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Ethylenecarbodiimide-coupled allogeneic antigen presenting cells induce human CD4⁺ regulatory T cells

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Abstract

Adoptive transfer of naturally occurring CD4⁺CD25⁺ regulatory T cells can tolerize transplantation alloresponses in animal models. However isolation of these cells in sufficient numbers from humans is cumbersome and prone to contamination with alloreactive CD25⁺ T-cells. Incubation of ethylenecarbodiimide-coupled antigen presenting cells (APC) with naïve T-cells and antigen has been shown to induce tolerance in various experimental models. We therefore investigated whether ECDI-coupled allogeneic APC were able to induce an expandable human CD4⁺ Treg population.

CD4⁺ and CD4⁺CD25⁺ cells cultured for five days with ECDI-treated human PBMC exhibited potent suppressive capacity in a mixed lymphocyte reaction. Induction of these ECDI-Tregs was associated with up-regulation of Foxp3 mRNA and protein expression and they maintained high expression of CD62L and CD27 as well as low CD127 expression. ECDI-treated APC displayed reduced expression of the co-stimulatory signaling molecules CD40 and CD80, and failed to stimulate proliferation and cytokine secretion in co-cultured CD4⁺ T cells. Restimulation in the presence of rapamycin and hrIL-2 led to expansion of ECDI-Tregs with increasing Foxp3 levels and suppressive activity significantly higher than expanded naturally occurring CD4⁺CD25⁺ Tregs.

In summary these findings support the hypothesis that ECDI-coupled APC can convert naïve CD4⁺ T cells into functional Tregs with different phenotypic characteristics than naturally occurring CD4⁺CD25⁺ Tregs. These inducible Tregs could provide a novel approach that might facilitate the translation of ex vivo generated and expanded Tregs into clinical settings.

Keywords

ECDI; Treg; Rapamycin; GVHD

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Introduction

Successful transplantation of hematopoietic stem cells or solid organ grafts is often associated with unwanted immune responses mediated by either donor or recipient T-lymphocytes which cause either graft-versus-host disease (GVHD) or graft rejection. It is now widely accepted that transplantation tolerance only ensues when a homeostasis between protective immunosuppressive regulatory T-cells (Treg) and aggressive effector T-cells (Teff) has been achieved. Naturally occurring Tregs received great research interest since their prediction decades ago by Gershon and Kondo [1] and their reintroduction as CD4⁺CD25⁺ Tregs in the mid 1990-ies by Sakaguchi et al [2]. These cells show great potential for the treatment of autoimmune disease [3], transplant rejection and GVHD [4;5]. Naturally occurring CD4⁺CD25⁺ Tregs (nTregs) express high levels of the transcription factor forkhead box p3 (Foxp3), which is believed to be their main developmental regulator [6;7]. However, several other molecules such as GITR have also been put forward as being specific for Tregs [8].

It has been shown that the transfer of CD4⁺CD25⁺ nTregs can tolerize alloresponses and prevent GVHD in murine models [9;10;11]. Cells expressing high levels of CD27 [12] or CD62L [13;14] have been demonstrated to be especially potent regulators of alloimmunity. However, due to the low frequency of CD4⁺CD25⁺ nTregs in the peripheral blood, efficient isolation and expansion protocols are a prerequisite for their successful transfer to clinical settings [15]. While technically challenging, isolation and therapeutic application of these cells also carries a substantial risk of contamination with activated and thereby CD25⁺ effector T cells [16;17]. Many different strategies to generate or expand regulatory T cells ex vivo have been investigated in recent years [18]. One interesting approach was proposed by Battaglia et al who successfully expanded freshly isolated human and murine CD4⁺CD25⁺ Tregs with high dose IL-2 and the immunosuppressive drug rapamycin (sirolimus) [18;19]. Nevertheless, feasible protocols to provide large amounts of pure Tregs with a high regulatory capacity are still in demand [15].

The tolerogenic potential of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (ECDI)-coupled antigen presenting cells was first described by Miller et al and demonstrated to result from effector T-cell anergy and antigen-specific unresponsiveness in various experimental in vitro and in vivo models [20;21;22;23;24;25]. Injection of ECDI-treated spleen cells promoted transplantation tolerance in a murine model [26].

The isolation of human CD4⁺CD25⁺ regulatory T cells in sufficient numbers and purity remains the major obstacle for their transfer into clinical settings. In this study we therefore pursued a unique strategy that facilitates the induction of regulatory T cells ex vivo with subsequent expansion to numbers adequate for adoptive immunotherapy. We have used ECDI-treated human PBMC as allogeneic antigen presenting cells (APC) to induce a regulatory T cell population, both functionally and phenotypically, from fresh human CD4⁺ cells in a mixed lymphocyte reaction. We further investigated whether these cells could be expanded for several weeks without loss of their regulatory activity.

Materials and methods

Cell isolation and cell culture

Peripheral blood mononuclear cells (PBMC) obtained from healthy donors were isolated from buffy coat by density gradient centrifugation over Ficoll-Hypaque 1077 (Pharmacia, Uppsala, Sweden). PBMC were washed twice in PBS and subsequently used for magnetic cell sorting. CD4⁺ cells, CD3⁺ and CD3⁻ cells were purified by positive and negative selection with anti-CD4 and anti-CD3 conjugated magnetic microbeads (Miltenyi Biotec,

Bergisch Gladbach, Germany). Purity was routinely between 97-99% for positive selections and 95-98% for negative selections as measured by flow cytometry. CD3 depleted PBMC were used as APC. Monocytes, B cells and dendritic cells were purified with anti-CD14 or anti-CD19 magnetic beads or a dendritic cell isolation kit (all from Miltenyi Biotec), respectively. Naturally occurring CD4⁺CD25⁺ T cells and CD4⁺CD25⁻ T cells were enriched using the MACS regulatory T cell isolation kit (Miltenyi Biotec). Purity of positive selected cells was between 94-97% and of negative selected cells above 99.7%. Cells were cultured in RPMI 1640 supplemented with 2mM glutamine, 1% non-essential amino acids, 1mM sodium pyruvate, 100U/ml penicillin, 100µg/ml streptomycin, 10% FCS (PAA-laboratories, Pasching, Austria) and 50µM 2-mercaptoethanol and maintained in a 5% CO₂ incubator at 37°C for the indicated time periods.

ECDI-treatment of APC

A 1M stock solution of ECDI (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide or ethylenecarbodiimide, Calbiochem, Darmstadt, Germany) was freshly prepared for each experiment in ice cold PBS and filter sterilized. After CD3 depletion by immunomagnetic negative selection, APC were treated with 10 to 80mM ECDI at a cell density of 5×10^6 cells per ml for 1 hour at 4°C in culture medium. Cells were washed three times with culture medium prior to irradiation (30Gy) and once more after irradiation.

Ex vivo generation and expansion of Tregs

Purified naïve CD4⁺ cells or CD4⁺CD25⁻ cells (1×10^6) were co-cultured for 5 days with 5×10^6 irradiated (30Gy) allogeneic APC or with 5×10^6 irradiated ECDI-treated APC. For expansion, cells were harvested over Ficoll-Hypaque 1077 (Pharmacia), washed twice and without further separation restimulated with freshly isolated irradiated allogeneic APC from the original donor for 3 weeks at one week intervals. Growth media was supplemented with 1000U/ml hrIL-2 (Proleukine®, Chiron, Amsterdam, Netherlands) and 100nM rapamycin (LC Laboratories, Woburn, USA) where indicated. During the generation and expansion period the cell numbers and Foxp3 mRNA levels were monitored weekly. After the third round of restimulation, cells were washed and allowed to rest for 24 hours in culture medium with hrIL-2 (50U/ml) and without rapamycin before being used for suppression experiments. Cell viability of cultured APC and ECDI-treated APC was determined using the trypan blue (Sigma-Aldrich, Munich, Germany) exclusion test. For transwell experiments, polyester 24-well transwell inserts were used (Corning, NY, USA). Purified CD4⁺ T cells (1×10^6) were co-cultured with 5×10^6 irradiated APC in the lower chamber of the wells. In the upper chamber 1×10^6 CD4⁺ T cells were co-cultured with irradiated ECDI-treated APC. After five days in culture, CD4⁺ T cells were positively selected with anti-CD4 microbeads and tested in a secondary mixed lymphocyte reaction.

Mixed lymphocyte reaction (MLR)

After resting 24 hours cells were harvested over Ficoll-Hypaque 1077 (Pharmacia), washed twice and without further separation added to a primary MLR consisting of CFSE-labeled responder CD3⁺ cells and allogeneic irradiated (30Gy), CD3 depleted stimulator APC from the original donors at a ratio of 5×10^4 to 2.5×10^5 cells per 96-well (Cellstar) in complete media. Staining of effector T cells with carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes, Eugene, USA) was carried out as previously described [27]. Briefly, cells were stained by mixing equal volumes of cell suspension with 6µM CFSE in PBS with 10% FCS for 5 minutes at room temperature. Proliferation was measured after 5 days in culture. T cells that had undergone at least one cell division were designated as alloreactive T cells.

Real-time PCR

Total RNA was isolated using QIAshredder and RNeasy Mini Kit (Qiagen, Hilden, Germany) from 2×10^5 cells. 40ng RNA was reverse transcribed to cDNA with QuantiTect (Qiagen) using oligo-dT primer. Real-time PCR was performed using SYBR Green (Bio-Rad, Munich, Germany) with the following intron spanning PCR primer pairs: Abl, 5'-CAACACTGCTTCTGATGGCAA-3' and 5'-CGGCCACCGTTGAATGAT-3'; Foxp3, 5'-TCACCTACGCCACGCTCATC-3' and 5'-GGCAAACATGCGTGTGAACC-3', CD25, 5'-ATCAGTGCCTCCAGGGATAC-3' and 5'-CTTGCCTGAGGCTTCTCTTC-3', CD127, 5'-GCAGAACTGGATGACTACTC-3' and 5'-TCCTTCCCGATAGACGACAC-3', CTLA-4, 5'-CTCTTCATCCCTGTCTTCTG-3' and 5'-CTTTGCCTGGAGATGCATAC-3' and GITR, 5'-TTCCACTGCGGAGACCCTTG-3' and 5'-CCGAGGCACAGTCGATACAC-3'. Relative expression was determined by normalization to the expression of abl-kinase. Oligonucleotides were purchased from MWG Biotech (Ebersberg, Germany).

Flow cytometric analysis

T cells were stained for cell surface markers with FITC-anti-CD27, FITC-anti-CD62L, FITC-anti-CD127, FITC-anti-PD-1, PE-anti-PD-L1, Alexa488-anti-GITR, PC5-anti-CD25, (eBioscience, San Diego, USA), PE-anti-CD4 (Dakocytomation, Glostrup, Denmark), FITC-anti-CD86, PE-anti-CD40, PE-anti-CD80, APC-anti-CD152 (BD-Bioscience, Franklin Lakes, USA) and APC-anti-CD4 (Miltenyi Biotec) prior to intracellular staining for Foxp3. Foxp3-anti-PE staining was carried out with PCH101 (eBioscience) according to manufacturer instruction. Apoptosis and cell death was quantified by Annexin V-PE and 7-AAD staining (BD Biosciences). Flow cytometry was performed on a FACSScan or a FACS-Calibur flow cytometer (BD Biosciences). Proliferation rates and staining of phenotypes were calculated by gating on live cells by forward and side scatter characteristics and on CD4⁺ cells and on indicated gates. Data were analyzed with CellQuest (BD Biosciences) and WinMDI (J. Trotter) software.

Cytokine analysis

Culture supernatants were assayed for IL-2, IL-4, IL-5, IL-6, IL-10, GM-CSF, IFN- γ TNF- α using the 8-Plex cytokine assay kit (Bio-Rad) according to the manufacturers instructions and analyzed on the Bio-Plex 200 System (Bio-Rad). TGF- β was assayed with the TGF- β kit (Upstate). Cytokine levels are indicated as pg/ml.

Statistical analysis

Comparison of means from real-time PCR analyzes, flow cytometry and cytokine assays were calculated using the two-tailed student's t test. p values of less than .05 were considered to be significant.

Results

Purified CD4⁺ T cells co-cultured with ECDI-coupled APC acquire regulatory capacity and up-regulate Foxp3 mRNA

Given the tolerogenic potential of ECDI-coupled APC, we hypothesized that a possible mechanism of tolerance induction was the conversion of CD4⁺ T cells into Tregs in the responding T cell population.

We therefore co-cultured fresh human CD4⁺ T cells with irradiated allogeneic, T cell depleted APC pre-treated with ECDI for 1 hour. After 5 days in culture cells were isolated and added to a primary mixed lymphocyte reaction with CFSE labeled responder cells and allogeneic stimulator cells from the original donors. Addition of cells cultured in the

presence of ECDI-coupled APC (ECDI) resulted in potent suppression of allogeneic T cell proliferation (Figure 1A, upper panel). This was in contrast to the control cells co-cultured with untreated allogeneic APC (medium) which exerted no regulatory potential (Figure 1A, upper panel). To test whether these regulatory cells were specific for alloantigens on the APC used for their generation we also tested them in an MLR against third party APC and found the same degree of suppressive potential (Figure 1B, lower panel). The suppressive activity of ECDI-induced regulatory T-cells (ECDI-Tregs) was equally strong as that of freshly isolated CD4⁺CD25⁺ cells (nTregs) at different Treg to Teff ratios (Figure 1C).

Next we addressed the question whether ECDI-coupled APC induce Treg conversion or rather expand pre-existing Treg populations. Therefore CD4⁺CD25⁺ Treg cells were depleted with immunomagnetic beads ($4.4 \pm 0.7\%$ CD25⁺ cells before selection, $0.2 \pm 0.16\%$ after selection, Figure 1B) and the CD4⁺CD25⁻ cells were co-cultured with ECDI-treated APC or untreated APC as described above. Even though naturally occurring CD25⁺ Tregs had been depleted, ECDI-APC induced a regulatory cell population in these CD4⁺CD25⁻ cells in identical fashion as in undepleted CD4⁺ T cells (Figure 1C). These data argue for Treg induction rather than expansion of nTregs. To exclude the possibility that unwanted transfer of ECDI-coupled APC into the secondary mixed lymphocyte reaction contributed to the observed suppressive effect, CD4⁺ T cells were positively selected to very high purity after primary co-culture with ECDI-APC as shown in figure 1D and surface stained to demonstrate that they were CD3⁺CD14⁻CD19⁻ (data not shown).

In order to evaluate the optimal dose of ECDI for induction of T cells with regulatory function, we incubated freshly isolated CD4⁺ T cells with allogeneic, T cell depleted APC, pre-treated at a range of ECDI concentrations, for 5 days and determined a concentration of 40mM as the optimal dose level (Figure 1E). In order to test whether Foxp3 as the key regulator in Treg development was involved in the generation of ECDI-Tregs, total RNA from these cells was isolated to determine the relative Foxp3 mRNA level by quantitative PCR. Co-incubation with ECDI-coupled APC resulted in an ECDI dose dependent induction of Foxp3 mRNA levels. Foxp3 expression in ECDI-Tregs was up to 9fold higher than in freshly isolated CD4⁺ cells, while cells co-incubated with uncoupled APC only about doubled their Foxp3 mRNA expression level (Figure 1F). The suppressive potential of ECDI-Tregs corresponded well with Foxp3 induction levels, except at a dose level of 80mM ECDI (Figure 1F). Based on these findings, a concentration of 40mM ECDI was determined as optimal and used for treating APC in the Treg generation phase for subsequent experiments. In summary these data show that ECDI-coupled APC induce a potent, antigen-non-specific regulatory T cell population characterized by strong Foxp3 mRNA induction from freshly isolated human CD4⁺ T cells.

Phenotypic characteristics of ECDI-Tregs

Next we examined the phenotype of these ECDI-Tregs to find out whether they shared phenotypic characteristics associated with other naturally occurring or induced Treg populations.

In addition to intracellular Foxp3, cell surface expression markers previously reported to have a characteristic pattern in regulatory T cells such as CD127, CD27, CD62L, CTLA-4 (CD152), GITR and PD-1 were analyzed by multicolor flow cytometry to define the phenotype of ex vivo generated regulatory T cells [10;12;13;28;29] After 5 days in culture live CD4⁺ T cells were separated from either ECDI-treated or untreated stimulator APC and compared to freshly isolated CD4⁺ cells. Gates were either set on total CD4⁺ (Figure 2A) or on the CD25⁺ subpopulation (Figure 2B).

When analyzing total CD4⁺ cells a significantly higher proportion of medium cultured cells displayed Foxp3 expression ($12.0 \pm 1.3\%$) compared to ECDI-induced Tregs ($3.2 \pm 1.6\%$), most likely representing activated T cells in the medium cultures, which was further supported by a slightly increased CD127 expression ($11.1 \pm 1.3\%$ versus $5.9 \pm 1.6\%$) than on ECDI-Tregs. Similarly CTLA-4 ($14.9 \pm 2.1\%$) and PD-1 ($31.2 \pm 3.2\%$) expression was markedly increased in medium cells compared to ECDI-Tregs, altogether suggesting a decreased level of activation in ECDI-cultured cells. GITR could not be detected on either cell type. As expected, high levels of CD27 and CD62L positive cells $90.6 \pm 1.9\%$ and $88.5 \pm 1.0\%$ respectively could be found in freshly isolated T cells. A similar naïve pattern was apparent when analyzing cells co-cultured with ECDI-coupled APC (ECDI) with $91.1 \pm 0.8\%$ and $91.4 \pm 1.2\%$ positive cells, respectively. This was in contrast to cells incubated with uncoupled APC (medium) which showed a decrease in CD27 and CD62L positivity to $82.8 \pm 1.8\%$ and $77.9 \pm 1.2\%$, respectively ($n = 5$, $p < .002$ for CD27 and $p < .001$ for CD62L). Interestingly, CD4⁺ T cells incubated with ECDI-treated APC always displayed a slightly reduced CD4 expression (MFI 511 ± 47 vs. 774 ± 67 for medium cells and 759 ± 52 fresh CD4⁺ T cells) which corresponds well with the low CD4 expression characteristics of naturally occurring CD4⁺CD25⁺ Tregs.

In medium cultured cells $31.8 \pm 3.4\%$ of CD4⁺ cells were CD25 positive as compared to $11.7 \pm 2.1\%$ ($n = 5$, $p < .0001$) for ECDI-cells, again suggesting a decreased level of activation in ECDI cultured cells. If gated on CD25⁺ cells (Figure 2B) ECDI cultured cells revealed a markedly different pattern of Foxp3 protein expression with $75.7 \pm 6.7\%$ positive cells as compared to $47.0 \pm 4.8\%$ ($n = 5$, $p < .001$) for medium cells. The Foxp3 protein expression pattern found in CD4⁺CD25⁺ ECDI-cells was very similar to the one seen in freshly isolated CD4⁺CD25⁺ nTregs at $78.2 \pm 2.2\%$ positive cells (Figure 2B, second panel) and was not different if CD25⁺ cells had previously been depleted from the original cell population (data not shown). CD127 expression was highest in medium cultured cells $22.3 \pm 4.3\%$, whereas CD127 was low in CD25⁺ ECDI-Tregs ($7.5 \pm 2.4\%$) and in freshly isolated CD4⁺ T cells ($11.7 \pm 0.5\%$). CTLA-4 was predominantly found on CD4⁺CD25⁺ nTregs with $26.4 \pm 3.8\%$ positive cells and on medium cultured cells with $18.3 \pm 2.0\%$ positivity but was absent on ECDI-Tregs. Again GITR was absent on all three cell types. Expression levels of CD27 and CD62L on CD4⁺CD25⁺ ECDI-cells with $85.2 \pm 3.4\%$ and $78.2 \pm 1.6\%$ positive cells were similar to that seen in freshly isolated CD4⁺CD25⁺ nTregs $85.5 \pm 0.8\%$ and $79.0 \pm 0.1\%$ but significantly reduced in medium cultured cells with $66.4 \pm 1.9\%$ and $58.4 \pm 2.6\%$ positive cells. We found a weak up regulation of PD-1 in medium cells as would be expected in activated T-cells but no change in ECDI Tregs (Fig. 2A). Gated on CD4⁺CD25⁺ cells, PD-1 was highest expressed in $49.2 \pm 3.7\%$ of medium cultured cells and to a lower extent in ECDI-Tregs ($39.4 \pm 2.8\%$) and CD4⁺CD25⁺ nTregs ($28.6 \pm 0.6\%$) (Fig. 2B). Although CTLA-4 and GITR were not detectable on a protein level in ECDI-cells, significantly stronger up-regulation of mRNA compared to medium-cells was observed. Similarly CD25 mRNA was up-regulated in ECDI-cultured cells (Figure 2C) although cell surface expression of CD25 was much lower in these cells.

To assess whether the regulatory phenotype of ECDI-Tregs was associated with a specific cytokine profile, we analyzed the supernatants of the medium culture and ECDI culture for seven different cytokines (IL-2, IL-4, IL-5, IL-6, IL-10, GM-CSF, IFN- γ , TNF- α and TGF- β) with a luminex-based quantification method. As shown in figure 2D, ECDI-treated APC displayed a reduced capacity to induce secretion of these cytokines in CD4⁺ cells as compared to cells cultured with uncoupled APC only.

Taken together these phenotypic analyses indicate that ECDI-Tregs exhibit a naïve phenotype similar to the one typically associated with naturally occurring regulatory T-cell

populations. They further lack a specific surface or intracellular marker and they are not characterized by a particular - i.e. anti-inflammatory - cytokine profile.

ECDI-treated APC fail to up-regulate co-stimulatory signaling molecules and require cell to cell contact to induce Tregs

To analyze a possible underlying mechanism for Treg induction by ECDI-treated APC the effect of ECDI-treatment on APC was further investigated.

When analyzing how ECDI affected APC viability, we found that ECDI-treated APC had an increased death rate during the 5 day culture period compared to untreated APC as determined by trypan blue exclusion (Figure 3A). As shown in Figure 3B a fluorescence-based viability assay was performed to discriminate between apoptotic and necrotic events. While $76.3 \pm 7.0\%$ of ECDI-treated APC passed through a single Annexin V positive to a double positive Annexin V and 7-AAD stage only $18.8 \pm 4.3\%$ of medium cultured APC underwent apoptosis within 5 days ($p = .0003$).

As we had seen reduced expression of T cell activation markers on ECDI-Tregs, we next analyzed the capacity of ECDI-treated APC to induce proliferation in $CD4^+$ T cells. $CD4^+$ T cells were CFSE stained and co-cultured with ECDI-treated and untreated APC. After 5 days cells were stained for CD25 and Foxp3 expression. In the medium culture $52.3 \pm 5.1\%$ cells had undergone at least one cell division cycle and displayed strong up-regulation of Foxp3 and CD25. In contrast cells co-cultured with ECDI-coupled APC remained in a non-proliferating state (Figure 3C).

To assess whether this effect could be due to a lack of functional stimulation exerted by ECDI-APC, changes in HLA-DR and co-stimulatory cell surface markers were determined by flow cytometry (Figure 3D). While medium cultured APC up-regulated CD40, CD80, CD86, HLA-DR and PD-L1 expression, ECDI-APC displayed a strongly reduced expression of CD40, CD80, HLA-DR and PD-L1 but not CD86, thus indicating a reduced co-stimulatory capacity of ECDI-treated APC.

In order to determine which subpopulation of APC was mainly responsible for Treg induction, isolated monocytes, B cells and dendritic cells were co-cultured with $CD4^+$ T cells. Suppressive potential of co-cultured $CD4^+$ T cells was assayed in a mixed lymphocyte reaction as described above. Highest suppressive activity was observed in $CD4^+$ T cells derived from co-cultures with ECDI-treated monocytes and ECDI-treated dendritic cells, whereas $CD4^+$ T cells derived from co-cultures with ECDI-treated B cells were less suppressive (Figure 3E). To determine whether ECDI Treg generation was based on cell to cell contact we performed transwell experiments. As shown in figure 3F, ECDI-treated APC were able to induce regulatory activity in $CD4^+$ cells when cultured in the same chamber, but not when separated by a semi permeable membrane.

In summary these data suggest that the generation of ECDI-Tregs could be due to a lack of TCR stimulation and proper delivery of co-stimulatory signals by ECDI-APC undergoing rapid apoptosis. Treg induction by ECDI-treated APC is cell contact dependent and mainly relies on peripheral blood monocytes and dendritic cells.

Expansion and Foxp3 induction of ECDI-induced Tregs

As shown above we were able to induce a potent Treg population from human $CD4^+$ cells after a simple 5 day culture with ECDI-coupled APC. Since Battaglia et al had demonstrated that human $CD4^+CD25^+$ Tregs could be nicely expanded ex vivo using a restimulation protocol with high dose hrIL-2 and rapamycin [19], we next addressed the question whether

ECDI-Tregs could be expanded ex vivo without losing their regulatory potential using a similar approach.

Following the first stimulation cycle of isolated CD4⁺ T cells with ECDI-coupled APC (ECDI) or uncoupled APC (medium), the unseparated cells harvested after 5 days of culture were restimulated three times at weekly intervals with fresh APC from the original donor in culture medium supplemented with hrIL-2 and rapamycin. The experimental setup for generation and expansion of Tregs in this report is summarized in figure 4A.

Foxp3 protein levels were determined at the indicated weekly time points. A marked increase in Foxp3 protein was evident in the ECDI-cultured cells compared to medium cultured cells. Removal of rapamycin during the fourth week of restimulation resulted in a similar drop in Foxp3 protein levels in both cultures (Figure 4B).

Although T cell numbers in ECDI cultures in contrast to the medium cultures declined about 30% during the generation phase, a 15 ± 1 fold expansion by week 3 was apparent in both expansion cultures ($n = 6$, $p = .75$) (Figure 4C). As a surrogate marker for regulatory capacity we determined changes in Foxp3 mRNA levels by quantitative real-time PCR during the generation and expansion process of both cultures as displayed in figure 4D. We initially observed a 6 fold induction of Foxp3 expression in ECDI cultures (ECDI, rapamycin) after the first week of the expansion phase, which remained constant during the expansion phase. In contrast, cells cultured with uncoupled APC (medium) exhibited only a modest induction of Foxp3 after the generation phase (2.0 ± 0.3 , $n = 6$, $p = .02$ compared to ECDI). Consistent with the potential for a selection of Tregs under culture conditions with rapamycin and hrIL-2 we found a constant increase of Foxp3 mRNA and protein levels during the expansion phase in medium cells. However these levels remained significantly lower than in ECDI-Tregs at all time points during the expansion.

We could therefore demonstrate satisfactory expansion capacity in ECDI-Tregs which was comparable to medium cells and was accompanied by a significantly higher level of Foxp3 mRNA and protein in expanded ECDI-Tregs promising a superior regulatory potential of these cells even after expansion.

Expanded ECDI-Tregs retain their suppressive activity independent of rapamycin

Since the Tregs were devoid of contact with ECDI-coupled APC during the three week expansion phase, it was crucial to determine whether the expanded ECDI-Tregs had retained their regulatory potential.

The suppressive activity of expanded cells was compared in a primary MLR with allogeneic stimulator and responder cells from the original donors. Although the difference of suppressive activity in ECDI cultures compared to medium cultures was less pronounced after the expansion phase than immediately after the generation phase (Figure 1A and C), we observed a significantly higher suppression of $79.2 \pm 5.3\%$ in cells derived from ECDI cultures (ECDI, rapamycin) compared to $60.3 \pm 3.1\%$ for medium cultured cells (medium, rapamycin) ($n = 6$, $p = .004$) and compared to cultured CD4⁺CD25⁺ nTregs (nTreg, rapamycin) ($61.0 \pm 3.6\%$, $n = 3$, $p = .004$) (Figure 5A and B).

The ability of rapamycin to selectively promote the growth of T cells with regulatory phenotype in culture was recently described by other groups [18;30]. Though, when rapamycin is removed from the culture the expanding cells lose their suppressive capacity [31]. Therefore we tested whether expanded ECDI-Tregs were independent of the presence of rapamycin in the expansion culture during the third week. Consistent with previous observations, the removal of rapamycin lead to a significant diminution of suppressive

activity in medium cultures (from $56.7 \pm 5.8\%$ to $15.0 \pm 6.9\%$, $n = 3$, $p = .001$) while, in contrast, the suppressive activity of ECDI-Tregs ($76.5 \pm 5.8\%$ versus $72.4 \pm 7.1\%$, $n = 3$, $p = .4$) remained almost unaffected after depletion of rapamycin (Figure 5C).

In summary we could demonstrate robust in vitro expansion of ECDI-Tregs facilitated by rapamycin and high dose hrIL-2. These expanded ECDI-Tregs fully retained their regulatory potential, even independent of the presence of rapamycin during the last week of culture.

Discussion

Efficiently using Tregs for adoptive immunotherapy will require larger numbers of cells than what can be reasonably obtained by isolating $CD4^+CD25^+$ nTregs from peripheral blood. Additionally such cell preparations will likely contain unwanted, alloreactive, $CD25^+$ effector T cells when used in an allogeneic stem cell or solid organ transplant setting. Therefore efficient methods for ex vivo generation and expansion of Tregs are in great demand. Here we report an alternative approach for conversion of non-regulatory $CD4^+$ cells into Tregs based on tolerance induction with ECDI-coupled allogeneic APC. Our findings show that in a mixed lymphocyte reaction ECDI-treated APC induce a regulatory T cell population that can be successfully expanded ex vivo without loss of regulatory capacity. This could potentially offer an inexpensive, fast and simple high-yield method to generate Tregs from small amounts of peripheral blood ex vivo instead of going through the laborious and low-yield process of isolating $CD4^+CD25^+$ nTregs.

The tolerizing potential of antigen coupled to APC with the chemical cross-linker ECDI has been shown in several – mostly murine – studies [22;23;26]. So far, the underlying mechanism is elusive, but there is evidence that ECDI exerts its function by modifying costimulatory signals and that its effect can be independent of MHC class II and T-cell receptor interaction [22;32]. We demonstrated here that freshly isolated $CD4^+$ T cells exposed to ECDI-coupled allogeneic APC gain regulatory function and keep a naïve phenotype during a 5 day culture. The complete fraction of $CD4^+$ cells exposed to ECDI-APC for five days were equally potent as freshly isolated naturally occurring Tregs to suppress allogeneic responses in a mixed lymphocyte reaction with effector cells from the original donor. This is a remarkable finding as these cells were not further separated, thereby indicating that even a potential contamination with allo-activated cells could not compromise their suppressive activity. The robust induction of Foxp3 expression in ECDI-Tregs further supports the concept that the tolerizing potential of ECDI-coupled APC is based on Treg induction rather than expansion, especially since prior depletion of $CD4^+CD25^+$ Tregs from the starting population did not diminish the tolerizing activity of ECDI-Tregs. Our study provides new means to promote the idea of de novo generation of Tregs from naïve $CD4^+CD25^-$ T-cells additional to what has been described for TGF- β [33] or ATG treatment [34].

Recent reports have shown that in vitro activated human T cells undergo transient induction of Foxp3 which is not necessarily associated with conversion to regulatory T cells [35;36;37]. The mechanism of Foxp3 induction in ECDI-Tregs seems to be due to a different mechanism since ECDI-APC were incapable of activating T cells as determined by CD25 induction and proliferation studies. Furthermore, ECDI-Tregs expressed higher mRNA levels of Foxp3 compared to stimulated allogeneic control cells. However there was an obvious discrepancy between Foxp3 mRNA levels and protein levels in both groups after 5 days. This might be explained by a delay between mRNA synthesis and protein expression where for the latter we observed a significant up-regulation in cells derived from the ECDI-culture following the first restimulation in the presence of rapamycin. The fact that this induction of Foxp3 protein depends on the presence of rapamycin, might point to specific

mechanisms involving impaired TCR and mTor signaling in rapamycin dependent Foxp3 expression or as recently proposed a specific resistance to rapamycin in Tregs [38]. Recent data published by Tran et al also suggest that there might not always be a strict correlation between induced Foxp3 expression and suppressive function in human T-cells [39]. We therefore suggest that Foxp3 expression fails to exclusively characterize ECDI-induced Tregs.

It had previously been shown that strong expression of the two surface markers CD27 and CD62L characterizes Treg populations with especially strong regulatory potential [12;13;40]. CD27 expression was also shown to be useful for distinguishing between regulatory and non-regulatory cells after expansion of human CD4⁺CD25⁺ cells [41]. Two recent studies suggested that expression of the IL-7 receptor CD127 inversely correlated with regulatory T-cell function and Foxp3 expression [28;42]. With respect to these three markers and Foxp3 expression ECDI-Tregs maintained a phenotype with high expression levels of CD27, CD62L and Foxp3 and low levels of CD127 and PD-1 which was (except for CD25) highly reminiscent of that of naturally occurring CD4⁺CD25⁺ Tregs. In contrast ECDI-Tregs differed from the phenotype of nTregs by their apparent lack of CTLA-4 and GITR up-regulation. Since these observations were most pronounced when gating on the CD25⁺ cell fraction, one might argue that this might be caused by an enrichment of nTregs during incubation with ECDI-APC. This hypothesis however fails to explain the potent regulatory potential of ECDI-Tregs since only a small proportion of these cells is CD25⁺ and displays the described phenotype. Another possible explanation could be that ECDI-APC effectively deplete alloreactive T-cells from the culture while sparing CD25⁺ nTregs. However the fact that we could demonstrate that prior depletion of CD4⁺CD25⁺ Tregs from the starting cell population did not alter the suppressive capacity of ECDI-Tregs and that we could not clearly show an expansion of cells sharing an nTreg phenotype after primary culture leads us to conclude that induction rather than expansion of Tregs is the prevailing mechanism of ECDI-Treg generation.

Cytokines are known to be important for function and generation of Tregs. We therefore investigated whether the induction of Tregs by ECDI-APC was dependent on cytokine secretion. Especially IL-2, TGF- β , IL-10, and other cytokines have been shown to control the generation of Tregs [43]. However in the culture setting used in this study we found generally decreased capacity of ECDI-treated APC to secrete a variety of cytokines including the ones above, thereby indicating a TGF- β and IL-10 independent, non-stimulatory environment in ECDI cultures and separating them from IL-10 induced T regulatory type 1 (Tr1) cells [4]. The very low levels of IL-2 might offer a possible explanation as to why ECDI co-cultured T cells don't proliferate at this induction stage. In addition we could demonstrate that the mechanism for Treg induction is cell contact dependent and is elicited mainly by monocytes and dendritic cells.

Interestingly we found an increased apoptotic rate of ECDI-APC, thereby supporting previous observations in mouse dendritic cells treated with ECDI [26;32] but also observed reduced expression of the co-stimulatory signaling molecules CD40, CD80 and PD-L1 and of HLA-DR (Figure 3D) on APC treated with ECDI, possibly implying defective co-stimulation as a mechanism of Treg induction by ECDI-APC. PD-L1 has been implicated in the induction of allogeneic regulatory T-cells and peripheral tolerance, but does not seem to play an important role in ECDI-Treg generation. Concomitantly these APC also failed to stimulate proliferation and cytokine secretion in co-cultured CD4⁺ T cells.

Ex vivo creation of antigen-specific Tregs is another intriguing strategy to reduce the need for large quantities of Tregs [21]. Tolerance induction by ECDI-coupled APC had already been demonstrated to result in only partially antigen-specific tolerance [22;24;26]. It was

therefore not surprising to find that human ECDI-Tregs would lack antigen-specificity and also suppress alloresponses against third-party APC. This is however an important detail as antigen specificity of human ex vivo created Treg has not been studied extensively [21]. Walker et al described a technique using tetramers to select antigen-specific Tregs from bulk Tregs generated against a nominal antigen [44].

ECDI-coupled APC had been shown to induce hyporesponsiveness and anergy in co-cultured T cells [22;26]. Battaglia et al had demonstrated that the immunosuppressive drug rapamycin selectively expands CD4⁺CD25⁺ Tregs, a cell population also characterized by poor proliferative responses in vitro [18;19]. Based on these findings we used a similar approach to demonstrate sufficient expansion of ECDI-Tregs. This would theoretically make it possible to generate a Treg graft big enough for adoptive immunotherapy from an allogeneic donor. ECDI-Tregs constantly expressed high levels of Foxp3 mRNA during the expansion phase, which was accompanied by constantly higher levels of Foxp3 protein expression than in expanded medium cells. Medium cells cultured under the same conditions also exhibited an increase in Foxp3 mRNA during expansion, but never reached the same levels. More importantly, expanded ECDI-Tregs revealed a significant higher suppressive capacity than medium cells in proliferation assays. Medium cells also acquired some regulatory potential during expansion which we assume was due to selective expansion of the small proportion of nTregs contained in the originating cell population or due to induction of Tregs from naïve T cells by rapamycin as recently described by Gao et al. [45]. We therefore investigated the dependency of suppressive activity on the presence of rapamycin. Interestingly ECDI-induced Tregs do not lose their regulatory potential even after withdrawal of rapamycin during the third week of expansion culture in contrast to medium cells. These results were consistent with recent findings from Valmori et al who reported that suppressive potential of expanded nTregs declined after removal of rapamycin [31]. Since rapamycin was not present during the induction phase in our system, it is likely that rapamycin facilitates expansion of induced Tregs in subsequent cultures, consistent with its capacity for preferential proliferation of nTregs [18]. However, because rapamycin contributes to Treg conversion as well [45;46], it cannot be completely excluded that Treg conversion could still occur during restimulation.

In summary we describe a simple method for inducing a functional Treg population from naïve CD4⁺ T cells within a short 5 day culture with ECDI-coupled APC. Moreover, these cells were efficiently expanded without loss of regulatory capacity. These inducible Tregs suggest a feasible approach that might facilitate the translation of ex vivo generated and expanded Tregs into clinical settings.

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Abbreviations

CFSE	carboxyfluorescein diacetate, succinimidyl ester
ECDI	ethylenecarbodiimide
GVHD	graft-versus-host disease
MLR	mixed lymphocyte reaction

APC antigen presenting cells

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Figure 2. Phenotypic characteristics of ECDI-Tregs

Purified CD4⁺ T cells were cultured for 5 days either with APC (medium) or with ECDI-treated APC (ECDI). These cells and freshly isolated CD4⁺ cells (nTreg) were stained for CD4, CD25, CD27, CD62L, CD127, CD152 (CTLA-4), GITR, PD-1 and intracellular Foxp3. **(A)** Gated on live CD4⁺ cells or **(B)** on live CD4⁺CD25⁺ cells. Data representative of five equivalent experiments. **(C)** Fold induction of CD25, CD127, CTLA-4 and GITR mRNA expression in cultured CD4⁺ T cells as determined by real-time PCR analysis normalized to the expression of abl-kinase (mean $\pm$ SD, n = 4). *p < .004. **(D)** Cytokine levels in the medium supernatant of CD4⁺ cells co-cultured with untreated APC (medium) and ECDI-treated APC (ECDI) (mean $\pm$ SD, n = 4). *p < .002; ns: not significant.

Figure 3. ECDI-treated APC fail to up-regulate co-stimulatory signaling molecules and undergo increased apoptosis

Untreated APC (medium) or ECDI-treated APC (ECDI) were cultured for 5 days. **(A)** Cell viability as determined by trypan blue exclusion. (mean $\pm$ SD, $n = 3$, $*p < .001$) and **(B)** apoptosis rate determined by Annexin V and 7-AAD staining. **(C)** CFSE-labeled CD4⁺ cells were co-cultured with untreated APC (medium) or ECDI-treated APC (ECDI) and stained for CD25 and Foxp3. **(D)** Untreated APC (medium), ECDI-treated APC (ECDI) and freshly isolated APC were stained for CD40, CD80, CD86, HLA-DR and PD-L1. **(E)** Treg induction potential of defined APC subpopulations. CD4⁺ T cells were co-cultured with isolated and ECDI treated peripheral blood monocytes, B cells and dendritic cells (DC). After 5 days in culture, CD4⁺ T cells were analyzed for suppressive activity in a MLR as above. ($n=3$) **(F)** Co-cultures of untreated APC (med-APC, lower chamber) with CD4⁺ T cells and ECDI treated APC (ECDI-APC, upper chamber) with CD4⁺ T cells were separated by transwell inserts. After 5 days in culture CD4⁺ T cells from the lower and upper chamber were isolated and analyzed for suppressive activity in a secondary MLR with CFSE-labeled effector T cells (Teff) and APC from the original donors. ($n=3$)

Figure 4. Expansion and Foxp3 induction of ECDI-induced Tregs

Following the first stimulation cycle of isolated CD4⁺ T cells with irradiated allogeneic APC (medium) or ECDI-treated APC (ECDI) (first week), both cultures were restimulated with freshly isolated APC in culture medium supplemented with hrIL-2 and rapamycin three times at weekly intervals. **(A)** Scheme of the generation and expansion protocol used in this study. **(B)** Foxp3 protein levels in medium and ECDI cultured cells during expansion. Gated on live CD4⁺ cells. **(C)** Expansion of ECDI-induced Tregs (ECDI) and medium cells (medium) (mean $\pm$ SD, n = 6). **(D)** Relative Foxp3 mRNA expression in both cell types during expansion. (mean $\pm$ SD, n = 6) *p < .02. rapa: rapamycin.

Figure 5. Ex vivo expanded ECDI-induced Tregs retain their suppressive activity independent of rapamycin

After expansion with rapamycin and hrIL-2, cells originating from medium (medium, rapamycin) or ECDI primary cultures (ECDI, rapamycin) were harvested and added to a primary MLR with CFSE-labeled effector T cells (Teff) and APC from the original donors. **(A)** CFSE dilution profile showing the relative suppression of Teff proliferation at a Treg:Teff ratio of 1:1 (data representative of six equivalent experiments; gated on live CD4⁺ and CFSE⁺ cells). **(B)** Relative suppression of Teff proliferation at various Treg:Teff ratios (mean $\pm$ SD, n = 6) *p < .02. **(C)** Relative suppression assessed as in (A) and (B) for medium and ECDI cultured cells after presence (+ rapamycin) or absence of rapamycin (- rapamycin) during the last week of expansion in culture (mean $\pm$ SD, n = 3). *p < .002; ns: not significant.