressing T helper cells, irradiated CD40L-transfected J558 cells were added at a concentration of 5 x 10^4 cells/ well. After 24 hours of stimulation, the supernatant was harvested. The production level of IL-6, IL-10, IL-12p70 and TNFα was determined by ELISA. For the detection of IL-6, IL-10 and TNFα the PeliKine-compact ELISA kit was used (Sanquin Reagents, Amsterdam, The Netherlands). For the detection of IL-12p70 a combination of BT-21 mAb (Diaclone, Besançon, France), recognizing the p35 subunit, and C8.6 mAb (BD Biosciences), recognizing the p40 subunit, was used in an ELISA.

**Isolation of naïve and memory CD4⁺ T cells**

Naïve CD4⁺CD45RA⁺CD45RO⁻ T cells were purified from PBMCs or from the T cell containing fraction of Elutra™ using CD4 T cell isolation kit II (Miltenyi Biotec), together with mAb CD45RO-PE and anti-PE beads (Miltenyi Biotec). Naïve T cells were used as responder T cells in an MLR and for priming to evaluate suppressive capacity. Memory CD4⁺CD45RA⁻CD45RO⁺ T cells were used as responder T cells in an MLR and in the suppression assay. Purities of T cell subsets were routinely around 92% for naïve and memory T cells.
Mixed lymphocyte reaction (MLR)

DCs were used to stimulate allogeneic memory or naïve CD4⁺ T cells. Varying numbers of DCs were co-cultured in 96 well flat bottom plates (Nunc) with 5 x 10⁴ T cells/well in culture medium. After 5 days of culture, 0.2 μCi [³H]-thymidine (Amersham Biosciences, Amersham, UK) was added to each well and the incorporation of radioactivity was measured after 16 hours using a beta liquid scintillation counter (1205 Betaplate Wallac).

Priming of T cells and cytokine production

1 x 10⁶ allogeneic naïve CD4⁺ T cells were primed in the presence of 1 x 10⁵ DCs in 24 well plates (Nunc) for 6 days. Alternatively, naïve T cells were primed by DCs for 13 days with addition of 10 U/ml IL-2 (PeproTech) and fresh medium on day 7. DC-primed T cells were harvested, washed and evaluated for their suppressive capacity.

Cytokines produced during the priming of T cells were measured by harvesting the supernatant of the DC and T cell coculture after 6 days of culture. Alternatively, 5 x 10⁴ DC-primed T cells were restimulated in 96 well flat bottom plates (Nunc) with PMA (10 ng/ml; Sigma-Aldrich) and ionomycin (1 μg/ml; Sigma-Aldrich), or with 4 x 10³ mDCs in culture medium. After 24 hours of stimulation, the supernatant was harvested. The production of IL-10, IL-13 and IFNγ was determined by ELISA using the PeliKine-compact ELISA kit (Sanquin Reagents).

Suppression assay

DC-primed T cells were added to a second culture consisting of responder T cells stimulated with mDCs. Responder T cells are CD4⁺ memory T cells of the same T cell donor and mDCs are from the same DC donor. To track proliferation of responder T cells in the suppression assay, cells were labelled with 0.5 μM of the ‘green’ fluorescent dye CFSE (Invitrogen, Eugene, Oregon, USA) for 15 minutes at RT. To discriminate between proliferated, CFSE negative, responder T cells and non-CFSE labelled primed T cells, the primed T cells were labelled with 6 μM of the ‘red’ fluorescent dye PKH26 (Sigma-Aldrich) for 5 minutes at RT. Primed T cells were irradiated (30 Gy) after labelling to prevent convergence of proliferated CFSE- and PKH26-negative cells. After labelling with the tracking dyes, 5 x 10⁴ responder T cells were incubated with 5 x 10⁴ primed T cells (1:1), or at ratio’s 1:2 and 2:1. 4 x 10³ mDCs were added as stimulation (1:12.5 ratio with the responder T cells), or in varying amounts as described. Cells were cultured in 96 well round bottom plates (Greiner Bio-One, Frickenhausen, Germany) for 6 days in culture medium. Prior to flow cytometry, cells were stained with mAb against CD4 and DAPI. The lymphocyte gate was established on basis of forward vs. sideward scatter plot, including blasting cells. DAPI⁺ dead cells and PKH26⁺ cells were excluded from analysis. Proliferation of CD4⁺ responder T cells was measured on an LSRII flow cytometer (BD Biosciences) and analysed with FACS Diva software (BD Biosciences).

For blocking experiments neutralizing Abs were used at 10 μg/ml against: TGFβ (R&D Systems, Minneapolis, USA), PD-1 (R&D Systems), IL-10 (anti-IL-10.8, Sanquin Reagents) and CTLA-4 (Ancell, Bayport, USA). For isotype controls an irrelevant IgG₁
Ab directed to FelD1 or a goat-anti-mouse Ab (Sanquin Reagents) were used. For transwell experiments, 96-well transwell plates were used (Corning, New York, USA) with a 0.4 μm pore size polycarbonate membrane. CFSE-labelled responder T cells and mDCs were cultured in the lower compartment. PKH26-labelled primed T cells were added to the upper compartment, with or without mDCs as stimulation. As a control, PKH26-labelled primed T cells were added directly to responder T cells and mDCs in the lower compartment.

Statistical analysis
Data are expressed as mean + SEM. The statistical significance of the data was analysed using a paired Student’s t test in Graphpad Prism 4.03 software.

Results
Effect of IL-10 on DC phenotype and cytokine production
Previously is shown that incubation of human monocyte-derived DCs with IL-10 promotes the generation of tolerogenic DCs which are able to induce Tregs. To generate DCs with a tolerogenic phenotype which meet the requirements for clinical application, we generated monocyte-derived immature (im)DCs from elutriated monocytes which were subsequently incubated with IL-10 and a maturation cocktail, consisting of IL-1β, TNFα and PGE2 (IL-10 DCs) under serum-free conditions. Alternatively, imDCs were treated with the maturation cocktail only to generate immuno-activatory mature (m)DCs, or were left untreated to keep them in their immature state. Tolerogenic DCs are characterized by low to intermediate expression of cell surface MHC molecules and costimulatory molecules, low production of pro-inflammatory cytokines and high production of IL-10. To determine the maturation status of the DCs, we performed flow cytometric analysis on typical DC markers (Figure 1A). IL-10 DCs clearly showed intermediate expression of the DC maturation marker CD83, the costimulatory molecules CD80 and CD86, and the MHC class II molecule HLA-DR, compared to imDCs and mDCs. To determine cytokine production, we stimulated the DCs with CD40L-transfected cells to mimic the interaction with CD40L-expressing T helper cells (Figure 1B). IL-10 DCs showed a significantly higher production of IL-10 and TNFα than mDCs. The ratio of IL-10:TNFα production was significantly higher for IL-10 DCs than for mDCs. The IL-6 levels were not significantly different between the two conditions. For both DC types IL-12p70 production was not detectable. These results show that, apart from the TNFα production, the IL-10 DCs have the characteristics of semi-mature tolerogenic DCs.

Effect of IL-10 DCs on T cells
It has been reported that IL-10 DCs have a reduced capacity to stimulate allogeneic T cells in an MLR and that the T cells rendered show a low production of IFNγ and a high production of IL-10. To determine the effect of IL-10 DCs on the proliferation of allogeneic T cells, DCs were cocultured with memory or naïve CD4+
T cells for 6 days and T cell proliferation was measured (Figure 2A). mDCs strongly stimulated proliferation of CD4+ memory and naïve T cells. IL-10 DCs showed a reduced stimulatory capacity compared to mDCs, although it was superior to that of imDCs. We measured the cytokine production of naïve T cells after 6 days of coculture with DCs (Figure 2B). Coculture of T cells with IL-10 DCs resulted in a significant decrease in IFNγ and IL-13 production and a significant increase in IL-10 production compared to the coculture of T cells with mDCs. Since IL-10 DCs produce IL-10 themselves (Figure 1B), it cannot be excluded that the observed IL-10 production is DC-derived. To demonstrate enhanced IL-10 production by the T cells cocultured with IL-10 DCs, we harvested the T cells after 6 days of coculture and measured cytokine production after 24 hours restimulation (Figure 2C). T cells previously cocultured with IL-10 DCs showed a significant higher IL-10 production upon restimulation with PMA and ionomycin than the T cells primed by mDCs. IL-10 DC-primed T cells restimulated with mDCs also showed this tendency, although it was not significant. Overall, these results show that the IL-10 DCs generated in these experiments have a reduced capacity to stimulate allogeneic T cells and...
that these T cells produce less IFNγ and IL-13, and a higher amount of IL-10 upon coculture with IL-10 DCs.

**IL-10 DC-primed T cells suppress proliferation of responder T cells**

One characteristic of tolerogenic DCs is their capacity to expand or activate regulatory T cells. However, as specific human Treg markers are lacking, human Tregs need to be assayed by their functionality. Most preferably this involves Treg mediated suppression of the proliferation of activated T cells. Therefore we have set up a suppression assay to test the functionality of human DC-induced Tregs. We have generated potential Tregs by priming naive T cells for 6 days with IL-10 DCs. Primed T cells were cocultured with responder T cells and mDCs as stimulation. In the control situation naive T cells were primed with the immuno-activatory mDCs as these are known to primarily induce effector T cells. Responder T cells were labelled with CFSE, a fluorogenic compound to detect cell division. To distinguish
primed T cells from proliferated, CFSE-negative responder T cells, primed T cells were labelled with PKH26 (see gating strategy in Figure 3A). Addition of IL-10 DC-primed T cells to the suppression assay caused a trend for suppression of the proliferation of responder T cells (Figure 3A and B). Compared to proliferation of mDC-stimulated responder T cells only, this however was not significant for the cell titrations used. The introduced control condition of mDC-primed T cells did not suppress the proliferation of responder T cells, as expected. Comparison of IL-10 DC-primed T cells with the mDC-primed T cells, however, did show significant suppression of proliferation of responder T cells. Thus, priming of naïve T cells with IL-10 DCs induces Tregs with significant suppression capacity when compared to the negative control condition of mDC-primed T cells.

Figure 3. IL-10 DC-primed naïve T cells suppress mDC-stimulated proliferation of responder T cells when compared to the mDC-primed T cells. Naïve CD4$^+$ T cells were primed by DCs (Tpr) for 6 days. DC-primed T cells were harvested, washed and labelled with PKH26. CFSE-labelled responder CD4$^+$ memory T cells (Tresp) were added in a 1:1 ratio together with mDCs as stimulation (Tresp:mDC = 12.5:1). (A) Gating strategy for read-out of suppression assays. Viable lymphocyte gates were based on FSC vs. SSC plot, including blasting cells. DAPI$^+$ cells were excluded from analysis (data not shown). Next, CFSE$^+$ cells were gated and analysed for proliferation. One representative experiment is shown. (B) Mean proliferation of responder T cells + SEM from 6 independent suppression experiments is shown.
Suppression by IL-10 DC-induced Tregs is dependent on the amount of APCs in the suppression assay

Our results show that responder T cell proliferation is significantly suppressed by IL-10 DC-primed T cells, but only when compared to the negative control condition with mDC-primed T cells and not when compared to the proliferation of mDC-stimulated responder T cells. As the suppression induced by the IL-10 DC-primed T cells in our assay was disappointing compared to their suppressive capacity described in literature 24,25, we optimized the suppression assay. Oberg and co-workers 29 found by using artificial APCs, in the form of anti-CD3/anti-CD28 coated beads that fewer beads resulted in more suppression by natural Tregs. Therefore, we investigated whether we could improve suppression by adjusting the number of APCs in the suppression assay (Figure 4). With lower numbers of mDCs as stimulation, IL-10 DC-induced Tregs showed more obvious suppression of proliferation of responder T cells. IL-10 DC-induced Tregs suppressed proliferation of responder T cells up to 44% with the lowest amount of mDCs compared to proliferation of mDC-stimulated responder T cells. These data show that the suppressive capacity of IL-10 DC-induced Tregs is sensitive to the number of APCs that provide T cell stimulation in the suppression assay.

Suppression by induced Tregs is dose-dependent

In addition to the number of APCs, we also investigated the dose-dependency of iTregs on the level of suppression in the suppression assay (Figure 5A). The degree of suppression of responder T cell proliferation by IL-10 DC-induced Tregs is indeed dose-dependent, whereas the control condition of mDC-primed T cells did not show suppression of proliferation of responder T cells. IL-10 DC-induced Tregs suppressed the proliferation of responder T cells up to 64% with the highest amount of Tregs, compared to proliferation of mDC-stimulated responder T cells.

Figure 4. IL-10 DC-induced Tregs suppress the mDC-stimulated responder T cell proliferation more effectively in conditions with lower numbers of mDCs. Naïve CD4+ T cells were primed by DCs for 6 days. DC-primed T cells were harvested, washed and PKH26 labelled. CFSE-labelled responder CD4+ memory T cells were mixed with PKH26-labelled primed T cells in a 1:1 ratio together with varying amounts of mDCs as stimulation (Tresp:mDC = 12.5:1, 25:1, 50:1). Mean proliferation of responder T cells + SEM from 6 independent suppression experiments is shown.
These data show that the degree of suppression of responder T cell proliferation is dependent on the concentration of iTregs in the suppression assay.

Finally, we investigated whether priming of naïve T cells with IL-10 DCs for a longer time period would induce Tregs with a higher suppressive capacity. We primed naïve T cells with DCs for 13 days and performed a suppression assay under optimal conditions of low mDC stimulation and included a titration of primed T cells (Figure 5B). Control T cells primed with mDCs for 13 days showed a trend to suppress the proliferation of responder T cells. As a resultant, there was no significant difference in suppression of proliferation of responder T cells between the two conditions of primed T cells. These data show that a longer priming period results in induction of generalised suppression in the negative control condition and does not result in improved read-out of the suppression assay.

**Suppression by induced Tregs is dependent on cell contact**

Naturally occurring Tregs are described to suppress proliferation of responder T cells via cell contact-dependent mechanisms and independent of secreted cytokines. This in contrast to the induced Tregs Tr1 and Th3, which are typically characterized by predominantly utilizing IL-10 and TGFβ in their respective mechanism of action.\(^{15,16}\) One report however, shows that IL-10 DC-induced Tregs mediate suppression in a fashion that is cell contact-dependent between Tregs and responder T cells and not dependent on IL-10 or TGFβ secretion.\(^{25}\) Here we investigated the mechanism behind the suppressive capacity of the IL-10 DC-induced Tregs generated in this study. We first set out to examine whether the IL-10 DC-induced Tregs mediate suppression via cytokine secretion (Figure 6A). A neutralizing IL-10 antibody did not inhibit the suppression of proliferation of responder T cells by IL-10 DC-induced Tregs. Also a neutralizing TGFβ antibody did not inhibit the suppression of proliferation of responder T cells by IL-10 DC-induced Tregs. The neutralizing antibodies were
tested for functionality and both are able to inhibit the respective cytokine (data not shown). Next, we investigated whether cell contact is important for suppression by IL-10 DC-induced Tregs by use of a transwell system (Figure 6B). Separating IL-10 DC-induced Tregs in the upper compartment from responder T cells and mDCs in the lower compartment completely prevented suppression of responder T cell proliferation. This was not due to the fact that the iTregs were not stimulated in this experimental setup, also upon stimulation of the iTregs with mDCs in the upper well, suppression of proliferation of responder T cells was still completely inhibited. As cell contact seems to be important for suppression by IL-10 DC-induced Tregs, we investigated whether the inhibitory molecules CTLA-4 or programmed death

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Figure 6. Suppression by IL-10 DC-induced Tregs is cell contact-dependent and not cytokine-dependent. Naive CD4+ T cell were primed by DCs for 6 days. DC-primed T cells were harvested, washed and PKH26-labelled. PKH26-labelled primed T cells were added 1:1 to CFSE-labelled responder CD4+ memory T cells together with mDCs as stimulation (Tresp:mDC = 50:1). (A) Blocking antibodies against IL-10, TGFβ or an isotype control were added. The assay was performed in duplicate and mean + SEM of 3 independent experiments is shown. (B) Suppression assay was performed in a transwell system where primed T cells were separated from mDC-stimulated responder T cells by a microporous membrane, or not separated (Control). Primed T cells in the upper chamber of the transwell were either not stimulated (No stim.) or stimulated with mDCs (Stim.). The assay was performed in duplicate and mean + SEM of 4 independent experiments is shown. (C) Blocking antibodies against CTLA-4, PD-1 or isotype controls were added. The assay was performed in duplicate and mean + SEM of 3 independent experiments is shown.
(PD)-1 are important for suppression by IL-10 DC-induced Tregs. Blocking CTLA-4 did not inhibit the suppression of proliferation of responder T cells by IL-10 DC-induced Tregs. Also blocking PD-1 did not inhibit the suppression of proliferation of responder T cells. Collectively, these results show that suppression by IL-10 DC-induced Tregs is dependent on cell contact, or dependent on close proximity, and not dependent on secretion of IL-10 or TGFβ.

**Discussion**

The induction of Tregs is an important measure of the tolerogenicity of DCs. The best read-out system for induction of human Tregs is a functional assay demonstrating suppression of proliferation of stimulated responder T cells. However the setup of suppression assays is often different and poorly defined. Therefore in this study we optimized a suppression assay with human tolerogenic DC-induced Tregs. For our studies we have induced Tregs from naïve T cells by IL-10-treated tolerogenic DCs. Treatment of human monocyte-derived DCs with IL-10 results in the generation of tolerogenic DCs. The data presented here confirm that IL-10 treatment of DCs strongly inhibits DC maturation and T cell activation (Figure 1 and 2). In agreement with these previous studies, our results show that IL-10 treatment during maturation of DCs results in no detectable production of IL-12p70 and in a higher IL-10 production than their normal matured counterparts (Figure 1B). In contrast to a previous report, IL-10 DCs generated in our experiments also have a higher TNFα production than mDCs. However, this can be explained by the fact that McBride and co-workers measured cytokine levels that were produced in culture, while we have measured cytokine production of DCs after CD40L restimulation. In addition, we have generated DCs under serum-free conditions, which is a requirement for generation of clinically-applicable DCs. Furthermore, we have used a cytokine maturation cocktail instead of LPS to mature the IL-10 DCs. Therefore, the discrepancy in TNFα levels might be due to different experimental designs or different cell preparations. In agreement with previous studies, we have shown that IL-10 treatment of DCs results in a reduced expression of the maturation marker CD83, costimulatory molecules CD80 and CD86 and the MHC class II molecule HLA-DR, as compared with their normal mature counterparts (Figure 1A). Furthermore, consistent with previous reports, we have shown that IL-10-treated DCs have a reduced capacity to stimulate allogeneic naïve and memory CD4+ T cells (Figure 2A).

Most assays for analysis of Treg suppressive capacity consist of a coculture setup of Tregs with antigen-specific or polyclonal stimulated responder T cells. We have chosen to use APCs in this case mDCs, as stimulators for the responder T cells to mimic the in vivo situation more closely. Tregs can suppress T cell responses both in a direct or an indirect way via the APC. In the absence of APCs the responder T cells are the only target of suppression. In the presence of APCs however, the target of Treg suppression can be the APC, the responder cell or both. If Tregs induce suppression of responder T cell proliferation only via the indirect
way, this suppressive capacity would be missed when using a suppression assay with a polyclonal stimulus. In addition, when using mDCs as T cell stimulation in a suppression assay, the assay can potentially also be used to test the antigen-specific suppressive capacity of the Tregs.

Our extensive analyses show that the number of mDCs used for the stimulation of the responder T cells in the suppression assay is important for optimal read-out of suppression by DC-induced Tregs. Less APC-mediated stimulation of responder T cells allows more effective suppression by the Tregs (Figure 4). This is consistent with the findings of Oberg and co-workers who previously showed that upon usage of anti-CD3/ anti-CD28 coated beads as T cell stimulation less beads resulted in more suppression by natural Tregs. The improved suppression we observed in the presence of less APCs may be related to data of two studies which suggest that the susceptibility of responder T cells for Treg-mediated suppression is determined by the strength of activation of those responder T cells. Antons and co-workers showed that increase of the strength of TCR signal can override the Treg-mediated suppression. George and co-workers addressed the question whether loss of suppression that is seen with increasing stimulation comes from inactivation of Tregs or whether increasing stimulation enables responder T cells to escape suppression. Their results show that Tregs are still functional with high dose antigen stimulation but potently stimulated responder T cells may produce sufficient levels of IL-2 to override Treg-mediated suppression and thereby drive their own proliferation.

In addition, we have shown that the use of a negative control condition, T cells primed by mDCs, is essential to discriminate specific suppression from generalised suppression by DC-primed T cells. Longer priming conditions coincided with stronger suppressive effects but also in the negative control condition (Figure 5B). One possible reason for this is that the primed T cells are in a different, more rested, stage of T cell activation after long priming periods, which may lead to more generalised mechanisms of suppression upon coculture with responder T cells.

Titration of iTregs into the suppression assay showed that suppression of proliferation of responder T cells by IL-10 DC-induced Tregs is dose-dependent (Figure 5A). The highest amount of iTregs, that is a ratio of one responder T cell to two iTreg cells, yielded a significant suppression of proliferation of mDC-stimulated responder T cells. For naturally occurring Tregs often lower amounts of Tregs are sufficient to suppress proliferation of responder T cells. The reason why we need a relatively high amount of iTregs most likely reflects the probability that our priming conditions did not yield a pure population of iTregs. Priming of naïve T cells by IL-10 DCs very likely results in a mixture of primed T cells with a relative enrichment of the Treg fraction.

Our results show that suppression by IL-10 DC-induced Tregs is not dependent on secretion of IL-10 or TGFβ (Figure 6A). Furthermore, transwell experiments suggest that suppression by IL-10 DC-induced Tregs is dependent on cell contact (Figure 6B). These data confirm a previous report of Steinbrink and co-workers. In addition, using supernatant of IL-10 DC-induced Tregs, Steinbrink and co-workers show that the suppression is not dependent on any soluble factor. To further address
which cell surface molecule is important for suppression, we used neutralizing antibodies against PD-1 and CTLA-4. Our results suggest that suppression was not mediated via these inhibitory molecules. However, Steinbrink and co-workers reported that interaction between CTLA-4 and CD86, but not CD80, is important for suppression by IL-10 DC-induced Tregs. The use of a transwell system does not necessarily rule out the possibility that cytokines or other soluble factors are important for suppression by Tregs. It might as well be that close proximity of cells, immunological synapse formation between cells, or cell contact, is important for efficient suppression via soluble factors. Alternatively, it is also possible that there is no longer competition between Tregs and effector T cells for stimulation by the DCs, a mechanism described by Onishi and co-workers. Absence of competition with Tregs then indeed results in proliferation of effector T cells. Further studies will be needed to clarify the mechanisms responsible for the inhibitory effect of IL-10 DC-induced Tregs.

In this report we show extensive fine-tuning of a CFSE-based suppression assay to determine the induction of human tolerogenic DC-induced Tregs. The suppression assay uses mDC-stimulated T cell proliferation as read-out through which it becomes broader applicable, e.g. to detect suppressive mechanisms which are APC dependent and to determine antigen-specific suppression. Titration of APCs in the assay is furthermore shown to be essential to define the ideal setting for optimal suppression. In addition, the use of a negative control condition, consisting of T cells primed by mDCs, ensures that generalised suppression phenomena can be discerned from only specific iTreg-mediated suppression. The assay described in this study can now be used as a tool for the comparison of different types of human tolerogenic DCs in order to identify the optimal and best suited DC for tolerance therapy.

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