Published in final edited form as:

Hum Immunol. 2005 March ; 66(3): 222–230. doi:10.1016/j.humimm.2004.12.006.

Modulation of monocyte/macrophage function by human CD4+CD25+ regulatory T cells

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SUMMARY

The suppressive effects of CD4+CD25+ regulatory T cells (Tregs) on T cells have been well documented. Here we investigated whether human CD4+CD25+ Tregs can inhibit the pro-inflammatory properties of monocytes/macrophages. Monocytes and T cells were isolated from peripheral blood of healthy volunteers by magnetic cell separation, and co-cultured for 40 hours. Monocytes were analyzed directly for cytokine production and phenotypic changes, or re-purified and used in T cell stimulation and LPS challenge assays. Co-culture with CD4+CD25+ Tregs induced minimal cytokine production in monocytes, whereas co-culture with CD4+CD25- T cells resulted in large amounts of pro-inflammatory (TNF- α , IFN- γ IL-6) and regulatory (IL-10) cytokines. Importantly, when these CD4+CD25+ Treg-treated monocytes were re-purified after co-culture and challenged with LPS, they were severely inhibited in their capacity to produce TNF- α and IL-6 compared to control-treated monocytes. In addition, monocytes that were pre-cultured with CD4+CD25+ Tregs displayed limited up-regulation of HLA class II, CD40 and CD80, and down-regulation of CD86 compared to control-treated monocytes. This altered phenotype had functional consequences, as shown by the reduction in T cell-stimulatory capacity of Treg-treated monocytes. Together these data demonstrate that CD4+CD25+ Tregs can exert direct suppressive effects on monocytes/macrophages, thereby affecting subsequent innate and adaptive immune responses.

Keywords

Suppression; antigen-presenting cell; rheumatoid arthritis

INTRODUCTION

CD4+CD25+ regulatory T cells (Tregs) are key components of our peripheral tolerance system. Depletion of CD4+CD25+ Tregs results in spontaneous organ-specific autoimmunity in mice, and enhanced in vitro T cell proliferation in humans [1-5]. Reconstitution of CD4+CD25+ Tregs prevents this immune hyper-reactivity. Recent data suggest that in humans these cells might play a role in the prevention and/or control of rheumatoid arthritis [6, 7], multiple sclerosis [8] and autoimmune polyglandular syndrome

type II [9]. Boosting of these cells by specific targeting might therefore become an interesting immunotherapeutic avenue for the treatment of autoimmunity.

Besides their role in autoimmune diseases CD4+CD25+ Tregs exert regulatory functions in the control of transplantation tolerance [10], tumor immunity [11-13] and infection [14, 15]. The broad manifestation of suppressive effects in a variety of experimental models and clinical diseases suggests that CD4+CD25+ Tregs are able to inhibit the function of many different cell types. Indeed CD4+CD25+ Tregs can suppress T cell proliferation of both CD4+ [3, 16-18] and CD8+ T cells [19, 20]. In addition, CD4+CD25+ Tregs inhibit autoantibody production [21, 22], which might be the result of inhibition of CD4+ T cell help to B cells or of direct inhibition of B cell-mediated Ig production [23]. A recent study demonstrated that pre-activated CD4+CD25+ Tregs could suppress proliferation and IFN- γ production of CD8+ T cells that were stimulated with MHC I-peptide tetramers in the absence of antigen-presenting cells (APC) [19]. This provided evidence that CD4+CD25+ Tregs mediate T cell suppression via direct T-T cell interactions, and that the presence of APC is not required for their suppressive effects on T cells.

However, this finding does not exclude the possibility that CD4+CD25+ Tregs have direct inhibitory effects on professional APC, such as monocytes/macrophages, dendritic cells (DC) or B cells, as was described previously for anergic/suppressive CD4+ T cell clones [24-26] and alloantigen-specific CD8+CD28- T suppressor cells [27]. Indeed, Cederbom et al. showed that murine CD4+CD25+ Tregs down-regulated CD80 and CD86 expression on bone marrow derived DC and splenic B cells, suggesting that their antigen-presenting function might be affected [28]. These findings have recently been confirmed in humans in a study showing decreased antigen-presenting function of DC upon interaction with Tregs which coincided with down-regulated expression of CD40, CD86 and MHC class II [29].

In chronic inflammatory conditions such as rheumatoid arthritis, monocytes/macrophages contribute extensively to the chronic inflammatory process and tissue destruction via the production of pro-inflammatory cytokines such as TNF- α and IL-6 and via their T cell-stimulatory capacity [30-33]. Down-regulation of monocyte/macrophage function is therefore an attractive aim in the treatment of rheumatoid arthritis. The current study shows that CD4+CD25+ Tregs are able to exert direct inhibitory effects on the activation and function of human monocytes/macrophages, thereby hampering both the innate and adaptive effector functions of these cells.

MATERIALS & METHODS

Cell isolation and purification

Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated using Ficoll-Isopaque centrifugation. CD4+ T cells were purified (>95% purity) via magnetic depletion of CD8+ T cells, B cells, NK cells and monocytes/macrophages as described previously [3, 4]. Purified CD4+ T cells were incubated with mouse-anti-human CD25-beads (Miltenyi Biotec, Bergisch-Gladbach, Germany) and separated into CD4+CD25+ and CD4+CD25- T cells on a positive selection column (Miltenyi Biotec). Purities of CD4+CD25- T cells and CD4+CD25+ T cells were >98% and >80%, respectively [3, 4]. Monocytes were isolated via positive selection using anti-CD14 mAb, followed by incubation with goat-anti-mouse IgG beads and selection on an positive selection column (average purity >95%).

Cell culture and T cell proliferation

Purified CD14+ monocytes ($2.5-5 \times 10^5$ cells in 1 ml in 24 well plates or 10^5 cells in 200 μ l in 96 well plates) were cultured without T cells, with autologous CD4+CD25- or autologous CD4+CD25+ T cells at a 1:1 ratio in the presence of 0.4 μ g/ml soluble anti-CD3 mAb (clone

CLB-T3/4.E,1XE, CLB, Amsterdam, the Netherlands). When both CD4+CD25⁻ and CD4+CD25⁺ T cells were used, equal numbers of monocytes, CD4+CD25⁻ and CD4+CD25⁺ T cells were added to the wells. Cells were cultured in RPMI-1640 medium, supplemented with 1% Penicillin/Streptomycin, 1% Glutamine and 10% heat-inactivated pooled human male AB⁺ serum (Bloodbank, Utrecht, the Netherlands). T cell proliferation was measured in 96 wells plates by addition of ³H-thymidine at 1 µCi per well (specific activity 6.7 Ci/mmol) during the last 18 hours of a 72 hour culture period.

Phenotypic analysis

For analysis of T cell or monocyte phenotype, cells were collected after 40 hours of culture and triple color stained for FACS analysis using anti-CD4 PECy5 (DAKO, Diagnostics BV, Glostrup, Denmark) and FITC- or PE-conjugated mAbs against CD25 (DAKO), HLA II (BD Biosciences, San Jose, USA), CD40 (BD Biosciences), CD80 (BD Biosciences), CD86 (BD Biosciences), CD95 (DAKO), CD32 (BD Biosciences) or ILT3 (Immunotech, High Wycombe, UK). Viable CD4⁺ T cells and monocytes were gated based on their forward scatter/side scatter (FSC/SSC) and their CD4^{high} and CD4^{low} expression respectively. The expression of cell markers was analyzed using CellQuest software and expressed either as percentage of positive cells or geometric mean fluorescence intensity (MFI).

Cytokine analysis

Cell culture supernatants were collected after 40 hours of culture and centrifuged at 8000 g to remove cell debris. Supernatants were snap frozen in liquid nitrogen and stored at -80°C until further use. TNF-α, IFN-γ, IL-6 and IL-10 were measured via specific solid-phase sandwich ELISA using pairs of specific mAbs and recombinant cytokine standards (Biosource, Etten-Leur, the Netherlands). Detection limits for these cytokines were 20, 10, 20 and 10 pg/ml, respectively.

Monocyte/macrophage modulation experiments

Modulation of monocyte effector cell function was investigated by pre-culturing monocytes (3-4×10⁶ cells) without or with CD4+CD25⁻ or CD4+CD25⁺ T cells at a 1:0.5 ratio in 15 ml tissue culture tubes. After 40 hours cells were collected and monocytes were re-purified via positive magnetic selection using anti-CD14 mAb (purity always >90%). The purified monocytes (5×10⁴/well) were cultured in triplicate without or with 5 µg/ml purified protein derivative (PPD, Statens Serum Institut, Copenhagen, Denmark) and autologous responder CD4+CD25⁻ T cells (1×10⁵ per well) or used as stimulators to allogeneic CD4+CD25⁻ T cells (1×10⁵ per well). Ag-specific and allospecific T cell proliferation were assessed after respectively 5 and 7 days by ³H-thymidine incorporation. Alternatively, purified monocytes/macrophages (5×10⁴/well) were cultured with LPS (50 ng/ml) for 48 hours in 96 well plates and culture supernatants were collected for cytokine analysis.

Statistical analysis

Data on proliferation, phenotype and cytokine production were analyzed by non-parametric one-way ANOVA for related samples (Friedman test) using SPSS10 software. Significant differences between groups were further analyzed using a non-parametric test for related samples (Wilcoxon Signed Ranks Test). Groups were considered to be significantly different when p<0.05.

RESULTS

CD4+CD25+ Tregs are anergic and do not induce pro-inflammatory cytokine production by monocytes

Monocytes were cultured in the presence of anti-CD3 mAb without T cells, with CD4+CD25⁻ T cells or with CD4+CD25⁺ Tregs in an autologous setting. Monocytes cultured alone did not proliferate or produce significant levels of cytokines (Figure 1). Cultures of monocytes and CD4+CD25⁻ T cells resulted in vigorous T cell proliferation, and production of both pro-inflammatory (TNF- α , IFN- γ) and regulatory (IL-10) cytokines. In contrast, cultures of monocytes with CD4+CD25⁺ Tregs resulted in low proliferative responses and low TNF- α and IFN- γ production, indicating that the CD4+CD25⁺ Tregs were anergic and did not induce pro-inflammatory cytokine production either by themselves or by monocytes. We did detect some IL-10 in CD4+CD25⁺ Treg-monocyte cultures, which was produced by both populations as revealed by intracellular cytokine staining (data not shown). However the IL-10 levels were significantly lower than those in co-cultures of CD4+CD25⁻ T cells and monocytes.

Importantly, the lack of response was not due to a lack of activation of the CD4+CD25⁺ Tregs, since up-regulation of the activation markers HLA II and CTLA-4 was observed on these cells after anti-CD3 mAb stimulation (data not shown).

CD4+CD25+ Tregs suppress proliferation and pro-inflammatory cytokine production by monocytes/macrophages and T cells

We next investigated the suppressive activity of CD4+CD25⁺ Tregs during co-culture with monocytes and CD4+CD25⁻ T cells. Figure 2 shows the results of 9 independent experiments using 9 different donors (IL-10 was not detected in 2 donors). Significant inhibition of CD4+CD25⁻ T cell proliferation and of TNF- α and IFN- γ production was observed in the presence of CD4+CD25⁺ Tregs (mean percentage inhibition: $41 \pm 6\%$, $44 \pm 8\%$ and $50 \pm 8\%$, respectively, $p < 0.05$). The suppressive effects were also observed when co-cultures of monocytes and CD4+CD25⁻ T cells were compared to co-cultures of monocytes and unseparated CD4⁺ T cells (i.e. the naturally occurring mix of CD4+CD25⁻ and CD4+CD25⁺ T cells) (closed symbols). Interestingly, the production of the immunoregulatory cytokine IL-10 was inhibited in three out of seven experiments, but increased in four experiments. Independent of this increase or decrease in IL-10, the addition of CD4+CD25⁺ Tregs still resulted in suppression of T cell proliferation and reduced TNF- α and IFN- γ production (see corresponding symbols in Figures 2A-C). This suggests that suppression and IL-10 production are not related *per se*, however IL-10 might contribute to the suppressive effects in particular individuals. Be that as it may, suppression of T cell proliferation was still observed when neutralizing antibodies to IL-10 were added to co-cultures of monocytes, CD4+CD25⁻ and CD4+CD25⁺ T cells ($n=3$, data not shown). We are currently investigating the implications of the presence of IL-10 on monocyte function, since it is very well possible that T cells and monocytes react differently to the presence of this immunosuppressive cytokine.

CD4+CD25+ Tregs inhibit monocyte/macrophage activation and APC function

We next investigated whether CD4+CD25⁺ Tregs exerted any direct suppressive effects on the activation of monocytes/macrophages. Purified monocytes were cultured without T cells, with CD4+CD25⁻ T cells or with CD4+CD25⁺ Tregs at 1:1 ratios. Anti-CD3 mAb was added to all cultures to activate the T cells. After 40 hours cells were collected and the phenotype of monocytes/macrophages was assessed by flow cytometry. In the absence of T cells, monocytes displayed a resting phenotype, characterized by low expression of CD40 and CD80, and high expression of HLA II and CD86 (Figure 3). This phenotype was similar

to that of monocytes that were cultured for 40 hours without anti-CD3 mAb but in the presence of any of the T cell subsets (data not shown), indicating that the presence of either T cells or anti-CD3 mAb alone did not lead to monocyte activation. Upon co-culture with CD4+CD25⁻ T cells and anti-CD3 mAb the monocytes obtained an activated phenotype. The expression levels of CD40, CD80 and HLA II were significantly increased and CD86 expression remained high. In contrast, when monocytes were co-cultured with CD4+CD25⁺ Tregs, limited up-regulation of CD40, CD80 and HLA II was observed which was significantly lower compared to monocytes that were cultured in the presence of CD4+CD25⁻ T cells. Interestingly, CD4+CD25⁺ Treg-treated monocytes expressed significantly decreased levels of CD86 compared to monocytes cultured without T cells. This finding indicated that the diminished activation of monocytes was not due to a lack of stimulation by CD4+CD25⁺ Tregs (in which case CD86 levels would remain high), but rather was due to active modulation of the monocyte. We also observed that the morphology of monocytes/macrophages was different when cultured in the presence of CD4+CD25⁺ Tregs. The monocytes appeared as small and resting cells similar to untreated monocytes, whereas in the presence of CD4+CD25⁻ T cells the monocytes were enlarged and granular (data not shown).

In addition to the investigation of molecules involved in Ag presentation we studied the expression of the inhibitory receptors ILT-3 and CD32 on monocytes after interaction with CD4+CD25⁺ Tregs. Immunoglobulin-like transcript (ILT)-3 belongs to a family of inhibitory receptors containing immunoreceptor tyrosine-based inhibitory (ITIM) motifs and was shown to be up-regulated on human monocytes and dendritic cells upon tolerization by CD8+CD28-suppressor T cells [27], whereas CD32 encompasses both the ITAM containing FcγRIIa and the ITIM-containing FcγRIIb [34]. No significant differences were found in the expression of ILT-3 or CD32 on monocytes upon interaction with CD4+CD25⁺ Tregs (n=4, data not shown).

Importantly, the altered expression of MHC class II and costimulatory molecules had functional consequences. We re-purified monocytes that were pre-cultured without T cells, with CD4+CD25⁻ T cells or CD4+CD25⁺ Tregs and investigated their antigen-presenting capacity by adding autologous CD4+CD25⁻ T cells and protein purified derivative (PPD), or by adding the monocytes as stimulators to allogeneic CD4+CD25⁻ T cells. The proliferative response of the responder T cells was assessed after 5 and 7 days, respectively. Monocytes/macrophages that were pre-cultured without T cells or CD4+CD25⁻ T cells induced profound antigen-specific and allo-specific T cell proliferation in the responder T cells (Figure 4). In contrast, monocytes pre-treated with CD4+CD25⁺ Tregs were severely reduced in their capacity to induce an Ag-specific or allo-specific T cell response. Similar results were found when tetanus toxoid was used as an Ag ($63 \pm 15\%$ inhibition relative to untreated monocytes, n=3, data not shown). The difference in APC capacity was not caused by cell death, since similar numbers of viable monocytes were found upon co-culture. Also no differences in the expression of Fas/CD95 on the monocytes were found (data not shown).

CD4+CD25⁺ Tregs modulate the LPS-induced pro-inflammatory cytokine profile of monocytes/macrophages

We next investigated whether the interaction between monocytes and CD4+CD25⁺ Tregs affected the monocyte/macrophage cytokine profile in response to a strong pro-inflammatory stimulus, i.e. LPS. Monocytes were co-cultured without T cells, with CD4+CD25⁻ T cells or CD4+CD25⁺ Tregs in the presence of anti-CD3 mAb. After 40 hours monocytes/macrophages were re-purified by magnetic cell separation, to exclude a possible effect of persisting regulatory T cells, and challenged with LPS (50 ng/ml). Supernatants were collected two days later and TNF-α, IL-6 and IL-10 production was

measured by ELISA. Figure 5 shows that monocytes/macrophages that were pre-cultured in the absence of T cells produced large amounts of TNF- α and IL-6 upon LPS challenge, as well as IL-10 (hatched bars). Monocytes/macrophages that were pre-cultured with CD4+CD25⁻ T cells produced increased or similar amounts of TNF- α and IL-6 compared to untreated monocytes and decreased amounts of IL-10 (black bars). In contrast, monocytes/macrophages that were pre-cultured with CD4+CD25⁺ Tregs showed a striking decrease in LPS-induced TNF- α and IL-6 production compared to untreated monocytes (47 ± 12 and $58 \pm 12\%$ reduction, respectively, $p < 0.05$) as well as relative to monocytes pre-treated with CD25⁻ T cells (54 ± 19 and $84 \pm 7\%$ decrease, respectively, $p < 0.05$). The production of IL-10 was not significantly skewed upon pre-culture with CD4+CD25⁺ Tregs. These data thus show that CD4+CD25⁺ Tregs modulate the behavior of monocytes/macrophages in response to LPS and that this effect persists even when the Tregs have been removed.

DISCUSSION

We here present a novel suppressive characteristic of human CD4+CD25⁺ Tregs, namely that these cells can modulate monocyte/macrophage function. We demonstrate that CD4+CD25⁺ Tregs modulate monocytes by altering the activation of the cells, leading to reduced pro-inflammatory cytokine production upon subsequent LPS challenge and hampered APC function. Since monocytes/macrophages and their mediators are known to be major contributors to chronic inflammatory conditions, these findings provide support for the potential use of CD4+CD25⁺ Tregs as an immunotherapeutic target.

The results presented here fit with the data presented in a recent study by Powrie and co-workers that mouse CD4+CD25⁺ Tregs suppressed innate immune pathology [35]. Using a model of T cell-independent intestinal inflammation caused by *Helicobacter hepaticus* infection in RAG^{-/-} mice it was shown that immune pathology was prevented by adoptive transfer of CD4+CD25⁺ Tregs. The presence of CD4+CD25⁺ Tregs resulted in reduced recruitment of monocytes/macrophages, granulocytes and NK cells to the site of inflammation, and it was shown that IL-10 production by CD4+CD25⁺ Tregs played a crucial role in this process. From our data we conclude that although IL-10 was variably present, it does not appear to play an essential role in monocyte/macrophage modulation by human CD4+CD25⁺ Tregs. First of all, direct activation of CD4+CD25⁺ Tregs and monocytes resulted in positive but low levels of IL-10 production, which were lower than the IL-10 levels produced in co-cultures of CD4+CD25⁻ T cells and monocytes (Figure 1). Secondly, although in some co-cultures of monocytes, CD4+CD25⁻ and CD4+CD25⁺ T cells IL-10 production was increased, this was not *per se* correlated with suppressive activity, e.g. TNF- α levels were still suppressed when IL-10 production was not increased. Thirdly, upon LPS challenge CD4+CD25⁺ Treg-treated monocytes were not definitively skewed towards an IL-10-producing phenotype compared to untreated monocytes, whilst their IL-6 and TNF- α production was strongly reduced (Figure 3).

Our data are supported by previous findings on the APC modulating effects of anergic T cell clones. These rat CD4⁺ T cell clones were rendered anergic *in vitro* through non-professional Ag presentation, i.e. peptide presented by MHC class II⁺ T cells. Upon co-culture of these anergic T cells with splenic APC, the T cell-stimulatory capacity of the APC was strongly inhibited [25]. In fact, these anergic T cell clones share many characteristics with the naturally occurring CD4+CD25⁺ Tregs, as we have discussed recently [36]. Both subsets express CD25 and CD152 and show explicit signs of T cell differentiation (CD45RB^{low} and short telomeres), their suppressive effects are cell contact-dependent and cytokine-independent, and both subsets can modulate APC function [3, 4, 24, 25, 37]. Recently it was shown that both the *in vitro* anergized T cell clones and the naturally occurring CD4+CD25⁺ Tregs can modulate DC function by affecting their phenotype and/

or survival [29, 38]. Using human DC, Misra et al. showed that upon co-culture with pre-stimulated CD4+CD25+ Tregs the expression levels of CD40 and HLADR on DC were reduced and that the percentages of CD86+ and CD83+ DC were decreased relative to untreated DC [29]. In support of the data presented here, the altered phenotype was associated with a reduction in the T cell-stimulatory capacity during subsequent allogeneic and PPD-specific T cell stimulation assays, even when the DC were incubated with rhCD40L prior to incubation with CD4+CD25+ Tregs. The modulatory effect was cell-contact dependent since virtually no changes in DC phenotype were observed when cells were separated in a transwell system, although some role for IL-10 and TGF- β was suggested. In addition to these data, we show in the current study that CD4+CD25+ Tregs also affect the cytokine profile of monocytes upon subsequent TLR4/CD14 triggering. We are currently investigating how CD4+CD25+ Tregs modulate monocyte/macrophage function, and whether this is dependent on cell-contact between Tregs and monocytes.

Due to the potent immunosuppressive effects of CD4+CD25+ Tregs, restrictions must be set on immunoregulation by these cells in order to allow natural immunity to occur. It was shown that high doses of IL-2 or anti-CD28 mAb could break CD4+CD25+ Treg-mediated suppression [17]. Thus under inflammatory conditions, when large amounts of IL-2 are produced and APC express high levels of CD80 and CD86, the suppressive effects might be temporarily reduced. This might explain why in rheumatoid arthritis patients joint inflammation persists despite the presence of CD4+CD25+ Tregs at the synovial site [6, 7]. It also suggests that in chronic inflammatory conditions therapy should be targeted at both down-regulation of excessive inflammation and boosting of natural immune regulatory processes. The latter might be of importance to restore a normal immunological balance in order to avoid prolonged or even lifelong treatment with immunosuppressive agents. It was shown recently that microbial induction of the Toll pathway in DC blocked immunosuppression by CD4+CD25+ Tregs, which was in part dependent on IL-6 [39]. These ‘danger’ signals could therefore override immunosuppression by Tregs, thus allowing immunity to infection to occur. Importantly our data indicate that at least in the case of monocytes/macrophages, interactions with Tregs before microbial (LPS) encounter might result in decreased effector cell function. The timing as well as the quantity of the interactions between monocytes/macrophages with Tregs might thus influence the development of an immune response. Disturbances in this process could contribute to the development of chronic inflammation or autoimmunity.

The findings presented here together with existing literature suggest that the immunosuppressive potential of CD4+CD25+ Tregs is extensive, affecting CD4+ and CD8+ T cells, cytokines, antibody production, B cells, DC, and as we describe here, also monocytes/macrophages. The challenge researchers now face is to develop means to specifically target these suppressive properties of CD4+CD25+ Tregs in vivo.

Acknowledgments

The work described in this paper was partly supported by a grant from the Dutch Arthritis Association. MMT was supported by grant BBS/B/03181 from the Biotechnology and Biological Sciences Research Council. The authors would like to thank Dr Helen Collins (King’s College London) for critical review of the manuscript.

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Figure 1. CD4+CD25+ Tregs are anergic and do not induce pro-inflammatory cytokine production.

CD14+ monocytes were cultured with anti-CD3 mAb in the absence (hatched bars) or presence of either CD4+CD25- T cells (black bars) or CD4+CD25+ Tregs (open bars).

A. T cell proliferation was measured by ^3H -thymidine incorporation (mean $\pm$ SEM, n=16 independent experiments).

B-D. IFN- γ (n=12), TNF- α (n=16), and IL-10 (n=13) production was measured in culture supernatants collected 40 hours after the start of the culture.

* p<0.05, ** p<0.005 and *** p<0.0005 compared to monocytes cultured with CD4+CD25- T cells.

Figure 2. CD4+CD25+ Tregs suppress T cell proliferation and pro-inflammatory cytokine production by monocytes/macrophages and CD4+CD25- T cells.

Monocytes were cultured with anti-CD3 mAb and autologous CD4+CD25- T cells with or without autologous CD4+CD25+ T cells at a 1:1 ratio (open symbols). Alternatively, responses were compared between monocytes cultured with CD4+CD25- T cells and monocytes cultured with unseparated CD4+ T cells i.e. the naturally occurring mix of CD4+CD25- and CD4+CD25+ T cells (solid symbols). T cell proliferation and cytokine production were measured as described in the legend to Figure 1. Each symbol reflects a different experiment using a different donor; the symbols correspond between the four analyses (IL-10 was not detected in two out of nine experiments).

Figure 3. CD4+CD25+ Tregs inhibit activation of monocytes/macrophages.

CD14⁺ monocytes were cultured in the presence of anti-CD3 mAb without T cells (hatched bars), with CD4+CD25⁻ T cells (black bars) or CD4+CD25⁺ T cells (open bars). After 40 hours the cells were collected and labeled for FACS analysis. Monocytes were gated based on their forward/side scatter and low expression of CD4.

A-D. Expression of CD40, HLA II, CD80 and CD86 was analyzed using CellQuest software and depicted as geometric mean fluorescence intensity (MFI). Data represent the mean $\pm$ SEM of 10 independent experiments each with cells of a different donor.

Figure 4. CD14⁺ monocytes were cultured in the presence of anti-CD3 mAb without T cells (hatched bars), with CD4⁺CD25⁻ T cells (black bars) or CD4⁺CD25⁺ Tregs (open bars). After 40 hours, CD14⁺ monocytes were re-purified (see Materials and Methods for details). The antigen-presenting capacity was investigated by adding re-purified monocytes to autologous responder CD4⁺CD25⁻ T cells in the presence of PPD (A) or by using the monocytes as allogeneic stimulators to CD4⁺CD25⁻ T cells obtained from a different donor (B). T cell proliferation was measured after 5 and 7 days respectively. T cell proliferation in the absence of stimulation was less than 1,000 cpm. One out of three independent experiments is shown.

Figure 5. CD4+CD25+ Tregs modulate the monocyte/macrophage cytokine profile in response to LPS.

CD14+ monocytes were cultured without T cells (hatched bars), with CD4+CD25- T cells (black bars) or CD4+CD25+ Tregs (open bars) in the presence of anti-CD3 mAb. After 40 hours monocytes were re-purified and LPS was added to the cultures. Culture supernatants were collected after 48 hours for detection of TNF- α (A, n=6), IL-6 (B, n=5) and IL-10 (C, n=6). Data are depicted as mean cytokine production $\pm$ SEM (pg/ml).