

## Original article

## Antibody blocking of MHC II on human activated regulatory T cells abrogates their suppressive potential

Natural regulatory  $CD4^+CD25^+Foxp3^+$  T cells control peripheral immune responses. Freshly isolated regulatory T-cell populations are regarded as being unable to suppress the proliferation of strongly stimulated effector T cells. We now provide evidence that it is not the strength of the proliferative signal to effector T cells but activation and accessibility of regulatory T cells that determine whether suppression may occur. Human regulatory T cells were initially cocultured with allogeneic monocyte-derived dendritic cells for a short time and were then rendered accessible for effector T cells by removal of the dendritic cells. That way activated regulatory T cells effectively suppressed the proliferation of autologous effector T cells which was strongly driven by cell-sized Dynabeads coated with CD3/CD28 antibodies. Although regulatory T cells are known to display MHC II molecules and to upregulate their expression along with activation, a role of MHC II molecules in forming the contact to effector T cells was not yet envisaged. However, blocking of MHC II on activated regulatory T cells abrogated their suppressive potential. It should not be excluded that self-MHC molecules on physically accessible activated regulatory T cells arrange the contact to effector T cells.

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Accepted for publication 10 January 2007

Central tolerance is established by negative selection of self-reactive thymocytes and by thymocyte differentiation to natural regulatory T cells ( $T_{reg}$ ) whose task is to control peripheral immune responses (1). Impaired  $T_{reg}$  activity can cause autoimmune diseases and allergy (2).  $T_{reg}$  are hypoproliferative and need activation through their T-cell receptor (TCR) to become suppressive. *In vitro* TCR stimulation in presence of high IL-2 concentrations or together with CD28 ligation, however, results in  $T_{reg}$  proliferation and simultaneous loss of suppressive activity. After removal of IL-2 or of costimulation, the suppressive state reappears (3). Suppression is not antigen specific, inhibition of IL-2 synthesis in effector T cells ( $T_{eff}$ ) is involved, and cell contact between  $T_{reg}$  and  $T_{eff}$  is required (4–8). The molecules arranging these T-cell to T-cell contacts are unknown until now. After detection and characterization of murine  $T_{reg}$ , an equivalent human cell population with similar phenotypic and functional properties has been defined (9–14).

CD25 expression was found to be necessary for generation and maintenance of  $T_{reg}$  and was used to distinguish them from dormant  $T_{eff}$ . In mice,

$CD4^+CD25^+$   $T_{reg}$  constitute a focussed cell population being comprised of about 10% of the  $CD4^+$  T cells. The level of CD25 expression on murine  $T_{reg}$  is defined enough to allow straightforward antibody sorting. In humans,  $T_{reg}$  are less abundant. Exact numbers are unclear because human  $CD4^+CD25^+$  cells show ample variations in their actual CD25 expression levels.

The suppressive potential of  $T_{reg}$  was found to depend on the strength of the stimulating signal provided. The potency of suppression of freshly isolated human  $T_{reg}$  appeared to be only modest when compared with murine  $T_{reg}$  (15). In addition, fresh human  $T_{reg}$  lost suppressive capacity when costimulation with CD28 was provided (14).

It has been noted that not all human  $CD4^+CD25^+$  cells but only a small subpopulation of cells with the brightest CD25 expression has suppressor function (14–16). In addition, MHC II expression on  $CD25^{bright}$  cells identified an activated  $T_{reg}$  subpopulation that exerted early contact-dependent suppression (17). Thus, the true  $T_{reg}$  population in human peripheral blood would consist of only 1–3% of the  $CD4^+$  T cells (15), or even far below (16–18).

In short, the number of  $T_{reg}$  in human peripheral blood seems to be quite low, and they are regarded as being unable to suppress the proliferation of T cells stimulated by strong T-cell receptor (TCR) and costimulatory signals.

**Abbreviations:**  $T_{reg}$ ,  $CD4^+CD25^+$  natural regulatory T cells;  $T_{eff}$ ,  $CD4^+$  effector T cells;  $T_{reg[sep]}$ , moDC-activated  $T_{reg}$  after removal of moDCs; moDC, monocyte-derived dendritic cell.

To regulate immune responses efficiently, the suppressive activity of such limited numbers of  $T_{reg}$  in peripheral blood might need amplification, possibly provided by a platform on which the suppressor cells may physically interact with multiple effector cells. Such a platform might be formed by the cell surface of antigen presenting cells (APC) (6, 19). We have cocultured freshly isolated human  $T_{reg}$  for a short time with allogeneic monocyte-derived dendritic cells (moDC). Then the  $T_{reg}$  were introduced either directly or after removal of moDC into a  $T_{eff}$  proliferation assay which was strongly driven by cell-sized Dynabeads coated with anti-CD3 and anti-CD28 monoclonal antibodies. Any suppressive potential was observable only when the  $T_{reg}$  were at first preactivated by moDC and were then re-isolated. Under such a condition,  $CD4^+CD25^+Foxp3^+$  cell preparations could effectively suppress maximally stimulated  $T_{eff}$  cells.

It is as yet unknown which molecules form the bridge between  $T_{reg}$  and  $T_{eff}$  that leads to suppression. We found that blocking of MHC II on activated  $T_{reg}$  abrogated their suppressive potential.

## Materials and methods

All cells were cultured in RPMI 1640 medium with ultraglutamine 1 (Cambrex, Verviers, Belgium) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% heat inactivated fetal calf serum (all from Biochrome, Berlin, Germany).

### Isolation of cells

Whole blood buffy coat preparations of anonymous healthy donors were obtained from the Red Cross Blood Donation Service. The buffy coats were diluted 1 : 2 with PBS (Gibco, Grand Island, NE, USA) and centrifuged over a Ficoll-Hypaque (Biochrome, Berlin, Germany) density gradient. PBMC were collected from the interphase and washed extensively with cold PBS until the supernatant was clear. After the second wash step, the cells were incubated for 5 min in Erythrocyte Lysis Buffer (Qiagen, Hilden, Germany) to eliminate contaminating erythrocytes.

### Generation of monocyte-derived dendritic cells

Monocytes were enriched by adherence on tissue culture dishes (Greiner, Frickenhausen, Germany) for 1 h at 37°C and by subsequently washing with cell culture medium. Monocyte-derived dendritic cells were generated by culturing monocytes for 6 days in 100 ng/ml IL-4 and GM-CSF (both from Immunotools, Friesoythe, Germany) which were added on alternate days. To develop a more mature phenotype, the moDC were treated on day 5 with 1 µg/ml LPS (*Escherichia coli* serotype 0128:B12; Sigma, Deisenhofen, Germany). FACS analyses documenting the differentiation of monocytes to moDC and their maturation after LPS treatment are provided in the Supplementary material.

### Enrichment of $CD4^+$ T-cell subsets

$CD4^+$  T cells were isolated from the nonplastic adhering PBMC-fraction by depletion using the  $CD4^+$  T Cell Isolation Kit II

(Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's guidelines. Magnetic sorting was performed on a LS-Column using a SuperMACS (Miltenyi Biotech).

$CD4^+/CD25^+$  T cells (regulatory T cells;  $T_{reg}$ ) were further isolated from the  $CD4^+$  T-cell fraction by positive selection via direct labeling with anti-CD25-coated microbeads (10 µl/ $10^7$  cells; Miltenyi Biotech). The labeled cells were passed twice over LS-Columns. The anti-CD25 antibody on microbeads recognized epitope A of CD25, the FITC-conjugated anti-CD25 antibody used for flow cytometry recognized epitope B.

$CD4^+/CD25^-$  T cells (effector T cells,  $T_{eff}$ ) were collected from the flow-through.

### Activation of $CD4^+/CD25^+$ T cells

Freshly isolated regulatory T cells were cocultured with allogeneic moDC at  $T_{reg}$  (–) moDC ratios of 40 : 1, 10 : 1, or 1 : 1. For most of the experiments  $10^7$   $T_{reg}$  were cocultured with  $10^6$  moDC. After 20 h of  $T_{reg}$ /moDC coculture,  $T_{reg}$  were introduced into the  $CD4^+$  T-cell proliferation assay, either directly or after removal of moDC. Separation was performed by MACS using the still bound anti-CD25 microbeads. Such  $T_{reg}$  cells, cocultured with moDC for 20 h and then re-isolated, are referred to as  $T_{reg[sep]}$ .

### $CD4^+$ T-cell proliferation assay

To examine the suppressive capacity of cocultured  $CD4^+/CD25^+$  T cells, an autologous CFSE-based proliferation assay was used.  $CD4^+/CD25^-$  T cells were stained with CFSE (Molecular Probes, Eugene, OR, USA) at a final concentration of 50 nM for 10 min at RT. Remaining CFSE was removed by washing with cell culture medium, and the stained cells were cultured in 96-well-plates at  $10^5$  cells per well.  $CD4^+/CD25^+$  T cells treated as indicated were added to the CFSE-stained responder T cells at ratios varying from 1 : 8 to 1 : 1. Also the suppressive capacity of cell-free supernatants from moDC/ $T_{reg}$  cocultures was tested by adding the supernatants to the CFSE-stained  $CD4^+/CD25^-$  cells together with nonactivated  $T_{reg}$  at a responder T cell :  $T_{reg}$  ratio of 1 : 1. Experiments were carried out with both LPS-matured and with immature moDC. Proliferation was induced by  $10^4$  anti-CD3/anti-CD28-coated Dynabeads (Dynal Biotech, Oslo, Norway) per well or by 5 µg/ml Phytohemagglutinin-M (PHA, from *Phaseolus vulgaris*; Calbiochem, San Diego, CA, USA).

Proliferation of the CFSE-stained responder T cells was determined by flow cytometry using a FACSCalibur (BD Biosciences, Heidelberg, Germany). CFSE-stained T cells without Dynabead treatment were used to set compensation.

### Blocking experiments with anti-HLA-antibody and anti-CD2-antibody

Effects of anti-HLA-antibody and anti-CD2-antibody were tested in the proliferation assay. For blocking HLA, we used mouse anti-human IgG2a, recognizing all major HLA-DR, HLA-DP and most HLA-DQ antigens (mouse IgG2a, clone TŮ39; BD Biosciences).  $T_{reg}$  cells were incubated with 50 µg/ml antibody for 45 min at 4°C and thereafter washed in RPMI 1640. Subsequently, the cells were cocultured in proliferation assays as described above. Monoclonal anti-CD2-antibody (mouse IgG1, clone TS2/18; Endogen, Woburn, MA, USA) was used to block CD2. Anti-CD2 was added to the  $T_{reg}$ /moDC coculture at 1 µg/ $10^5$  T cells. After 20 h the cells were washed with RPMI and introduced into the proliferation assay.

## FACS analyses

Antibody staining was measured with a FACSCalibur (BD Biosciences). Analysis was performed with WinMDI software (Scripps Research Institute, La Jolla, CA, USA). Antibodies used were: Cy5-conjugated anti-CD4 (mouse IgG1, clone RPA-T4), FITC-conjugated anti-CD25 (mouse IgG1, clone M-A251), FITC-conjugated anti-CD86 (mouse IgG1, clone 2331[FUN-1]), FITC-conjugated anti-CD14 (mouse IgG1, clone M5E2), all from BD Biosciences; PE-conjugated anti-HLA-DQ (IQ Products, Groningen, the Netherlands), PE-conjugated anti-HLA-DR (IgG2b, clone B8.12.2, Immunotech, Marseille, France). PE anti-human Foxp3 PCH101 was from eBioscience (Kranenburg, Germany), and for intracellular staining, cells were fixed using BD Cytofix/Cytoperm (BD Biosciences). Isotype controls were: Cy5-conjugated mouse IgG1 (clone MOPC-21), FITC-conjugated mouse IgG1 (clone MOPC-21), PE-conjugated mouse IgG2b (clone 49.2) (all from BD Biosciences).

## ELISA

Interleukin-6 was measured in supernatants of 20-h  $T_{reg}$ /moDC cocultures with a DuoSet ELISA kit (R&D Systems, Wiesbaden-Nordenstadt, Germany).

## Microarray

RNA was isolated using an RNeasy Kit (Qiagen). According to Affymetrix' target preparation and hybridization protocols, cDNA was synthesized using HPLC-purified T7-dT24-primer (MWG, Ebersberg, Germany) and Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany). Second strand synthesis was performed with *E. coli* DNA ligase, DNA polymerase I, and RNase H (all from Invitrogen). Clean up of double-stranded cDNA was done by phenol/chloroform extraction. Biotinylated cRNA was prepared using the BioArray High Yield RNA Transcription kit (Enzo Diagnostics, Farmingdale, NY, USA). The amplified cRNA was purified with an affinity resin column (Qiagen), heat-fragmented, quantified by UV-spectroscopy and analyzed on a LabChip Bio-analyzer (Agilent, Santa Clara, CA, USA). HG-U 133 A 2.0 chips were hybridized and analyzed according to standard Affymetrix protocols. Data were deposited in the NCBI-GEO depository under accession number GSE3996.

## Results

Anti CD3/CD28-coated Dynabeads most strongly induce the proliferation of effector T cells

The suppressive potential of natural regulatory T cells ( $T_{reg}$ ) was reported to depend on the strength of the stimulating signal provided to  $CD4^+CD25^-$  effector T cells (Fig. 1A). We therefore compared at first the  $T_{eff}$  proliferation rates resulting from activation by allogeneic moDC, by the mitogen PHA, and by cell-sized Dynabeads coated with anti-CD3 and anti-CD28 monoclonal antibodies. Stimulation of  $T_{eff}$  with moDCs induced 37% of the  $T_{eff}$  to proliferate. With PHA, 67% were induced to divide (Fig. 1B). The Dynabeads induced a maximal and sustained proliferation of  $T_{eff}$  (Fig. 1B). About 90% of the CFSE-labeled cells divided and most of them did so several times.

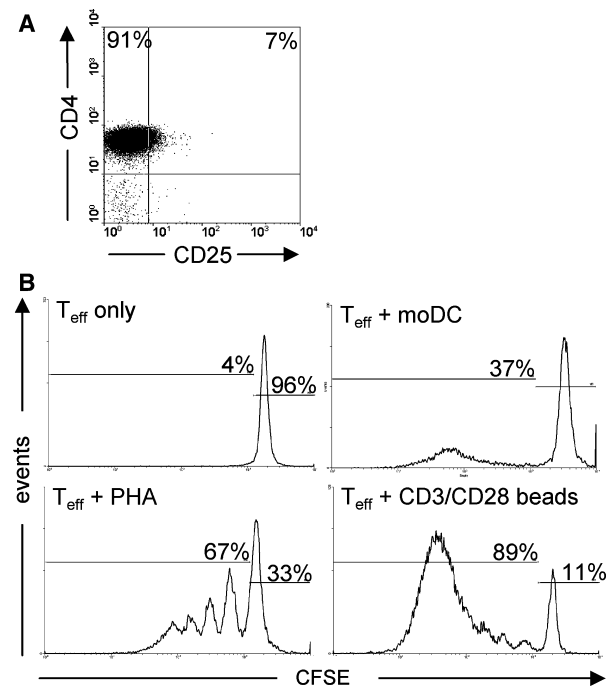


**Figure 1.** CD3/CD28-coated beads induce a maximal and sustained proliferation of effector T cells ( $T_{eff}$ ). (A) FACS analysis of magnetically sorted  $T_{eff}$ . (B) To compare the strength of proliferation signals, CFSE-stained  $CD4^+CD25^-$   $T_{eff}$  were cultured without further treatment ( $T_{eff}$  only), or were stimulated by coculture with allogeneic immature monocyte-derived dendritic cells (moDC) (10 : 1) ( $T_{eff}$  + moDC), with 5  $\mu$ g/ml phytohemagglutinin ( $T_{eff}$  + PHA), or with  $10^4$ /well cell-sized Dynabeads coated with anti-CD3 and anti-CD28 monoclonal antibodies ( $T_{eff}$  + CD3/CD28 beads). Shown are representative results of one of five  $T_{eff}$  donors.

Short-time coculture with allogeneic moDCs followed by re-isolation strongly activates the suppressive capacity of  $CD4^+CD25^+Foxp3^+$  regulatory T cells

Purification of  $CD4^+/CD25^+$  T cells yielded coherent cell populations. The majority of such freshly isolated cells expressed the markers of natural regulatory T cells ( $T_{reg}$ )  $CD4$ ,  $CD25$ , and  $Foxp3$  (Fig. 2A). The cell numbers of  $CD4^+CD25^+Foxp3^+$  T-cell populations varied from 10% to 14% of total  $CD4^+$  cells. One buffy coat prepared from 500 ml whole blood yielded about  $10^7$  of such  $CD4^+CD25^+Foxp3^+$  T cells.

For activation of regulatory T cells, fresh  $CD4^+CD25^+Foxp3^+$  cells were cocultured with immature or with LPS-matured allogeneic moDCs (for characterization of moDC please see Fig. S1A,B). Then, the  $T_{reg}$  were re-isolated from the moDC. FACS analyses showed that fresh  $T_{reg}$  and re-isolated  $T_{reg}$  were constituted of cell-populations alike in their  $CD4/CD25/Foxp3$ -expression levels, in purity and in vitality (Fig. 2A/B; Fig. S1C,D). Also, overnight culture of  $T_{reg}$  alone did not alter the

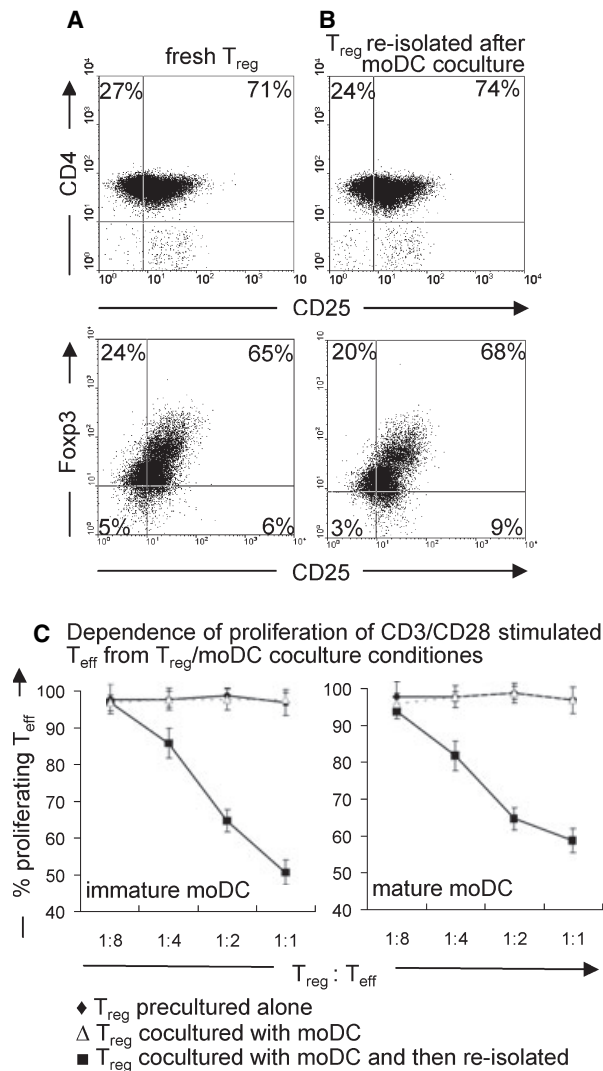


**Figure 2.** The proliferation of strongly stimulated  $T_{eff}$  is suppressed by those regulatory T cells ( $T_{reg}$ ) which were at first activated by short-time coculture with monocyte-derived dendritic cells (moDC) and were then re-isolated. (A, B) FACS analysis of magnetically isolated  $T_{reg}$ . Freshly isolated  $T_{reg}$  (A) and  $T_{reg}$  cocultured with moDC for 20 h followed by re-isolation (B). Analyses were performed without using gates. (C) To analyze the suppressive potential of  $T_{reg}$ , CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>  $T_{reg}$  were mixed with CFSE-stained CD4<sup>+</sup>CD25<sup>-</sup>  $T_{eff}$  at indicated ratios and stimulated with cell-sized Dynabeads coated with anti-CD3 and anti-CD28 monoclonal antibodies. Before the onset of coculture with  $T_{eff}$ ,  $T_{reg}$  either have been precultured alone (◆), or have been cocultured with moDC at a 10 : 1 ratio for 20 h and were then introduced into the proliferation assay together with the moDC (Δ), or after removal of the moDC (■). Activating moDC were immature or matured by LPS treatment. The results are shown as triplicates of one donor and are representative for eight of 10  $T_{reg}$  donors. In two of 10 donors, suppression by  $T_{reg[sep]}$  was not detectable.

cell's appearance (not shown). There was no definable population of activated CD25<sup>+</sup>Foxp3<sup>+</sup> T cells which later might give rise to T-cell anergy (Fig. 2A/B).

The  $T_{reg}$  were introduced either together with the moDCs or after removal of the moDC (Fig. 2B) into the  $T_{eff}$  proliferation assay (Fig. 2C). The proliferation of  $T_{eff}$  was suppressed only by those  $T_{reg}$  which were activated by the moDC and were then re-isolated. At a 1 : 1  $T_{reg}/T_{eff}$  ratio, the re-isolated  $T_{reg}$  reduced the proliferation of  $T_{eff}$  to about 50% (Fig. 2C). Both, immature and LPS-matured moDCs were comparably effective in  $T_{reg}$  activation (Fig. 2C).  $T_{reg}$  that had not been activated by moDCs, and  $T_{reg}$  that had not been separated from activating moDCs showed no suppressive capacity (Fig. 2C).

For  $T_{reg}$  activation by moDCs, we tested  $T_{reg} : \text{moDC}$  ratios of 40 : 1, 10 : 1, and 1 : 1. All these ratios yielded comparable results regarding suppression of  $T_{eff}$  proliferation (not shown).

The activated and then re-isolated  $T_{reg}$  are further referred to as  $T_{reg[sep]}$ .

Suppression of T-cell proliferation depends on cell contacts and on MHC II

Cell free supernatants of  $T_{reg}/\text{moDC}$ -cocultures were introduced into the  $T_{eff}$  proliferation assay together with nonactivated  $T_{reg}$  or with  $T_{reg[sep]}$  re-isolated from the preactivating coculture (Fig. 3). The media conditioned by coculture with immature and with LPS-matured moDCs were ineffective in suppression of  $T_{eff}$  proliferation. The conditioned media neither contained any molecules able to activate the  $T_{reg}$ , nor any molecules able to act directly on  $T_{eff}$ . This indicates that both, activation of  $T_{reg}$  by moDCs and suppression of  $T_{eff}$  proliferation by  $T_{reg[sep]}$  depend on cell contacts.

Looking for pairs of cell contact molecules and surface receptors/ligands, which are shared by moDCs,  $T_{reg}$ , and  $T_{eff}$  and which therefore might give rise to competition for intercellular binding, we came across the pair CD2/CD58. CD2 antibody blocking on  $T_{reg}$  before coculturing them with moDCs did, however, not affect the suppressive capacity of the resulting  $T_{reg[sep]}$  (not shown).

We then compared the nonactivated  $T_{reg}$  without moDC-contact with  $T_{reg[sep]}$  in their global gene expression using Affymetrix HG-U 133 A 2.0 chips. Fifty-four percent of the genes represented on the chip were active in regulatory T cells, and only a limited number of genes were differentially up- or down-regulated in response to moDC-contact (Fig. 4). Surprisingly, activated  $T_{reg}$  themselves seemed to transcribe chemokines as CCL17 and CCL22, which might influence the trafficking of activated  $T_{eff}$  (Fig. 4). Grouping showed that the expression of a striking number of MHC II related genes was up-regulated with  $T_{reg}$  activation (Fig. 4).

FACS analysis of MHC II molecules revealed in  $T_{reg[sep]}$  a HLA-DR<sup>bright</sup>/CD25<sup>+</sup> population consisting of about 4% of total cells which were not present in nonactivated  $T_{reg}$  (Fig. 5A). In addition,  $T_{reg}$  activation detectably increased HLA-DQ expression (Fig. 5A).

Dependence of proliferation of CD3/CD28 stimulated  $T_{eff}$  from  $T_{reg}$ /moDC coculture-conditioned media

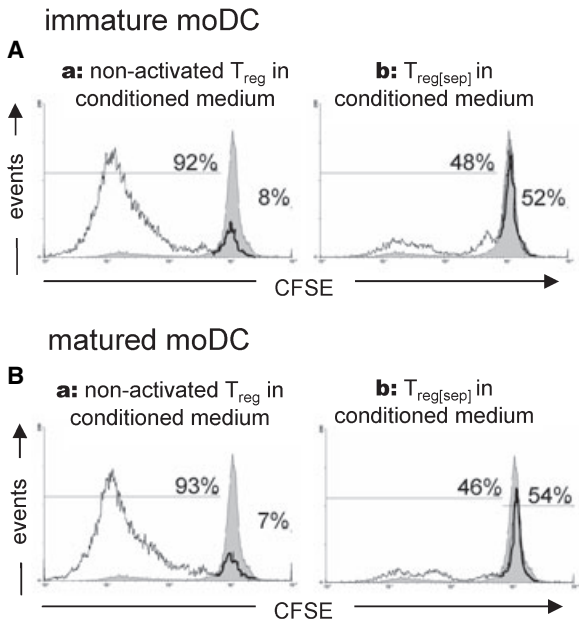


**Figure 3.** Media conditioned by coculturing of  $T_{reg}$  and monocyte-derived dendritic cells (moDC) did not affect  $T_{reg}$  activation and  $T_{eff}$  proliferation. (A)  $T_{reg}$  were cocultured with immature moDC for 20 h at a 10 : 1 ratio. Then, cell-free conditioned media of such cocultures were gathered, and activated  $T_{reg}$  were re-isolated. Two sets of Dynabead-driven  $T_{eff}$  proliferation assays were initiated: in the conditioned media, nonactivated  $T_{reg}$  (a), or the activated and re-isolated  $T_{reg[sep]}$  (b) were cocultured with CFSE-stained  $T_{eff}$  at a 1 : 1 ratio. CFSE fluorescence intensity was determined on day 6. (B) Same as in A, but moDC have been matured by LPS. Shaded histograms refer to CFSE-stained  $T_{eff}$  without Dynabead stimulation. Shown are the results of one experiment representative for five independent experiments with varying blood donors (MFI in% correspond to the mean of triplicates).

The staining of MHCII cannot be assigned to potential residual nonseparated moDC, because these did not express CD25, and in addition, the level of MHCII staining on  $T_{reg[sep]}$  was much brighter than on moDC (Fig. S1E).

MHC II antibody blocking on  $T_{reg[sep]}$  before using them in the proliferation assay abolished their suppressive capacity (Fig. 5B). Blocking with an irrelevant antibody did not effect  $T_{reg[sep]}$ 's regulatory potential (Fig. 5B).

Discussion

The immunosuppressive potential of  $T_{reg}$  in therapy of autoimmune diseases and in inhibition of undesirable allogeneic responses after transplantation could be demonstrated in animal model systems (1) (20–23). *In vivo*,  $T_{reg}$  even displayed antigen specificity, which would

HG-U 133A 2.0 array: 14500 genes

in  $T_{reg}$  active: 54 %

differentially regulated in response to moDC-contact:

upregulated > x 2 : 3.4 %  
> x 5 : 0.5 %  
downregulated < x 0.5 : 3.1 %  
< x 0.2 : 0.1 %

| HLA-related |        | Chemokine-related |        |
|-------------|--------|-------------------|--------|
| Gene name   | Factor | Gene name         | Factor |
| HLA-DQ A1   | 73,6   | CCL17 (TARC)      | 42,0   |
| HLA-DQ α    | 4,6    | CXCL9 (MIG)       | 24,9   |
| HLA-DQ β    | 4,6    | CCL18 (MIP-4)     | 19,6   |
| HLA-DR A    | 4,4    | CCL13 (MCP-4)     | 13,0   |
| HLA-DP A1   | 2,8    | CCL22 (MDC)       | 8,8    |
| HLA-DP ω3α1 | 2,8    | CCL5 (RANTES)     | 0,2    |
| HLA-DM α    | 2,5    | S100-related      |        |
| HLA-DM β    | 2,7    | Gene name         | Factor |
| HLA-DR ω6   | 2,2    | S100 A12          | 0,09   |
| HLA-DR B6   | 2,1    | S100 A8           | 0,14   |
| HLA-DR B4*  | 2,0    | S100 A9           | 0,20   |
| CD74        | 5,4    | S100 A14          | 0,34   |

**Figure 4.** Gene expression analysis of  $T_{reg[sep]}$  vs nonactivated  $T_{reg}$  using Affymetrix HG-U 133 A 2.0 chips. Quantitative removal of monocyte-derived dendritic cells before RNA preparation was proven by FACS-analysis (not shown, but comparable with Fig. 2B). Additionally, expression of the typical myeloid DC-markers CD11c and CD33 was found to be absent. Complete microarray data are available under accession-number GSE3996 in the NCBI-GEO depository.

permit a  $T_{reg}$ -based therapy with limited risk of development of general immunosuppression (24).

One of the difficulties in the clinical use of  $T_{reg}$  would be to obtain sufficient number of competent cells from a single donor. We have isolated human  $T_{reg}$  using a routinely applicable technique. The number of real  $T_{reg}$  in this preparation is unknown. However, after short-time coculture with moDC followed by re-isolation, the  $T_{reg}$  achieved the potential to suppress the expansion of strongly stimulated  $T_{eff}$  (Fig. 2C).

Populations of about  $10^7$  cells containing suppressive  $T_{reg}$  were routinely obtained from 500 ml blood donations (Fig. 2A/B). The percentage of true  $T_{reg}$  is unclear. However, dilution of nominal  $T_{reg}$  in the proliferation assay did not result in a quick loss of suppressive function

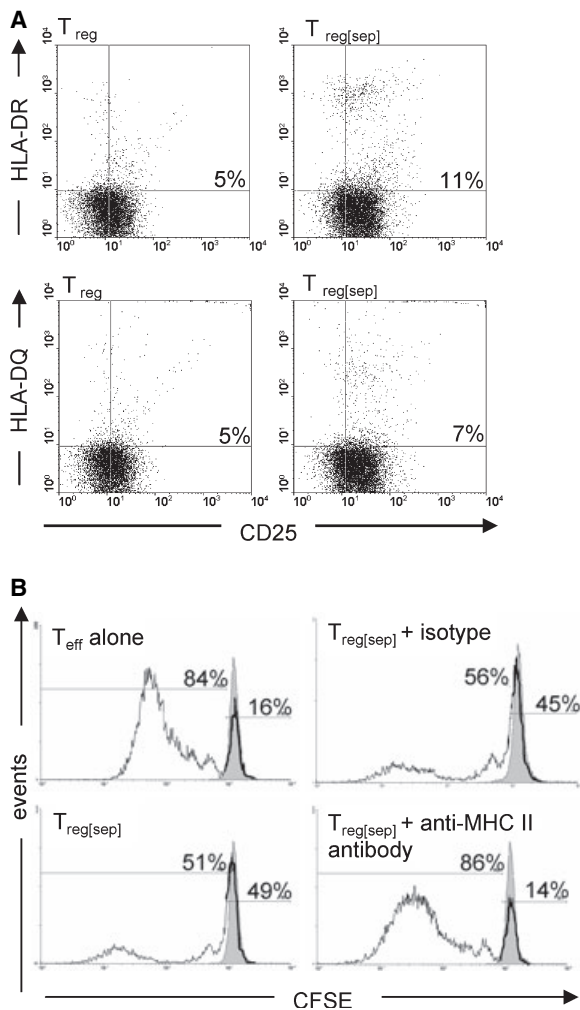


**Figure 5.** Activation of T<sub>reg</sub> by monocyte-derived dendritic cells (moDC) increased HLA-DR and HLA-DQ levels, and antibody blocking of MHC II abolished the suppressive potential of T<sub>reg[sep]</sub>. (A) FACS analysis of nonactivated T<sub>reg</sub> (T<sub>reg</sub>) and of T<sub>reg</sub> separated from cocultured moDC (T<sub>reg[sep]</sub>). Note the HLA-DR<sup>bright</sup>/CD25<sup>+</sup> population in T<sub>reg[sep]</sub>. (B) CFSE-stained T<sub>eff</sub> were stimulated with CD3/CD28-Dynabeads, and after 6 days of cell culture, staining intensity was determined. T<sub>eff</sub> were either cultured alone (T<sub>eff</sub>), or with T<sub>reg[sep]</sub> at a 1 : 1 ratio (T<sub>reg[sep]</sub>), or with T<sub>reg[sep]</sub> at a 1 : 1 ratio plus irrelevant antibody control (T<sub>reg[sep]</sub> + isotype), or with T<sub>reg[sep]</sub> at a 1 : 1 ratio plus anti-MHC II blocking monoclonal antibody (T<sub>reg[sep]</sub> + anti-MHC II). Shown in A and B is a typical of five independent experiments.

(Fig. 2C). This indicates that the T<sub>reg</sub> population contained fair numbers of suppressive cells (15). It is unknown which T<sub>reg</sub> cell numbers might be sufficient to evoke a clinical effect. The activity of T<sub>reg</sub>-numbers therapeutically applied may be amplified as T<sub>reg</sub> are able to induce secondary suppressor cell populations (25).

It is well documented that T<sub>reg</sub> need activation through their TCR to become suppressive. This activation may be performed by allowing contact with various types of allogeneic or antigen-pulsed autologous APC or to

surrogate APC composed of beads coated with anti-CD3 or anti-CD3/CD28 antibodies (15, 18, 24, 26, 27). All these applications are also involved in T<sub>eff</sub> stimulation. We have used allogeneic moDC. These activated only alloantigen-specific T<sub>eff</sub> and induced only low proliferation rates as compared to those after polyclonal T<sub>eff</sub> activation using CD3/CD28 beads (Fig. 1B). In difference, the suppressive capacity of T<sub>reg</sub> was more effectively activated by moDC than by beads (Fig. 2C). Although IL-2 was not exogenously added, it might be argued that moDC were superior, because CD28 costimulation supplied by the beads might render the T<sub>reg</sub> rather proliferative than suppressive. However, matured moDC with increased CD86 levels (Fig. S1A,B) were comparably successful in T<sub>reg</sub> activation as immature moDC (Fig. 2C).

It might be conceivable that the suppression of T<sub>eff</sub> proliferation might be caused by activation induced cell death (AICD). Residual nonregulatory T cells present in the T<sub>reg</sub> population might have been repeatedly stimulated, at first by moDC and then by the beads. Such T cells could then have induced AICD and could have hindered the growth of nearby cells in culture. However, if AICD would have been involved in the suppression of T<sub>eff</sub> proliferation, suppression would have occurred also in those proliferation assays which used T<sub>reg</sub> nonseparated from preactivating moDC. In addition, there were no typical activated CD25<sup>+</sup>Foxp3<sup>-</sup> T-cell populations detectable in the T<sub>reg</sub> preparations (Fig. 2A/B).

The moDC provided the T<sub>reg</sub> with a coactivating factor that was missing on the Dynabeads which served as surrogate APC. This missing factor seemed not to be soluble, because it could not be passed to the T<sub>reg</sub> in the proliferation assay by addition of moDC/T<sub>reg</sub> conditioned medium (Fig. 3). Murine bone marrow-derived DC have been found to block T<sub>reg</sub>-mediated suppression partially via IL-6 secretion (28). For human moDC, a role of IL-6 in blocking of T<sub>reg</sub> suppressive activity could be excluded, as IL-6 was not detectable in moDC/T<sub>reg</sub> cocultures as determined by ELISA (not shown).

A possible candidate for stabilizing moDC/T<sub>reg</sub> contacts might be CTLA-4 (CD152), which is highly expressed on T<sub>reg</sub> and which could be ligated by moDC-surface molecules but not by the beads used (1). A preferential binding of CD80/CD86 on moDC to CTLA-4 at the expense of CD28 on T<sub>reg</sub> might explain why mature moDC did not shift the T<sub>reg</sub> to a proliferative/nonsuppressive state. Among further candidates for the unknown molecular interaction between moDC and T<sub>reg</sub> might be targeting of DC-SIGN which was shown to be involved in T<sub>reg</sub> induction (27).

Our results indicated that the avidity between T<sub>reg</sub> and moDC was higher than that between T<sub>reg</sub> and T<sub>eff</sub>. It was necessary to separate the activated T<sub>reg</sub> from the moDC by magnetic sorting to get observable suppression in the proliferation assay (Fig. 2C). The interaction of moDC/T<sub>reg</sub> centered around the TCR interfered with the assembly of functional T<sub>reg</sub>/T<sub>eff</sub> contacts. This must not

necessarily indicate that the TCR of  $T_{reg}$  is involved in forming  $T_{reg}/T_{eff}$  contacts. Like the TCR itself (29), many cell surface molecules are not equally distributed, and cellular contacts between  $T_{reg}$  and moDC might physically hinder the formation of the bridge between  $T_{reg}$  and  $T_{eff}$ . If so,  $T_{reg}$  sticking on moDC would modulate the function of moDC by constricting access and activation of  $T_{eff}$ . In addition, only  $T_{reg}$  unattached to moDC might act as direct suppressors of  $T_{eff}$  expansion.

Murine bone marrow-derived dendritic cells blocked the suppressive activity of  $T_{reg}$  (28), and murine  $T_{reg}$  were shown to mediate their suppressor function in the absence of APC via a T–T cell interaction (30). Differential gene expression analysis after  $T_{reg}$  activation pointed to an upregulation of MHC II (Fig. 4). This result was supported by FACS analysis (Fig. 5A). A subpopulation of moDC-activated  $T_{reg}$  reached levels of HLA-DR and also of HLA-DQ that exceeded even those typical of mature moDC (Fig. 5A). It might be assumed that it is this MHC II<sup>bright</sup> subpopulation that represents true regulatory T cells in our preparation. In fact, it was reported that humans harbor an activated  $T_{reg}$  subpopulation of  $CD4^+CD25^+MHCII^+FoxP3^{high}$  cells involved in early contact-dependent suppression (17).

Human  $T_{reg}$  are already known to express HLA-DR directly *ex vivo* (15) and to upregulate it with activation (10, 17). However, a direct role of MHC II molecules on  $T_{reg}$  in forming the contact to  $T_{eff}$  was never envisaged. At a first glance, this might appear curious, because all of the T cells in the proliferation assay were autologous. But still, antibody blocking of MHC II on  $T_{reg[sep]}$  using an antibody with proven blocking capacity (31) abrogated their suppressive capacity (Fig. 5B). The TCR is committed to recognition of MHC molecules. Only positively selected thymocytes harboring a useful TCR with weak to intermediate avidity to self-MHC continue their development. A substantial body of evidence indicates that survival and expansion of peripheral naïve T cells continuously depend on recognition of self MHC II complexes (32–35). This means that the TCR repertoire is able to interact with self-MHC molecules. It might be conceivable that increased MHC II epitope densities on

activated  $T_{reg}$  could exceed a threshold allowing a productive interaction between MHC of  $T_{reg}$  and TCR of  $T_{eff}$ . Such an interaction would be independent from the presented peptide and would be in line with the nonspecific activity of  $T_{reg}$ .

One should not exclude the possibility that the activated  $T_{reg}$  represents a kind of antigen-presenting cell that attracts  $T_{eff}$ , adheres to them via increased self-MHC II – TCR avidity, and renders them anergic.

### Acknowledgment

We thank cand. med. Maximilian Schreiner for technical help.

### Supplementary material

The following supplementary material is available for this article:

Figure S1. FACS analysis documenting the differentiation of monocytes to moDC (A) and their maturation after LPS treatment (B). Grey, isotype controls. The analyses were performed routinely (more than 25 times). Shown are representative results. Forward/sideward scatter analysis of fresh  $T_{reg}$  (C) and of re-isolated  $T_{reg[sep]}$  (D). Cells were analysed without gating. Shown are typical results of 10 experiments. FACS analysis of CD25 and HLA-DR expression on moDC (E). The quadrant lines demarcate the isotype controls. Shown is a typical result of three independent experiments.

This material is available as part of the online article from: <http://www.blackwell-synergy.com/doi/abs/10.1111/j.1398-9995.2007.01339.x> (This link will take you to the article abstract).

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