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Costimulation blockade and regulatory T-cells in a non-human primate model of kidney allograft transplantation

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Citation

Haanstra, K. G. (2008, March 13). *Costimulation blockade and regulatory T-cells in a non-human primate model of kidney allograft transplantation*. Retrieved from <https://hdl.handle.net/1887/12636>

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Chapter 6

Characterisation of naturally occurring CD4⁺CD25⁺ regulatory T-cells in rhesus monkeys

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Transplantation, accepted

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Abstract

Background Translational research in a relevant preclinical model is recommended before Treg-inducing protocols can be implemented in humans. We have characterised rhesus monkey CD25⁺ cells phenotypically and functionally.

Methods The phenotype of CD4⁺CD25^{high} cells was determined by FACS, focusing on established markers of mouse and human Treg cells. Percentages of cells positive for CD45RA, CD62L and intracellular CTLA-4 and FOXP3 were compared between CD4⁺CD25^{high} and CD4⁺CD25⁻ cells. CD25⁻ cells stimulated with anti-CD3, ConA and/or allogeneic PBMC were mixed with freshly isolated CD25⁺ cells. The suppressive activity of the CD25⁺ cells *in vitro* was assessed using several experimental conditions.

Results Rhesus monkey CD4⁺CD25^{high} cells expressed high intracellular levels of CTLA-4 and FOXP3, while expression was negligible in CD4⁺CD25⁻ cells. The CD25^{high} population was mostly CD45RA⁻, indicative of a memory phenotype. The CD25⁺ cells were anergic, as they showed low proliferative responses, no IL-2 production and some IFN- γ and IL-10 production. Proliferation of CD4⁺CD25⁻ cells stimulated by anti-CD3 or allogeneic cells was decreased when CD4⁺CD25⁺ cells were added at a 1:1 ratio. In addition, we found that CD25⁺ cells inhibited the IL-2 and IFN- γ production by anti-CD3 stimulated CD25⁻ cells in a dose-dependent fashion, through a cell-cell contact dependent mechanism.

Conclusions Rhesus monkey CD4⁺CD25⁺ cells have similar phenotypic and functional characteristics as natural Tregs in humans. These findings allow testing of Treg expansion and/or induction protocols in a relevant preclinical model, the rhesus monkey.

Introduction

Naturally occurring CD4⁺CD25⁺ regulatory T-cells (Tregs) are important mediators of immune-tolerance in mice and humans. Mice lacking CD4⁺CD25⁺ cells after neonatal thymectomy develop various autoimmune diseases [1]. Adoptive transfer of CD4⁺CD25⁺ Tregs can prevent disease development in a variety of mouse autoimmune models. In contrast to Tregs of mice, those in humans form only a small part of all CD25 expressing cells; only the CD4⁺CD25^{high} population has regulatory properties [2, 3]. Another important difference between mouse and human Tregs is that human Tregs are antigen-experienced cells. Tregs of specific pathogen-free mice are immunologically naive (CD45RB^{low}) [4]. In humans, most Tregs have a memory phenotype [2], although Tregs with a naive, CD25^{int} phenotype have also been described [3, 5]. Further phenotypic characteristics of human and mouse natural Tregs include high intracellular expression of CTLA-4 and Foxp3 (mice) or FOXP3 (humans). Foxp3 expression in the mouse is clearly correlated with suppression [6]. However, in humans, CTLA-4 and FOXP3 are also expressed in activated T-cells without regulatory capacity [7–10]. This precludes CTLA-4 and/or FOXP3 expression as exclusive markers for human Tregs. Currently, the only reliable identification of Tregs in humans is the assessment of their functional phenotype. Tregs maintain a state of unresponsiveness when stimulated with polyclonal stimuli or alloantigens. They do not proliferate nor produce cytokines such as IFN- γ , IL-2 or TNF- α in response to these stimuli [2, 5, 11]. Production of the Th2 cytokines IL-4 and especially IL-10 has been reported in some cases [11, 12]. Furthermore, when added to a CD4⁺CD25⁻ population exposed to a polyclonal or an allogeneic stimulus, natural Tregs dose-dependently inhibit proliferation as well as cytokine production [2, 11, 12]. The mechanism by which Tregs suppress the response of CD4⁺CD25⁻ cells remains elusive, but Tregs can only suppress when cell-cell contact is present between responder cells and Tregs [2, 5].

Natural Tregs are most extensively studied *in vivo* as part of the immune response towards autoantigens and alloantigens. Natural Tregs maintain a state of self-tolerance [13], but can in an experimental system also downregulate an ongoing autoimmune response, leading to disease remission [14]. The response towards alloantigens can easily be inhibited by natural Tregs in mice [15, 16]. Functional CD4⁺CD25⁺ cells are also present in human kidney graft recipients, but it remains unclear whether they play a role in the recipient's unresponsiveness towards donor alloantigens [17, 18]. Although many other types of Tregs have been described, the naturally occurring CD4⁺CD25^{high} cells remain the golden standard with which phenotypic and functional characteristics of other types of Tregs are compared [19, 20].

Non-human primates (NHP) can serve as relevant preclinical models to translate a new therapeutic principle developed in lower species to the clinical setting [21]. The rhesus monkey has been our preferred model for skin and organ transplantation in which a wide variety of new therapeutic principles has been investigated [22–28]. Characterisation and *in vitro* expansion of naturally occurring Tregs in NHP was reported previously [29, 30], but a full evaluation of naturally occurring Tregs in

rhesus monkeys is still lacking. The aim of this study was therefore to phenotypically and functionally characterise the natural CD4⁺CD25⁺ T-cell population in the NHP species of rhesus monkeys.

Materials and methods

Phenotypic characterisation of rhesus monkey CD4⁺CD25⁺ cells

EDTA or heparinised blood samples were collected from healthy rhesus monkeys (*Macaca mulatta*) either imported or born and raised at the BPRC, according to institutional guidelines. As a control, freshly isolated PBMC from healthy human volunteers were used. 50 µl EDTA whole blood samples were stained for CD3, CD4, and CD25 (SP34, SK3 and 2A3, respectively; all BD Biosciences, San Jose, CA; clone 4E3, Miltenyi, Bergisch Gladbach, Germany, was also used to stain CD25). For phenotypic analysis, cells were additionally stained for CD62L (FMC45, Serotec, Oxford, UK), CCR7 (FAB197, R&D Systems, Abingdon, UK) and CD45RA (5H9, BD Biosciences). After lysis of the red blood cells, the remaining cells were washed and fixated using paraformaldehyde, or processed for intracellular staining with CTLA-4 (CD152, clone BNI3, BD Pharmingen, San Diego, CA, USA). The cells were permeabilised using the Cytotfix/Cytoperm kit (BD), according to the manufacturer's instructions. After washing, cells were fixated using 1% paraformaldehyde. For analysis of FOXP3 expression (PCH101, eBioscience, San Diego, CA, USA), PBMC were used instead of whole EDTA blood. The staining protocol was essentially the same as described for EDTA, except for the red cell lysis and permeabilization, which was performed using the kit provided with the antibody according to the manufacturer's instruction. Staining patterns were visualized by flow cytometry (FACS Aria, BD). Data analysis was performed using FACSDiva software (BD).

PBMC isolation and culture conditions

Heparinised blood samples were collected from healthy rhesus monkeys from the BPRC colony. Monkeys originated from different geographical origins, i.e. India, China or Burma. As a control, freshly isolated PBMC from healthy human volunteers were used. PBMC were isolated using gradient centrifugation. (LSM, MP Biomedicals, Aurora, OH, USA). Cells were stimulated with either ConA (5 µg/ml, Amer sham Pharmacia, Uppsala, Sweden), plate-bound anti-CD3 (SP34, BD Pharmingen) 1 or 10 µg/ml in PBS, one hr at 37 °C, and subsequently washed twice with PBS, or with plate-bound anti-CD3 (10 µg/ml) plus 2.5 µg/ml soluble anti-CD28 (CD28.2, BD Pharmingen). PBMC were cultured in RPMI-1640 supplemented with 10% foetal bovine serum (Invitrogen, Paisley, UK), penicillin-streptomycin solution, glutamax and β-mercaptoethanol (all from Invitrogen). Antigen-presenting cells (APC; 5 x 10⁴/well unseparated total PBMC, 30 Gy γ-irradiated) were added to all cultures. For cytokine assays culture supernatants were taken between 48-72 hrs after initiation of the cultures. Proliferation was measured by pulsing of the cell cultures with 0.5 µCi/well [³H]-thymidine for the last 18 hours of a 6-day culture. Counts

per minute (cpm) were determined using a Packard TopCount liquid scintillation counter (PerkinElmer, Groningen, The Netherlands).

Isolation of rhesus monkey CD4⁺CD25⁺ cells

Unmanipulated CD4⁺ T-cells were isolated from PBMC using two different methods. Method 1 comprised of depletion of CD8⁺ and CD20⁺ cells by incubation with anti-CD20 (clone L27, BD) followed by incubation with anti-mouse Dynabeads and anti-CD8 Dynabeads (Invitrogen). Method 2 consisted of negative CD4⁺ T-cell isolation with biotin-conjugated monoclonal antibodies against CD8, CD11b, CD16, CD20, CD56 and CD66abce, all cross-reactive with rhesus monkey cells (Miltenyi). Antibody-binding cells were removed with MACS anti-biotin MicroBeads (Miltenyi), leaving the CD4⁺ cells untouched. CD4⁺CD25⁺ cells were separated from CD4⁺CD25⁻ cells by CD25 isolation with anti-CD25 MicroBeads (Miltenyi). Flow cytometry was performed on a FACSaria with FACSDiva software to assess purity of each of the fractions.

Cell cultures

The suppressive activity of CD4⁺CD25⁺ cells was assessed by their ability to suppress proliferation of CD4⁺CD25⁻ cells stimulated with plate-bound anti-CD3. CD4⁺CD25⁻ cells (2.5×10^4 /well) were cultured with CD4⁺CD25⁺ cells in 96-well round-bottom plates. The stimulation indices (SI) of these cultures (cpm of stimulated cultures divided by cpm of unstimulated cultures) ranged between 13 and 178. CD4⁺CD25⁺ were added to the cultures at a 1:1 ratio. To determine dose-dependent inhibition of cytokine production, titrated numbers of CD4⁺CD25⁺ cells were added to 2.5×10^4 CD4⁺CD25⁻ cells/well, stimulated with plate-bound anti-CD3. CD4⁺CD25⁺ cell numbers decreased stepwise in ratios from 1:1 to 1:0.13. Culture supernatants were taken after 48 hrs. In addition, the ability of CD4⁺CD25⁺ cells to suppress CD4⁺CD25⁻ cells stimulated with allogeneic cells was investigated. A positive mixed lymphocyte reaction (MLR) with SI > 5 (range 5.0 - 55.1) was obtained by stimulating CD4⁺CD25⁻ responders (2.5×10^4 /well or 5×10^4 /well) with 30 Gy irradiated allogeneic stimulator PBMC (5×10^4 /well or 1×10^5 /well, respectively). APC were added as described above. CD4⁺CD25⁺ cells were added to these cultures in a 1:1 ratio to the CD4⁺CD25⁻ cells. To assess whether suppression in CD4⁺CD25⁻ / CD4⁺CD25⁺ co-cultures was mediated by suppressive cytokines, such as IL-10 and TGF- β , neutralising anti-IL10 (10 μ g/ml QS-10, U-CyTech, Utrecht, The Netherlands) and anti-TGF- β (8 μ g/ml 1D11, R&D Systems) antibodies, or mouse IgG₁ isotype control (18 μ g/ml 11711, R&D Systems) antibodies were added.

Transwell assays

CD4⁺CD25⁻ cells (2.5×10^5 /well) were stimulated with either ConA or plate-bound anti-CD3 in 24-well plates and were either mixed with CD4⁺CD25⁺ cells at a 1:1 ratio or separated by a membrane (0.4 μ m pore size, Corning, Schiphol-Rijk, The

Netherlands) from the CD4⁺CD25⁺ population. APC were present in the lower compartment (5×10^5 /well) and in the transwell inserts (5×10^4 /well).

Cytokine determinations by ELISA

Supernatants were taken after 48-72 hrs and were diluted 1:2.5 in PBS/1%BSA and stored frozen until further analysis. IFN- γ , IL-2 and IL-10 levels were determined with monkey-specific ELISAs (U-CyTech), following the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed with Prism 4 for Macintosh (GraphPad, San Diego, CA USA). Data are expressed as mean $\pm$ SEM. Significance of phenotypical differences was analysed using Student's paired *t* test. The effect of anti-IL-10 and anti-TGF- β was compared with the effect of control IgG using the Wilcoxon signed rank test. Data were considered significant if $p \leq 0.05$.

Results

CD4⁺CD25⁺ cells in rhesus monkeys

CD25 expression was detected on $10 \pm 1\%$ of CD3⁺CD4⁺ T-cells from 5 randomly selected rhesus monkeys. CD4⁺CD25⁻ and CD4⁺CD25⁺ cells (putative Treg cells) were analyzed for the presence of naive (CCR7⁺CD45RA⁺) cells, for surface expression of CD62L and for intracellular expression of CTLA-4 (CD152) or FOXP3. In humans, up to 40% of CD4⁺ cells may be positive for CD25, but only CD25^{high} cells have regulatory properties. We have used two different monoclonal antibodies specific for CD25; however, a separate CD25^{high} population was never observed when staining rhesus monkey cells (Fig. 6.1A), while this was clearly detected when staining human cells (Fig. 6.1B). We have therefore analyzed all CD4⁺CD25⁺ cells to investigate if they resemble human CD4⁺CD25^{high} or if they are more similar to helper T-cells. A clear phenotypic difference was observed between CD4⁺CD25⁻ and CD4⁺CD25⁺ populations (Fig. 6.1C). CD4⁺ T-cells were $29 \pm 4\%$ CD45RA⁺ of which 93% were CCR7⁺CD45RA⁺ (true naive, see ref. [31]), indicating that the remaining majority of CD4⁺ T-cells had a memory phenotype. The CD4⁺CD25⁺ population contained less naive cells than the CD4⁺CD25⁻ population ($20 \pm 3\%$ versus $29 \pm 4\%$; $p = 0.0158$, paired *t*-test). CD25⁺ cells expressed less CD62L, a homing marker for peripheral lymph nodes ($50 \pm 3\%$ versus $71 \pm 3\%$; $p = 0.0032$). Expression of the Treg markers CTLA-4 and FOXP3 was almost absent from CD4⁺CD25⁻ cells, while these were expressed at high levels on CD4⁺CD25⁺ cells (CTLA-4 $46 \pm 5\%$ versus $9 \pm 1\%$; $p = 0.0015$ and FOXP3 $59 \pm 4\%$ versus $8 \pm 2\%$; $p = 0.0005$).

To investigate if we could find phenotypic differences between cells expressing CD25 at intermediate or at high levels, an arbitrary threshold of CD25 expression was set in the CD4⁺CD25⁺ population, identifying two equally sized populations, a CD25^{high} and a CD25^{int} population. Both populations were analysed for the markers

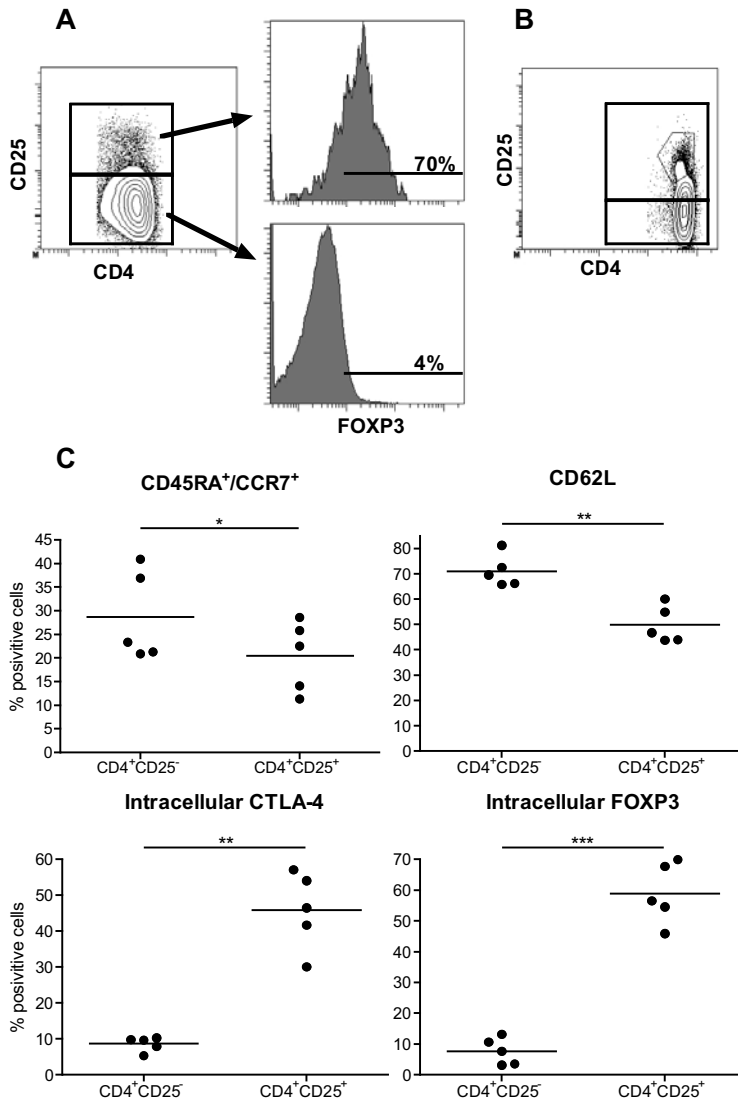


Figure 6.1: Phenotypic analysis of CD4⁺CD25⁻ and CD4⁺CD25⁺ cells. (A) Rhesus monkey CD3⁺CD4⁺ T-cells ($n = 5$) were gated. Gates were set around CD4⁺CD25⁻ and CD4⁺CD25⁺ populations (rectangular gates, thick lines). CD4⁺CD25⁺ made up approximately 10% of all CD4⁺ cells. No distinct CD25^{high} population was detected. CD4⁺CD25⁻ and CD4⁺CD25⁺ populations had clear phenotypic differences, of which intracellular FOXP3 staining is shown as an example. (B) A CD25^{high} population is clearly detected in human PBMC ($n = 3$) (polygon gate, thin line), stained and gated under identical conditions. (C) Less CD4⁺CD25⁺ cells were naive (CCR7⁺CD45RA⁺) ($20 \pm 3\%$ versus $29 \pm 4\%$; $p = 0.0158$, paired t -test) and expressed less CD62L ($50 \pm 3\%$ versus $71 \pm 3\%$; $p = 0.0032$). Expression of the Treg markers CTLA-4 and FOXP3 was very high on Tregs, but almost absent on CD25⁻ cells (CTLA-4 $46 \pm 5\%$ versus $9 \pm 1\%$; $p = 0.0015$ and FOXP3 $59 \pm 4\%$ versus $8 \pm 2\%$; $p = 0.0005$).

discussed above. CD25^{high} cells express even higher levels of CTLA-4 and FOXP3 than CD25^{int} cells (Table 6.1). Interestingly, although the total percentage naive cells and the percentage CD62L⁺ cells is lower in the CD4⁺CD25⁺ population as compared to CD4⁺CD25⁻ population, the CD25^{high} population contains more naive and CD62L⁺ cells than the CD25^{int} population (Table 6.1).

	CD45RA ⁺ CCR7 ⁺	CD62L ⁺	CTLA-4 ⁺	FOXP3 ⁺
CD4 ⁺ CD25 ⁻	29 ± 4	71 ± 3	9 ± 1	8 ± 2
CD4 ⁺ CD25 ⁺ total	20 ± 3	50 ± 3	46 ± 5	59 ± 4
CD4 ⁺ CD25 ^{int}	15 ± 3	43 ± 2	37 ± 5	43 ± 6
CD4 ⁺ CD25 ^{high}	27 ± 2	58 ± 4	58 ± 6	80 ± 3

Table 6.1: *Phenotype of CD4⁺CD25⁻ and CD4⁺CD25⁺ cells. The percentage of CD4⁺ cells positive for indicated markers or subsets are indicated (mean ± SEM).*

Suppressive capacity of CD4⁺CD25⁺ cells

Two different methods were used for CD4⁺ T-cell isolation, CD8⁺ plus CD20⁺ cell depletion with Dynal beads and negative CD4 isolation with MACS beads. No difference was found between the two methods with regard to the purity or activity of the cells. Subsequently, CD25⁺ cells were separated from CD25⁻ cells. The purity of the CD4⁺CD25⁺ population was on average 82 ± 4%. Although some residual CD25^{int} cells could be detected in the CD25 depleted fraction, CD25 expression was significantly higher in the CD25 enriched fraction as compared to the CD25 depleted fraction (MFI CD25 2541 ± 1262 versus 1125 ± 458), indicating a preferential enrichment of CD25^{high} cells in the CD4⁺CD25⁺ fraction (Fig. 6.2).

Human Tregs typically do not respond themselves towards polyclonal and allogeneic stimuli and they inhibit the response of CD4⁺CD25⁻ cells towards these stimuli. Rhesus monkey CD4⁺CD25⁺ cells were in general unresponsive towards activation signals via the TCR, such as plate-bound anti-CD3 (Fig. 6.3A, left panel, and Fig. 6.3B) and allogeneic cells (Fig. 6.3C). The response towards polyclonal stimuli was very comparable with the response of human cells (Fig. 6.3A, right panel). However, the unresponsiveness was strongly dependent on the strength of the activation signal (Fig. 6.3A). Addition of soluble anti-CD28 to the cultures induced proliferation of CD25⁺ cells to similar levels as of CD25⁻ cells, and proliferation of CD25⁻ cells was not inhibited in these anti-CD3 + anti-CD28 stimulated co-cultures with CD25⁺ cells. This effect was not seen when cells were stimulated with soluble anti-CD28 alone (Fig 6.3A). Proliferation of CD4⁺CD25⁻ cells stimulated by plate-bound anti-CD3 or by allogeneic cells was reduced by the addition of CD4⁺CD25⁺ cells added at a 1:1 ratio (Fig. 6.3B and 6.3C, respectively). For unknown reasons and seemingly not related to the effectiveness of the isolation procedure, CD4⁺CD25⁺ cells were not equally anergic in all individuals. When such increased responsiveness in CD4⁺CD25⁺ cells was seen, inhibition of CD25⁻ cells was much less effective.

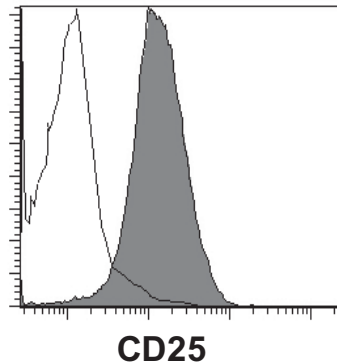


Figure 6.2: CD25⁺ cell separation. CD4⁺ T-cells were isolated either by depletion of CD8⁺ and CD20⁺ cells with Dynal beads or with the negative CD4 MACS isolation kit. The resulting CD4 enriched cells were separated into CD25⁻ (open histogram) and CD25⁺ (filled histogram) fractions. These fractions were stained for CD25 and purity of the CD4⁺CD25⁺ fraction was on average $82 \pm 4\%$ CD25⁺ ($n = 23$). CD25 expression was higher in the CD25 enriched fraction (MFI CD25 2541 ± 1262) as compared to the CD25 depleted fraction (1125 ± 458).

Cytokine production and dose-dependent inhibition by CD4⁺CD25⁺ cells

CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were stimulated with plate-bound anti-CD3. IL-2, IL-10 and IFN- γ production of both populations was measured in culture supernatants taken after 48-72 hrs of culture (Fig. 6.4A). CD4⁺CD25⁺ cells did not produce IL-2. Production of IFN- γ was very low, only 16% (117 ± 83 pg/ml) of the IFN- γ production by CD4⁺CD25⁻ cells and production of IL-10 was 38% (34 ± 6 pg/ml) of CD25⁻ cells. We investigated if the cytokine production by CD25⁻ cells is dose-dependently inhibited when titrated numbers of CD25⁺ cells were added to the cultures. CD25⁻ cells (2.5×10^4) were mixed with CD25⁺ cells at ratios ranging from 1:1 to 1:0.13. Cytokine production was measured after 48 hrs. IL-2 and IFN- γ production of CD25⁻ cells cultured without CD25⁺ cells was inhibited dose-dependently upon addition of CD25⁺ cells (Fig. 6.4B). Interestingly, IL-2 production was most effectively inhibited; with over 30% of IL-2 production by CD25⁻ cells inhibited at ratios of 1:0.13, while IFN- γ production was inhibited only at ratios of 1:1 and 1:0.5. The effect of addition of CD25⁺ cells on the production of IL-10 was variable between animals. Inhibition of IL-10 production was seen only at ratios of 1:1, but two animals showed increased IL-10 production when low numbers of CD25⁺ cells were present.

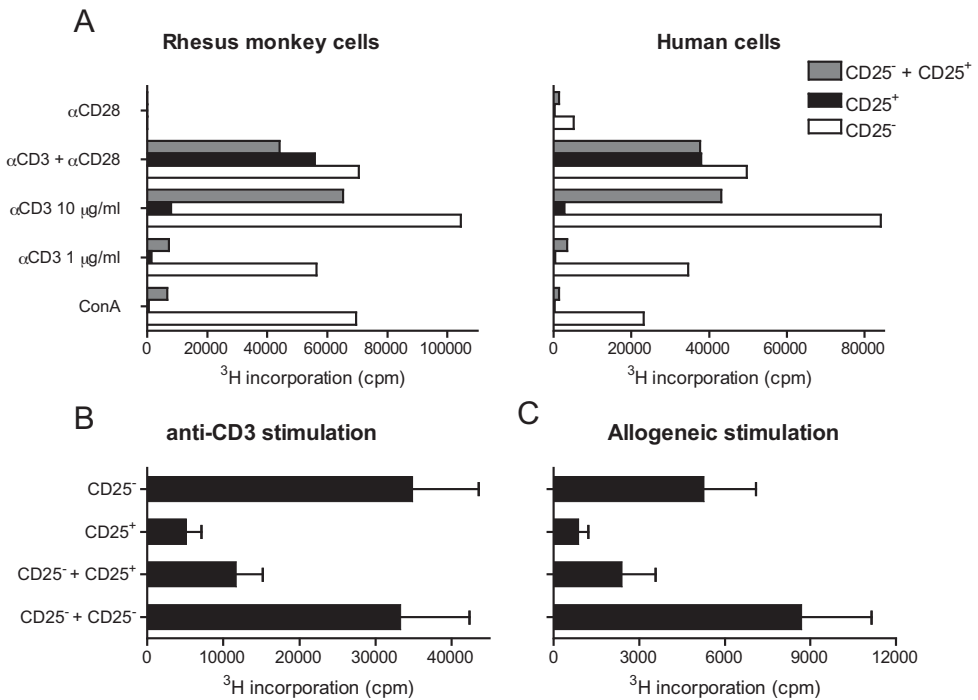


Figure 6.3: Functional analysis of $\text{CD4}^+\text{CD25}^+$ cells. (A) $\text{CD4}^+\text{CD25}^-$ cells (2.5×10^4 /well), $\text{CD4}^+\text{CD25}^+$ cells, or both, mixed in a 1:1 ratio were stimulated with ConA ($5 \mu\text{g/ml}$), plate-bound anti-CD3 (1 or $10 \mu\text{g/ml}$), or plate-bound anti-CD3 ($10 \mu\text{g/ml}$) plus soluble anti-CD28 ($2.5 \mu\text{g/ml}$), in the presence of irradiated APC. The left panel shows a representative experiment of five, using rhesus monkey cells, the right panel shows a representative experiment of three, using human cells. Both rhesus monkey and human CD25^+ cells proliferate much less than CD25^- cells. Increasing the strength of the stimulus leads to increased proliferation of CD25^+ cells (black bars) and concomitant decreased suppression in the co-culture (grey bars) in both species. Soluble anti-CD28 stimulation alone did not induce proliferation in rhesus monkey CD25^- cells and only to a limited extent in human CD25^- cells. (B) Rhesus monkey $\text{CD4}^+\text{CD25}^-$ and $\text{CD4}^+\text{CD25}^+$ cells were stimulated with plate-bound anti-CD3 ($1 \mu\text{g/ml}$) as described ($n = 8$). ^3H -thymidine incorporation of co-cultures of CD25^- and CD25^+ cells is only 31% of CD25^- cells alone. In contrast, ^3H -thymidine incorporation of 5×10^4 CD25^- cells/well is 97%. (C) CD25^- cells were stimulated with allogeneic irradiated PBMC ($n = 6$). CD25^+ cells can inhibit proliferation of allo-antigen activated CD25^- cells, although considerable variation is seen between animals.

Suppression by rhesus monkey $\text{CD4}^+\text{CD25}^+$ cells is soluble factor independent and cell contact dependent

$\text{CD4}^+\text{CD25}^-$ cells were either stimulated with plate-bound anti-CD3 or ConA, and cultured with $\text{CD4}^+\text{CD25}^+$ cells that were either separated or unseparated by a

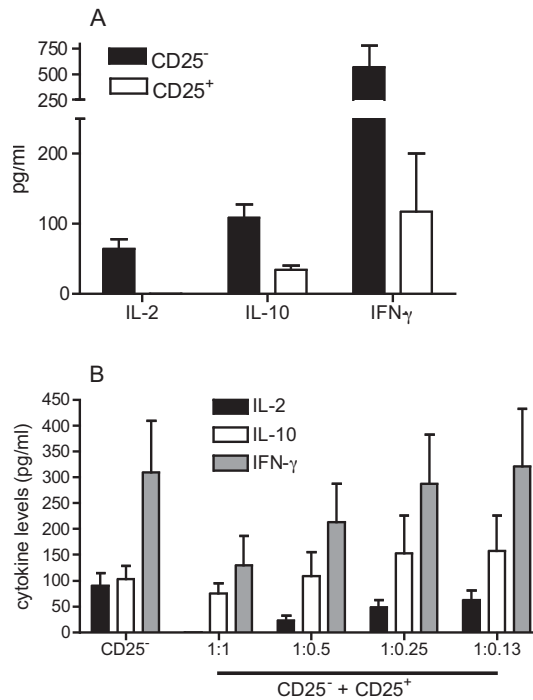


Figure 6.4: Cytokine production and dose-dependent inhibition by CD4⁺CD25⁺ cells. (A) CD4⁺CD25⁻ cells (black bars) and CD4⁺CD25⁺ cells (open bars) were stimulated with plate-bound anti-CD3 and cytokine production was measured after 48-72 hrs ($n = 9$). CD4⁺CD25⁺ cells do not produce IL-2, they produce 34 ± 6 pg/ml IL-10 ($38 \pm 7\%$ of the production by CD25⁻ cells), and they produce 117 ± 83 pg/ml IFN- γ ($16 \pm 6\%$ of CD25⁻ cells). (B) CD25⁻ cells were stimulated with anti-CD3 and CD25⁺ cells were added to the CD25⁻ cells in 1:1 ratios and were titrated down to ratios of 1:0.13 ($n = 4$). IL-2 production was most effectively inhibited, with over 30% of IL-2 production by CD25⁻ cells inhibited at ratios of 1:0.13, while IFN- γ production was inhibited only at ratios of 1:1 and 1:0.5. The effect of addition of CD25⁺ cells on the production of IL-10 was variable between animals, with low IL-10 production in the presence of CD25⁺ cells in two animals, and an additive effect on IL-10 production in the presence of (low numbers of) CD25⁺ cells in two different animals.

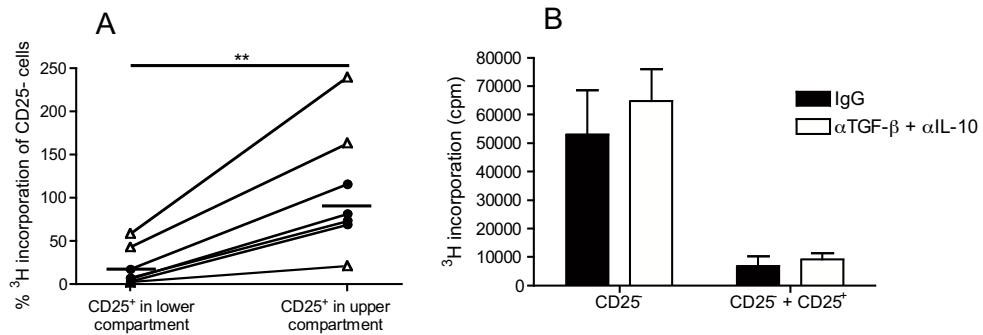


Figure 6.5: Inhibition by CD25⁺ cells is cell contact dependent. (A) CD25⁻ cells were stimulated either with plate-bound anti-CD3 (open triangles) or ConA (closed rounds) in 24-well transwell plates. CD25⁻ cells were cultured without, or with CD25⁺ cells either separated or unseparated by a semi-permeable membrane, at a 1:1 ratio. Responses of the co-cultures are expressed as a percentage of ³H-thymidin incorporation of CD25⁻ cells alone. Proliferation of co-cultures not separated by a membrane were reduced to 23 ± 11%, while proliferation in co-cultures separated by a membrane was 116 ± 38% ($p = 0.0280$, paired t -test). (B) CD25⁻ cells and CD25⁻/CD25⁺ co-cultures were stimulated with ConA and cultured in the presence of either IgG control mAb 18 µg/ml or antibodies against TGF-β 8 µg/ml and IL-10 (10 µg/ml; $n = 4$). Addition of anti-IL-10 and anti-TGF-β increased proliferation of CD25⁻ cells and of CD25⁻/CD25⁺ co-cultures, but suppression was still present; 11 ± 2% in the presence of IgG control mAb and 17 ± 6% in the presence of anti-IL-10 and anti-TGF-β ($p = 0.8750$, Wilcoxon signed rank test).

semi-permeable membrane from the CD25⁻ population. When both populations were not separated by the membrane, proliferation of CD4⁺CD25⁻ cells was reduced to a mean of 20 ± 8% of proliferation of CD25⁻ cells cultured without CD25⁺ cells. When a membrane separated both populations, proliferation of the co-culture was restored to the mean level of proliferation of CD25⁻ cells alone (109 ± 27%) (Fig. 6.5A).

To further investigate if the mechanism of suppression involves the suppressive cytokines IL-10 or TGF-β, CD4⁺CD25⁻ cells were stimulated with ConA in the presence of antibodies against IL-10 and TGF-β or control IgG (Fig. 6.5B). Addition of anti-IL-10 and anti-TGF-β did not significantly alter proliferation of CD25⁻ cells and of CD25⁻/CD25⁺ co-cultures, and suppression was still present; 11 ± 2% in the presence of IgG control mAb and 17 ± 6% in the presence of anti-IL-10 and anti-TGF-β (not significant, Wilcoxon signed rank test).

Discussion

Protocols for expansion of CD4⁺CD25⁺ Tregs, or *in vitro* induction of Tregs from CD4⁺CD25⁻ cells, compare the characteristics of these Tregs with the characteristics of natural CD4⁺CD25⁺ Tregs. In addition, naturally occurring Tregs are also the standard to which other types of Tregs such as Tr1 and CD8⁺CD28⁻ Tregs are compared [19, 20]. Treatment protocols aimed at the *in vivo* or *in vitro* induction and/or expansion of Tregs need to be tested and optimised. Since mouse models of tolerance induction are of low predictive value for tolerance induction in primates [32], the development of a relevant preclinical NHP model for Treg based therapies is needed [33]. It is crucial for the further understanding how to use Tregs to (re)induce a state of tolerance.

Analysis of rhesus macaque and baboon regulatory T-cells showed that CD4⁺CD25⁺ are present in the normal repertoire of NHP, and that these are indeed very similar to human Tregs [30, 34]. In addition, naturally occurring Tregs of cynomolgus monkeys have been successfully expanded [29]. Furthermore, we have previously reported the presence of T-cells with a CD4⁺FOXP3⁺ phenotype in kidney allografts, although they are mainly present in rejected kidneys [35]. However, thus far a thorough description of rhesus monkey CD4⁺CD25⁺ cells is lacking.

We report here the phenotypical and functional characteristics of CD4⁺CD25⁺ cells in normal healthy rhesus monkeys. In contrast to human Tregs, rhesus monkey CD4⁺CD25⁺ cells cannot be separated in a clearly distinct CD4⁺CD25^{high} population and CD4⁺CD25^{int} cells, while CD25^{high} cells can be discerned in human PBMC using the same settings (Fig. 6.1A and B). We cannot exclude that this is due to a difference in binding kinetics of the antibodies between human and rhesus monkey cells, but possibly, the CD25 staining pattern resembles the uniform CD25 expression in mouse spleen cells or human cord blood cells [3], although Wing et al. describe that human cord blood cells are almost all CD25^{high} [36]. The human cord blood and mouse CD25⁺ cells have a naive phenotype [3, 36]. The CD25⁺CD45RA⁺ population in human peripheral blood decreases with age and might well represent a subset of thymus-derived naturally occurring Tregs [3, 5]. These cells were identified as CD25^{int}, in contrast to the CD25^{high} cells, which are mostly CD45RA⁻ [2, 3, 5, 36]. The human CD45RA⁻CD25^{high} population might represent a population of antigen-experienced cells, which have differentiated from CD4⁺CD25⁻ cells into regulatory T-cells *in vivo* [37]. Both CD25^{int} and CD25^{high} populations have regulatory activity, but functional differences may exist with regard to the life span and possibilities of expanding the population [37, 38]. Similar to human Tregs, most rhesus monkey CD25⁺ cells have a memory phenotype (CD45RA⁻, Fig. 6.1C). Whether expression of L-selectin (CD62L) is highest on human CD25^{int} or CD25^{high} cells, remains a matter of controversy [2, 5, 36]. In the rhesus monkey highest CD62L expression is found on CD25^{high} cells, but this is still lower than expression found on CD25⁻ cells. In conclusion, rhesus monkey CD25⁺ cells have a CD25 expression pattern that resembles naive Tregs as found in mice and human cord blood, but they are not naive. Their antigen-experienced state is more similar to adult human peripheral blood Tregs.

We have investigated the phenotype of the CD25⁺ cell population, including CD25^{int} cells and CD25^{high} cells. Currently, the most specific marker for Tregs is FOXP3, although in humans FOXP3 is also expressed in non-regulatory cells [7–9]. We found that rhesus monkey CD4⁺CD25⁺ cells express FOXP3 at much higher levels as compared to CD4⁺CD25[−] cells. CTLA-4 is also expressed at high levels in rhesus monkey CD4⁺CD25⁺ cells, and expression of both FOXP3 and CTLA-4 is even higher in the CD25^{high} population, which is similar in humans [11, 12, 36, 39]. However, CD25^{int} cells also express FOXP3 and CTLA-4 at much higher levels than CD25[−] cells (Table 6.1). Some reports describe that FOXP3 and CTLA-4 expression is found in human CD25^{int} cells as well, while others report that this is not the case [3, 36].

When identifying Tregs, it is most important to evaluate their capacity to suppress responder cells. We investigated the functional activity of monkey CD4⁺CD25⁺ cells as isolated by MACS beads. This yielded a population enriched with CD25^{high} cells, but also contained CD25^{int} cells. This population is anergic to TCR-mediated stimulation, and can inhibit proliferation and production of IFN- γ and IL-2 of CD4⁺CD25[−] responder cells, which were stimulated via plate-bound anti-CD3 or allogeneic cells (Fig. 6.3 and 6.4). The inhibition is mediated through cell-cell contact, and is CD25⁺ cell dose dependent (Fig. 6.4 and 6.5). Variation between animals was seen with regard to the effectiveness of suppression. Some individuals display proliferation of CD25⁺ cells and then low inhibition of proliferation of CD25[−] cells was seen. Possibly, in these individuals non-regulatory CD25^{int} cells may have contaminated the isolated CD25⁺ suppressor population. The level of suppression of IL-2 and IFN- γ production is very similar to what has been observed in humans [2, 11].

Beacher-Allan et al. reported that increasing signal strength of the TCR stimulus might overcome anergy of CD25^{high} cells, with loss of suppressive function [2]. We have also seen this, when soluble anti-CD28 was added to the cultures stimulated with plate-bound anti-CD3, suppression was lost, with subsequent proliferation of CD25⁺ cells (Fig. 6.3A). Irradiated APC were present in the cultures. These were added in accordance with a report on the analysis of human Tregs [37]. The amount of cells available did not allow for the use of T-cell depleted accessory cells as reported by others [2, 5, 11, 12]. The APC may have presented the anti-CD28 antibodies via their Fc receptors, thereby cross-linking CD28 on the responder cells. However, anti-CD28 alone (with APC present) did not stimulate proliferation in rhesus monkey CD25[−] cells and induced only very little proliferation in human CD25[−] cells. This indicated that the anti-CD28 antibodies used are not agonistic, and most likely, it is the combined signal from plate-bound anti-CD3 and soluble anti-CD28 that induced proliferation in CD25⁺ cells.

The sensitivity of the cells stimulated with plate-bound anti-CD3 alone, may vary between individuals, possibly explaining the observed inter-animal variation. Disruption of the anergy of CD25⁺ cells can be used to expand Tregs. After a period of expansion, the cells need to be cultured in the absence of a stimulus, a resting period, to regain their suppressive capacity [19, 40]. In conclusion, rhesus monkey CD25⁺ cells resemble human Tregs. The highest expression of FOXP3 and CTLA-4 is found

in the CD4⁺CD25^{high} population, but also CD25^{int} cells express considerable levels of FOXP3 and CTLA-4. The CD25⁺ cells have a memory phenotype. CD4⁺CD25⁺ cells isolated by MACS are anergic and can suppress CD4⁺CD25⁻ cells via a cell-cell contact dependent mechanism. These results warrant further development of the rhesus monkey model as model to study the induction of tolerance by Tregs.

Acknowledgements

The authors thank Prof.Dr. F. Claas for critical review of the manuscript.

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