

Human Cytomegalovirus-specific regulatory and effector T cells are clonally identical

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ZUSAMMENFASSUNG

Die Mehrzahl der im Thymus generierten CD4+CD25high regulatorischen T-Zellen (Treg) besitzt hohe Affinität gegenüber körpereigenen Antigenen. Es ist bekannt, dass T-Zell Rezeptoren (TCR) auf Treg Zellen in der Peripherie zusätzlich auch fremde Antigene verschiedener Pathogene wie Parasiten, Bakterien und Viren erkennen.

Wenig ist bekannt über das klonale T-Zell Rezeptor Repertoire dieser Treg Populationen und ihre Beziehung zu CD4+CD25low effektor T-Zellen (Teff) im Menschen. In dieser Studie analysieren wir humane TCR auf expandierten Treg and Teff Zellen mit definierter Antigen Spezifität für Haupthistokompatibilitätskomplex (MHC) Klasse II restringierte „fremde“ Epitope des Cytomegalovirus (CMV).

Bemerkenswerterweise fanden wir, dass der gleiche TCR V β -CDR3 Klon in beiden funktionell unterschiedlichen Subpopulationen *in vitro* dominant expandiert ist.

Im Unterschied zu ihren klonal-identischen Teff Gegenspielern, exprimieren die suppressiven Treg Zellen kaum CD127 und IL-2, aber hohe Mengen an IFN γ und IL-10. Zusammen mit der signifikant erhöhten FOXP3 Expression, trotz unvollständiger *foxp3*-DNA Demethylierung, lassen sich die CMV-spezifischen CD4+CD25high Treg Zellen einem induzierten Treg (iTreg) Phänotyp zuordnen mit Ähnlichkeit zum beschriebenen Tr-1 Phänotyp.

Darüber hinaus konnten wir die klonale TCR Identität auch in frisch isolierten CD4+CD25low und CD4+CD25high Subpopulationen bestätigen, was die Entstehung von CMV-spezifischen Treg Zellen bereits *in vivo* nahe legt. Periphere CD25high Treg Zellen supprimieren die antivirale Immunantwort in Patienten mit häufigen CMV-Reaktivierungen, was auf ihre Bildung als Reaktion chronischer Antigenexposition interpretiert werden kann.

Unsere Ergebnisse beweisen erstmals direkt, dass aus dem gleichen humanen T-Zell Klon Teff und Treg Zellen mit identischer Spezifität entstehen können und lassen vermuten, dass die Treg Induktion in der Peripherie durch häufige Antigenexposition vorangetrieben wird.

SUMMARY

The majority of thymically arised regulatory CD4⁺CD25^{high} T cells (Treg) show high affinity to self-antigens. It has been proposed that T-cell receptors (TCR) on Treg cells in the periphery also recognize foreign-antigens from pathogens, such as bacteria and viruses. Studies in mice have shown that peripheral Treg cells can be generated not only from naïve T cells but also from effector T cells (Teff).

However, in humans the clonal TCR-repertoire of these Treg populations and their relation to effector CD4⁺CD25^{low} Teff is not sufficiently known up to date. Here, we analyzed human TCRs derived from expanded Treg and Teff cells with defined specificity to MHC class-II restricted “foreign” epitopes of Cytomegalovirus (CMV).

Remarkably, we found that both functionally distinct subsets share the same dominant TCR-CDR3 clones *in vitro*. In contrast to their Teff counterparts, the Treg cells express low CD127 and IL-2, but high IL-10 upon antigen stimulation. Therefore, together with increased FOXP3 expression, but incomplete *foxp3* DNA-demethylation, human CMV-antigen specific Treg cells exhibit an induced phenotype (iTreg) *in vitro* with similarity to recently described Tr-1 phenotype.

Moreover, the clonal identity was confirmed in freshly isolated CD4⁺CD25^{low} and CD4⁺CD25^{high} subsets, suggesting their generation occurred already *in vivo*. Peripheral CD25^{high} Treg cells suppress the anti-viral immune response in patients with frequent CMV-reactivations, implying their development as reaction on chronic antigen-exposure.

Our results demonstrate directly for the first time, that the same human T-cell clone can possess the phenotype of Teff and Treg cells with specificity to identical foreign epitopes and suggest that Treg-induction in the periphery is supported by frequent antigen-exposure.

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ABBREVIATIONS

AA	Amino Acid
Ag	Antigen
APC	Antigen presenting cell
CD	Cluster of differentiation
CDR3	Complementary determining region 3
CFSE	Carboxy fluorescein succinimidyl ester
CMV	Cytomegalovirus
CNI	Calcineurin inhibitors
CpG	Cytosine and guanine separated by phosphate
DMSO	Dimethyl Sulfoxide
DNA	Desoxyribonucleic acid
EBV	Epstein-Barr virus
FW	Forward primer
FACS	Flow cytometry
<i>foxp3</i>	Forkhead box P3 (DNA)
Foxp3	Forkhead box P3 (murine protein)
FOXP3	Forkhead box P3 (human protein)
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HLA	Human leukocyte antigen
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
IE1	Immediate early protein 1
IFN	Interferon
IFN γ	Interferon-gamma
IL	Interleukin
IL-2	Interleukin 2
IL-10	Interleukin 10
IU	International unit
iTreg	induced regulatory T cell
J β	Junction region of TCR β -chain
LCL	Lymphoblastic cell line
MHC	Major histocompatibility complex
N	Non-recurring
ND	Not detectable (under limit of detection)
nTreg	natural regulatory T cell

PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
pp65	phospho-protein 65
PTLD	Post-transplantation-lymphoproliferative disease
R	Recurring
RV	Revers primer
rIL-2	recombinant IL-2
RNA	Ribonucleic acid
S.D.	Standard deviation
SEM	Standard error of the mean
SO	Solid-organ
SOT	Solid-organ-transplantation
TCR	T-cell receptor
Teff	effector T cell
Th1	T-helper cell type 1
Treg	regulatory T cell
TGF- β	Transforming growth factor β
Tr1	T-regulatory cell type-1
Tx	Transplantation
V β	variable TCR β -chain

1 INTRODUCTION

1.1 ORIGIN OF HUMAN REGULATORY T CELLS

Human regulatory CD4+CD25^{high} T cells (Treg) play a crucial role in preventing autoimmunity, but they can also weaken the organism's immune response to infection (Shevach, 2002) (Thompson and Powrie, 2004) (Powrie, et al., 2003). Treg cells arise from two major sources; immature T cells committed to the lineage of natural Treg cells (nTreg) in the thymus and mature T cells recruited by antigenic induction to the regulatory population in the periphery (iTreg) (Sakaguchi and Powrie, 2007).

1.2 RELATIONSHIP OF TEFF AND Treg CELLS

Studies in mice and humans have shown that (Larkin, et al., 2008) peripheral Treg cells can be generated not only from naïve CD4⁺ cells (Apostolou and von Boehmer, 2004) but also from CD4⁺CD25⁻ effector T cells upon suboptimal activation of autoantigens (Knoechel, et al., 2005) (Seddon and Mason, 1999) or foreign antigens (Curotto de Lafaille, et al., 2004) (Kretschmer, et al., 2005) (Vukmanovic-Stejic, et al., 2006).

1.3 ANTIGEN SPECIFICITY OF Treg CELLS IN INFECTION

Recently, several groups reported elevated percentages of CD4+CD25+ Treg cells in the total T-cell population isolated from peripheral blood of patients undergoing diverse chronic infections of microbes (Belkaid and Rouse, 2005), parasites (Suffia, et al., 2006) and viruses, such as Herpes simplex virus (Suvas, et al., 2003), Hepatitis C Virus (Boettler, et al., 2005) (Rushbrook, et al., 2005) and Cytomegalovirus (Vukmanovic-Stejic, et al., 2006).

Mostly, the role of human Treg cells in response to infections has been examined by *in vitro* suppression assays. One study reports that during chronic Hepatitis C Virus (HCV) infection, cells with a CD4+CD25+ regulatory phenotype suppress *in vitro* proliferation of virus-specific CD8+ T cells (Boettler, et al., 2005). Importantly, CD4+CD25+ T cells from person recovered HCV infection and from healthy blood donors exhibited significant less suppressive activity. This was associated with a higher frequency of circulating CD4+CD25+ T cells.

Of particular interest was the finding that the suppression, mediated by the freshly isolated polyclonal CD4+CD25+ Treg cells, was not restricted to HCV-specific CD8+ T cells but also to Influenza virus specific CD8+ T cells.

Although, this experiment and other studies (Boettler, et al., 2005) (Belkaid and Rouse, 2005) clearly verify the ability of human CD4+CD25+ Treg cells to down-modulate T-effector responses specific for exogenous antigens, it is still unclear whether the Treg antigen-recognition is specific or rely on inherent cross-reactivity and bystander effect (Larkin, et al., 2008) (Ochando, et al., 2005).

1.4 T-CELL RECEPTOR (TCR) REPERTOIRE OF Treg CELLS

Some studies conclude that Treg cells express a diverse repertoire of TCRs that overlap with the TCR repertoire of CD4⁺ naïve T cells or CD4⁺CD25⁻ memory T cells (Kasow, et al., 2004) (Taams, et al., 2002) (Fujishima, et al., 2005), whereas other studies in mice conclude that peripheral Treg cells express different TCRs that are usually self-reactive and intersect with TCR expressed by thymic-derived Treg cells (Hsieh, et al., 2006). Moreover, a recently published report in mice has shown that thymically arised Foxp3⁺ Treg recognize foreign antigenic peptides as often as non-regulatory T cells (Pacholczyk, et al., 2007).

So far, analysis of human T-cell receptors (TCR) expressed on peripheral CD4⁺CD25⁺ Treg cells reached no uniform conclusion.

1.5 PHENOTYPIC MARKERS OF HUMAN CD4⁺ Treg CELLS

Although, the expression of CD25 (the γ -chain of high-affinity IL-2 receptor) on T cells can be a useful marker of Treg cells on peripheral blood cells, its expression is not necessarily associated with Treg cell function as it is also expressed by activated non-regulatory effector T cells. Therefore, in freshly isolated antigen non-exposed PBMCs CD4⁺CD25^{high} Treg cells are distinguished in many current studies from CD25^{low} – CD25^{intermediate} effector T-cell populations. Other molecules, including the glucocorticoid-induced TNFR family related gene (GITR) and the cytotoxic T-lymphocyte antigen-4 (CTLA-4) have been used as markers. However, both are activation markers also expressed on activated effector lymphocytes. More recently, two groups found that CD127 expression (the α chain of the interleukin-7 receptor) inversely correlates with suppressive function of CD4⁺ Treg cells (Seddiki, et al., 2006) (Liu, et al., 2006).

The transcription factor Foxp3 (Forkhead box P3) is currently considered to be the most reliable marker for CD4⁺ Treg cells in mice and men. Although, human FOXP3 expression is not as restricted to Treg cells as the murine counterpart, because it is also expressed in effector T cells at a relatively low level. A notable difference between humans and mice is that FOXP3 expression in human CD4⁺ T cells is tightly linked to TCR-mediated activation (Roncarolo and Gregori, 2008). Recent studies further revealed, that human nTreg cells express FOXP3 stable, whilst induced Treg cells assemble transient upregulation. These CD4⁺CD25^{high} Treg cells are induced in the periphery by MHC-peptide stimulation, secrete low levels of IL-2, in some studies TGF- β and/or IFN γ and high levels of IL-10 (T-regulatory cells type-1, Tr-1) (Roncarolo and Gregori, 2008).

1.6 FOXP3 IN HUMAN ANTIGEN INDUCED Treg CELLS

Foxp3 expression has been proposed to determine the fate of thymic precursors to the natural Treg-cell lineage (Fontenot, et al., 2005) and to be a crucial master transcription factor in the induction of peripheral Treg-cell populations (Apostolou and von Boehmer, 2004) (Hori and Sakaguchi, 2004). Conversely, in humans not all CD4⁺ FOXP3⁻ T cells that are induced to express FOXP3 also acquire regulatory function (Gavin, et al., 2006) (Allan, et al., 2008; Allan, et al., 2007), and further some but not all of the induced Treg cells that obtained suppressive function also expressed stable FOXP3 (Levings, et al., 2005) (Roncarolo, et al., 2006) (Roncarolo and Gregori, 2008) (Allan, et al., 2008).

Recently, a conserved region within the locus of *foxp3* has been identified comprising complete demethylation of CpG motifs in freshly isolated CD4⁺CD25⁺ Foxp3⁺ Treg cells but not in naïve CD4⁺CD25⁻ T cells (Floess, et al., 2007). In contrast to these natural Treg cells, peripheral Tregs in mice and humans induced *in vitro* by TGF- β display incomplete demethylation despite high Foxp3 expression.

Additionally, these *in vitro* induced Tregs lose Foxp3 expression and correspondingly suppressive activity upon further restimulation in absence of TGF- β (Floess, et al., 2007) (Polansky, et al., 2008) (Baron, et al., 2007). However, whether human peripheral antigen-induced Tregs need epigenetic modification of the *foxp3* locus for lineage stability and suppressive function has thus far not been investigated. Recent studies on human IL-10+ Tr1-like Treg cells suggest unstable FOXP3 expression (Roncarolo and Gregori, 2008).

1.7 Treg CELLS IN CHRONIC CYTOMEGALOVIRUS INFECTION

Cytomegalovirus (CMV) infection is a major clinical problem in immunosuppressed patients, particularly in organ transplant recipients. About 65 % of adults are CMV seropositive, and T cells play a central role in controlling the persistent virus. Some transplant patients repeatedly suffer from active infection that can result in CMV disease and graft injury (Reinke, et al., 1999). In these patients the frequent crosstalk between CMV and T cells might favour the generation of iTreg. Notably, to date this putative coherency has not been investigated.

1.8 IMPACT OF Treg CELLS ON ANTI-VIRAL T-CELL THERAPY

In contrast to the importance of viral-induced Treg cells in preventing overwhelming inflammatory effector T-cell response, their presence in chronic infected person might diminish an effective viral clearance and could explain, at least in part, why current clinical trials using viral-peptides, induce only weak or transient immune response or fail to produce therapeutic benefit.

In haematopoietic stem cell transplantation (HSCT) adoptive immunotherapy with allogeneic donor-derived T cells directed against e.g. Epstein-Barr virus (EBV) has been proven to prevent EBV-related lymphoma and treat the clinical manifestation of the virus (Rooney, et al., 1998). In solid organ transplantation (SOT) a recent approach in field of adoptive anti-viral therapy is the generation of autologous T-cell lines, directly isolated and expanded from peripheral blood of the immunosuppressed organ transplant recipient, suffering from the viral disease. Recently, in cooperation with Savoldo et al. we published a multicenter study in which SOT recipients at high risk for EBV-associated post-transplantation lymphoproliferative disease (PTLD) were treated with autologous EBV-specific cytotoxic T lymphocytes (CTLs) (Savoldo, et al., 2006). Thereby, we have investigated the *in vivo* safety, efficacy, and persistence of the infused autologous EBV-CTLs. None of the treated patients developed PTLD. However, despite the monitored transient increase in plasma EBV-DNA after T-cell infusion suggestive of lysis of EBV-infected cells, there was no consistent decrease in virus load in peripheral-blood mononuclear cells (50% relapse). Further, returned the initial increase in the frequency of EBV-responsive T cells, detected by IFN γ enzyme-linked immunospot assays, to preinfusion levels after 2 to 6 months.

In summary, these data are consistent with an expansion and persistence of adoptively transferred EBV-CTLs, but with limited long-term efficacy in the presence of continued immunosuppression and active chronic viral infection. We just started to introduce this promising therapy option also for SOT patients suffering from chronic CMV disease. Using adoptive T-cell therapy we presume that targeting the presence of regulatory T-cell, their development, clonal identity and antigen-specificity might be of main interest to improve the *in vitro* and *in vivo* efficacy of viral-specific T-cell lines.

1.9 MAIN SUBJECTS OF THE STUDY

To investigate foreign-antigen specific iTreg in humans, we used the immunologically well-characterized CMV *in vivo* “infection model”. We determined CD4+ T-cell response to CMV immediate-early protein 1 (IE1) and phosphoprotein 65 (pp65) epitopes by using our recently established method, employing protein-spanning overlapping peptide pools and matrix-analysis methodology (Kern, et al., 2000; Kern, et al., 1998). Individual epitope-specific CD4+ T cells were selected, expanded, and functionally characterized according to their subsets expressing low or high CD25 levels, respectively.

The following questions were addressed:

1. Are T cells with regulatory properties detectable among CMV-specific T-cell lines?
2. What are the phenotypes and functional characteristics of Treg cells specific for CMV?
3. Are Treg and Teff cells with specificity for CMV clonally related?
4. Are CMV-specific Treg cells detectable before *in vitro* expansion?
5. Is there a correlation between chronic CMV and Treg frequency and function *in vivo*?

2 RESULTS

2.1 SUBSETS OF CMV-SPECIFIC CD4+ T-CELL LINES

To isolate human CMV antigen-specific CD4+ T-cell subsets, we firstly mapped (Kern, et al., 1998) various seropositive donors regarding their CD4-epitope response to the immunodominant CMV antigens, IE1 and pp65. This had led to successful identification of several viral epitopes recognized by CD4+ T cells (Table 1).

Tab. 1: CD4-Epitopes.

Antigen	First AA	Sequence (≥15AA)	Last AA	Potential HLA-Restriction
IE1	89	IKVRVDMVRHRIKEHMLKK	107	DR13
IE1	269	LTHIDHIFMDILTTCVETM	288	DR3
pp65	25	AVFSRGDTPVLPHET	39	DR3
pp65	45	LLQTGHIVRVSQPSL	59	DR13, DR15
pp65	245	TLGSDVEEDLTMTRNPQPF	263	DR3

AA: amino acid; HLA: Human leukocyte antigen; IE1: Immediate early protein 1; pp65: Phospho-protein 65 of Cytomegalovirus.

Secondly, we isolated cells responding to these CD4-epitopes (0.2–0.5 %) from PBMC by IFN γ secretion assay (Hammer, et al., 2005). Specific T cells were expanded *in vitro* in the presence of irradiated autologous PBMC over 14 days (Figure 1A) to cell numbers of 0.5-1.0 x10⁸ CD3+ T cells, and as expected predominantly consisted of CD4+ T cells.

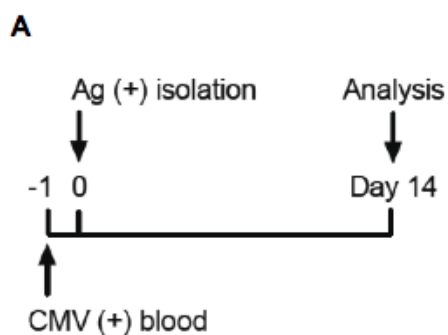


Figure 1A. Isolation and Expansion Protocol of CMV Epitope-Specific T Cells

PBMC from CMV seropositive donors are short-term stimulated with peptides coding for identified CD4-epitopes on the CMV antigens (Ag) pp65 and IE1. Specifically isolated cells are subsequently expanded in the presence of peptide-loaded APC and rIL-2 over 14 days.

Re-stimulation of expanded T cells with autologous antigen presenting cells (APC) and the specific antigen induced a strong IFN γ response of epitope-specific CD4 $^{+}$ T cells, exemplary shown for pp65 $_{<245-263>}$ specific CD4 $^{+}$ T cells (Figure 1B) (mean frequency \pm S.D.: 34.6 ± 24.7 %, n=5).

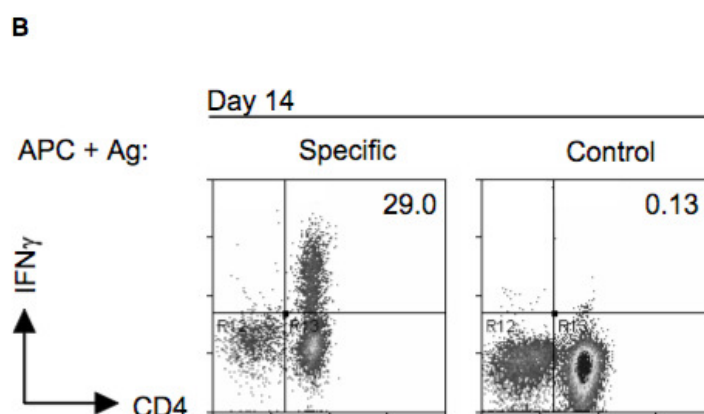


Figure 1B. CMV Epitope-Specific CD4 $^{+}$ T cells Can Be Expanded In Vitro.

Percentage of epitope-specific CD4 $^{+}$ T cells at day 14 of expansion, as demonstrated by intracellular IFN γ staining after TCR stimulation with autologous APC and the specific antigenic peptides or unspecific control. Data are representative of five independently expanded epitope-specific T-cell lines, exemplary shown for pp65 $_{<245-263>}$ specific T cells.

Flowcytometric analysis on day 14 of epitope driven expansion determined different subsets regarding the CD25 expression levels – low, intermediate, and high. Intensity of CD25 expression and frequency of CD4+CD25high T cells in the T-cell lines (Figure 2A, left) (mean frequency \pm S.D.: 4.5 ± 2.6 %, n=5) was comparable to those of natural CD4+CD25high Treg observed directly in peripheral blood (Baecher-Allan, et al., 2004).

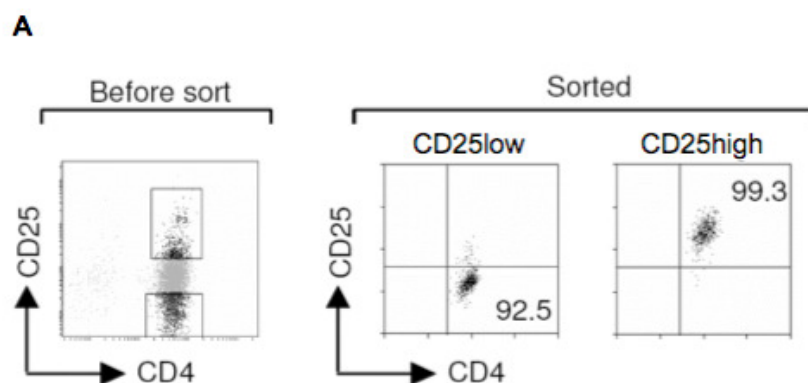


Figure 2A. Expanded Epitope-Specific CD4+ T cells Comprise Cells Possessing CD4+CD25high Treg Phenotype.

Epitope-specific CD4+ T cells expanded over 14 days in vitro were sorted according to CD25 expression. Numbers in dot plots indicate the percentage of gated CD4+CD25low (CD25low) and CD4+CD25high (CD25high) cells after sorting. Data are representative of five independent experiments.

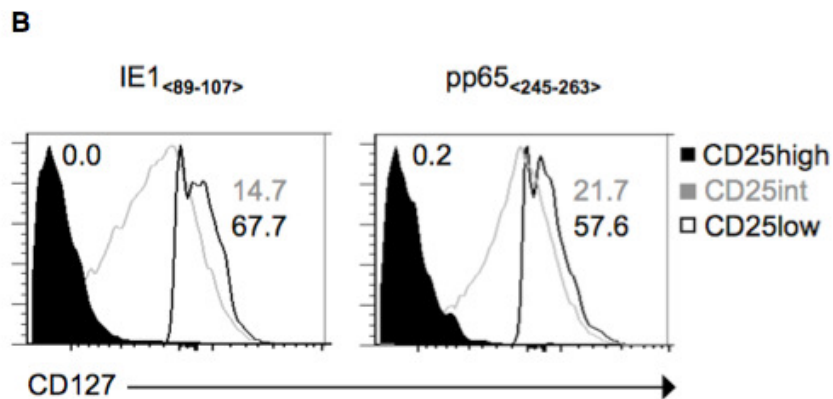


Figure 2B. Expanded CD4+CD25high T Cells Express CD127low.

Numbers in histograms indicate the percentage of CD127 expression on gated CD25high, CD25int and CD25low CD4+ T cells. Two representative data (specificity for IE1_{<89-107>} or pp65_{<245-263>}) of five independent expanded T-cell lines are shown.

However, in conditions involving deliberate T cell activation CD25 is a poor indicator for human regulatory T cells. Thus, we additionally analyzed CD127 and correlated it with CD25 expression. Conversely to CD25low and CD25intermediate CD4+ T cell populations, the CD4+CD25high T-cell subset expressed CD127low after specific re-stimulation (Figure 2B), indicating the presence of putative iTreg cells distinct from recently activated effector T cells (Seddiki, et al., 2006).

2.2 CMV-SPECIFIC EXPANDED CD4+CD25HIGH ARE SUPPRESSIVE

We next asked whether these specifically expanded CD25high T cells do also bear regulatory capacity. Therefore, five independent CD4+ T-cell lines with distinct CMV-epitope specificities from the two immuno-dominant antigens (IE1, pp65) were expanded to sufficient CD4+CD25high cell numbers to perform functional analysis.

On day 14, CD4+CD25^{low} (CD25^{low}) and CD4+CD25^{high} (CD25^{high}) T cells were sorted to high purity (Figure 2A, right) and restimulated by their specific peptide or an unspecific control peptide. Notably, as depicted in Figure 3A and Figure 4A upon specific re-stimulation CD25^{low} (CD127⁺) T cells produced high levels of IL-2, whereas IL-2 secretion in CD25^{high} (CD127^{low}) T cells was significantly lower (mean \pm S.D.: 1,237.0 \pm 340.1 compared to 122.5 \pm 119.5 pg/ml, n=5, p=0.0001 *t*-test, two tailed). Furthermore, CD25^{low} and CD25^{high} T cells were co-cultured at different ratios 1:0, 1:1, 1:0.5, 1:0.1 and 0:1 for 24h in the presence of the indicated specific peptide and APC. Indeed, CD25^{high} T cells suppressed the IL-2 secretion of CD25^{low} T cells in a dose-dependent manner.

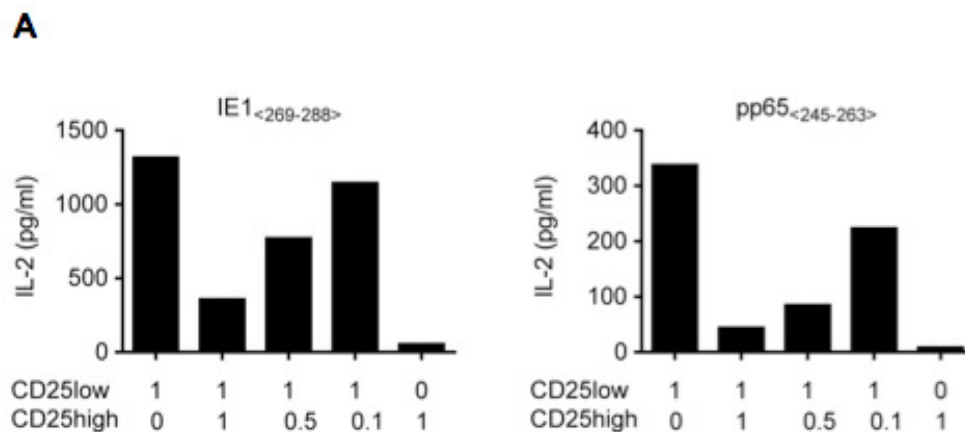


Figure 3A. Suppression of Specific IL-2 Secretion by CD4+CD25^{high} T Cells.

Sorted CD4+CD25^{low} (CD25^{low}) and CD4+CD25^{high} (CD25^{high}) populations were subsequently co-cultured at different ratios in the presence of APC and the respective peptide. After 24 h, secretion of IL-2 was measured by flowcytometric bead array. Unspecific stimulation showed background cytokine release near or under limit of detection (data not shown). Two representative data (specificity for IE1<269-288> or pp65<245-263>) of four independent experiments are shown.

This regulatory function *in vitro* was not only established for CD25^{high} cells with different CMV antigen and epitope specificity, exemplary presented for IE-1_{<269-288>} and pp65_{<245-263>} (Figure 3A) but also reproducible over time (>1yr), as three independently isolated and expanded CD25^{high} T-cell lines from the same donor and of the same Ag-specificity to pp65_{<245-263>} consistently possessed the ability to suppress IL-2 secretion (n=3, p=0.031, paired *t*-test, two tailed) (Figure 3B).

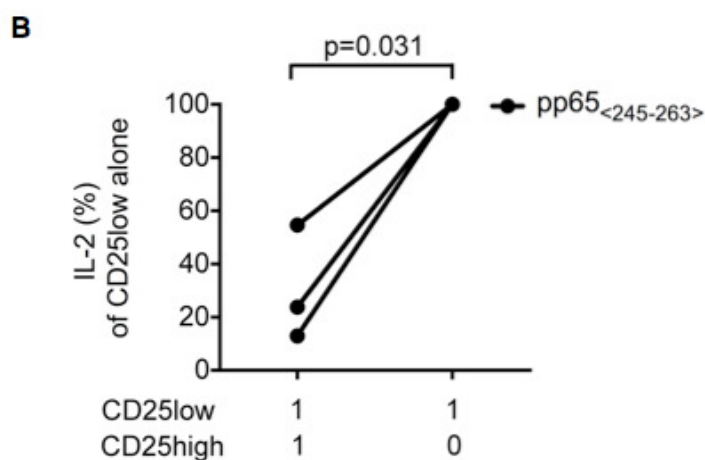


Figure 3B. Stable Suppression of Epitope-Specific IL-2 secretion by CD25^{high} Treg.

CD25^{high} T cells specific for pp65_{<245-263>} isolated and expanded at three different time points stably suppressed IL-2 secretion of CD25^{low} cells upon antigen-specific stimulation. IL-2 secretion was measured by flow-cytometric bead array. Data are representative of two independent epitope specificities.

Additionally, we found that peptide-driven proliferation of CD25^{low} T cells *in vitro* (Figure 3C) was suppressed when they were co-cultivated with CD25^{high} T cells recognizing the same epitope (n=3, p=0.003, paired *t*-test, two tailed). The inhibition on CD25^{low} proliferation was also dose-dependent and even evident at a tenfold smaller proportion of CD25^{high} cells.

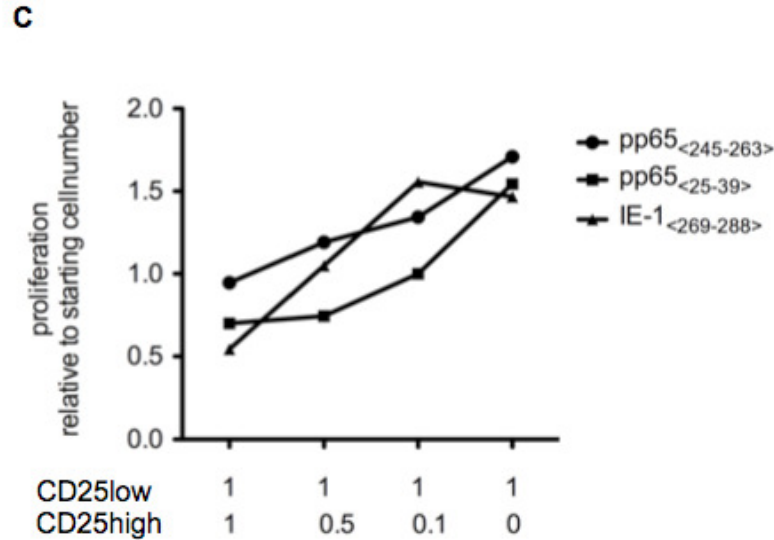


Figure 3C. Suppression of Epitope-Specific Proliferation by CD25high Treg.

Expanded CD25^{low} T cells were cultured alone or together with CD25^{high} T cells at different ratios over 5 days in the presence of APC and the indicated specific peptides. Cells that were dividing in the last 24h were measured by [³H] thymidine incorporation. Data are shown for three independent experiments with different epitope specificities (pp65_{<245-263>}, pp65_{<25-39>}, IE1_{<269-288>}).

On the basis of these evaluations, we assumed that expanded CD25^{high} T cells suppress the proliferation and IL-2 secretion of CD4⁺CD25^{low} T cells with the same antigen-specificity in a dose-dependent fashion. These findings are consistent with the defined *in vitro* properties of Treg cells in several previous reports (Lohr, et al., 2006), where the involvement of Treg cells in controlling immune responses to tissue transplants, tumors and various infectious agents (Sakaguchi, 2005) (Belkaid and Rouse, 2005) has been firmly established. These Treg cells are purported to use many cellular processes to control immune responses.

2.3 CD4+CD25HIGH Treg NEED SPECIFIC EPITOPE RECOGNITION FOR SUPPRESSIVE FUNCTION

It has been reported in mice, that once activated, Treg cells suppress in an antigen-unspecific way called 'bystander suppression' (Tarbell, et al., 2007) (Qin, et al., 1993). To investigate this issue for the human CMV-specific iTreg cells in this study, we tested whether IE-1_{<269-288>}CD25high iTreg cells can suppress the IL-2 response of pp65_{<245-263>}CD25low non-regulatory memory T cells with a different antigen specificity (Figure 3D). Importantly, both subsets were isolated and expanded from the same donor, and could thereby be restimulated in the presence of the same autologous APC.

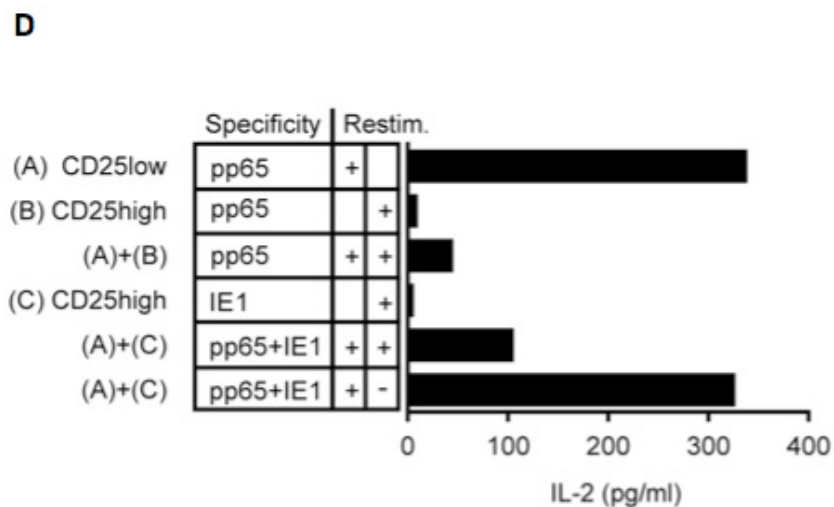


Figure 3D. Suppressive Capacity of CMV-Antigen Specific CD25high Treg Requires Activation by Their Cognate Epitope.

CD25low and CD25high T-cell populations from the same donor but of different antigen-specificity were cultured alone or co-cultured at a ratio of 1:1. The CD25high cells were cultured with (+) or without (-) their specific antigens (IE-1_{<269-288>} or pp65_{<245-263>}). IL-2 secretion was measured by flow-cytometric bead array. Data are representative of two independent experiments.

For this purpose, we firstly confirmed IL-2 suppression of pp65_{<245-263>}-specific CD25^{low} non-Tregs by pp65_{<245-263>}-specific CD25^{high} iTreg cells after re-stimulation with their respective peptide (pp65_{<245-263>}) as established previously. Next, pp65_{<245-263>}-specific CD25^{low} cells were cultured together with CD25^{high} iTregs specific for IE-1_{<269-288>} (also tested to express respective peptide-specific suppressive function; data not shown) in presence of both cognate antigens, pp65_{<245-263>} and IE-1_{<269-288>}. Under this conditions, IL-2 secretion of pp65_{<245-263>}-specific CD25^{low} cells was also diminished, contributing to `bystander suppression` of CD25^{high} iTreg cells *in vitro*. However, this suppressive ability was lost, when IE-1_{<269-288>}-specific CD25^{high} iTreg cells did not see their respective specific antigen during this co-culture.

Consequently, we demonstrated that although human CMV-specific CD25^{high} iTreg cells can suppress antigen-unspecifically (bystander suppression), they require prior specific activation through their TCR, comparable to other models (Roncarolo, et al., 2001) (Levings, et al., 2005).

2.4 CMV-SPECIFIC Treg EXPRESS LOW IL-2, HIGH IFN γ AND IL-10

Having shown that expanded foreign-antigen specific Treg cells can be isolated by CD25^{high} expression correlating with suppressive function *in vitro* we sought to further characterize this population for phenotypic and functional characteristics reported particularly for iTreg. Following re-stimulation with peptide *in vitro*, comparable IFN γ release was observed in both CD25^{high} and CD25^{low} T cells (IFN γ : mean \pm S.D.: 19.39 \pm 7.01 pg x10e3/ml vs. 17.60 \pm 8.74 pg x10e3/ml, respectively, n=5, p=0.730, *t*-test, two tailed). On the contrary, production of IL-10 was significantly higher in CD25^{high} compared to CD25^{low} T cells (IL-10: mean \pm S.D.: 74.47 \pm 42.49 pg/ml vs. 23.91 \pm 9.69 pg/ml, respectively, n=5, p=0.031, *t*-test, two tailed) (Figure 4A, middle, right).

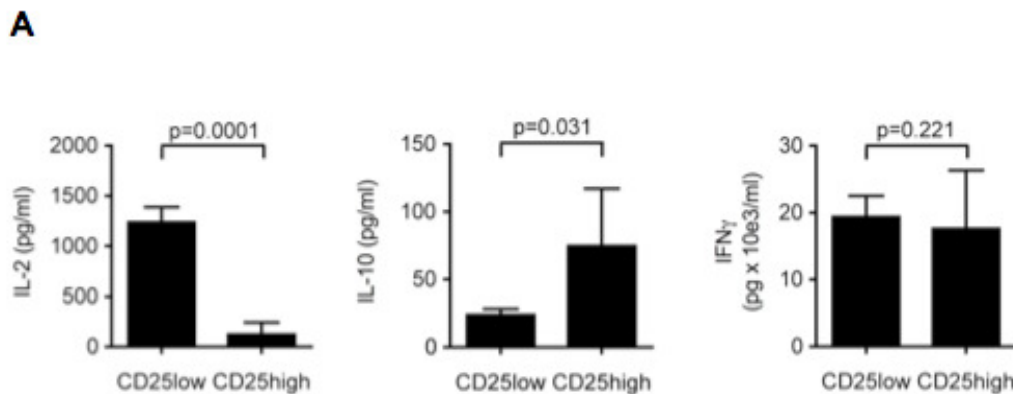


Figure 4A. Expanded CD25high Treg Cells Secrete Upon Epitope-Specific Stimulation Low IL-2, High IFN γ and IL-10.

CD25low or CD25high T cells were stimulated specifically for 24h. Concentrations (pg/ml) of secreted IL-2, IL-10 or IFN γ were measured by flow-cytometric bead array. DMSO control showed a background near or under the limit of detection (data not shown). Data are mean \pm S.D. of five independent epitope specific T-cell lines (t-test, tow-tailed).

IL-10 has been considered to be the key trait of an iTreg subset, classified as CD4+ T-regulatory type 1 (Tr1) cells. Upon activation via the TCR human Tr1 cells produce, besides IFN γ , high levels of IL-10 but very low levels of IL-2. They are described to suppress T-cell responses and maintain immunological tolerance (Levings, et al., 2005).

Whilst the development and function of nTreg cells is correlated with Foxp3 expression, studies in humans proved that Tr1 cells do not constitutively express FOXP3 (Vieira, et al., 2004) (Roncarolo and Gregori, 2008). Therefore, we were interested to provide details of FOXP3 expression status within IFN γ \square + compared to IL10+ CD25high iTreg on single cell level (Figure 4B) and importantly to determine the FOXP3-expression stability in the human CMV-specific iTreg subset.

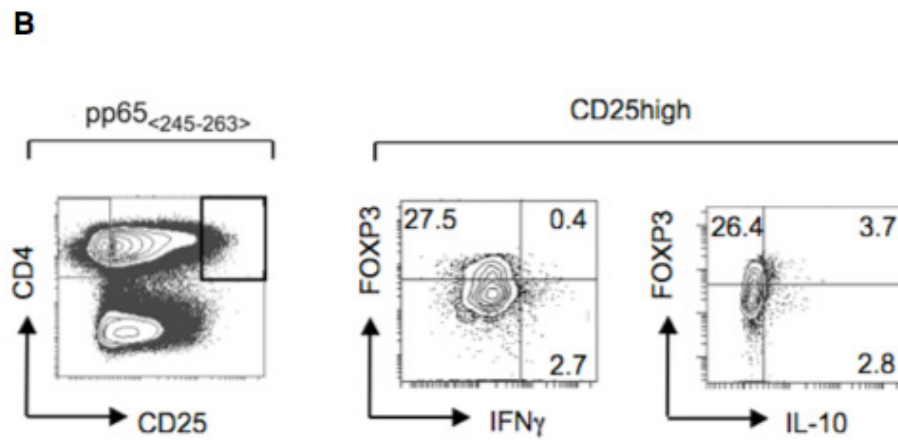


Figure 4B. FOXP3⁺ Expression in CD25^{high} Treg Cells is Not Correlated with IL-10 and Inversely Correlated with high IFN γ Production.

Expanded epitope-specific T cells were stimulated for 16h in the presence of autologous APC and the respective specific peptides. Brefeldin A was added to the culture after 2h of stimulation. IL-10, IFN γ and FOXP3 were measured by intracellular flowcytometry. Numbers in dot plots indicate the percentage of gated CD4⁺CD25^{high} cells (CD25^{high}). One representative data (specificity for pp65_{<245-263>}) of four independent experiments are shown.

2.5 METHYLATED FOXP3-DNA STATUS SUGGESTS TRANSIENT FOXP3 UPREGULATION IN CMV-INDUCED Treg CELLS

The total expression of FOXP3 on the different functional CD4⁺ subsets was determined by intracellular staining, and approximately 53% of CD25^{high} and 29% of CD25^{low} cells were FOXP3⁺ on day 14 of expansion. The gating strategy for FOXP3⁺ T cells and an exemplary dot plot is shown for IE1_{<269-288>}-specific CD4⁺ T cells (Figure 5A).

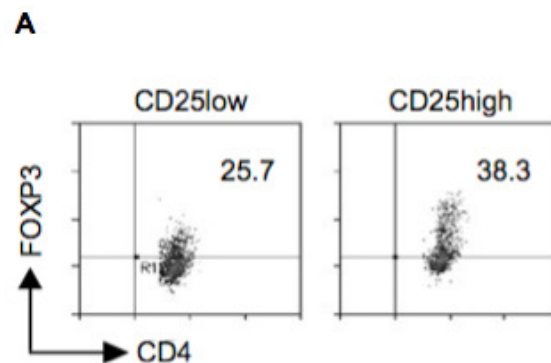


Figure 5A. FOXP3 Protein Expression Analyzed by Intracellular FACS Staining.

Numbers in dot plots indicate the FOXP3⁺ percentage of gated CD25^{low} or CD25^{high} CD4⁺ cells. Isotype-control antibody showed background staining of less than 0.2% (data not shown). Data are representative for five independent experiments and are shown exemplary for IE1_{<269-288>} specific T cells.

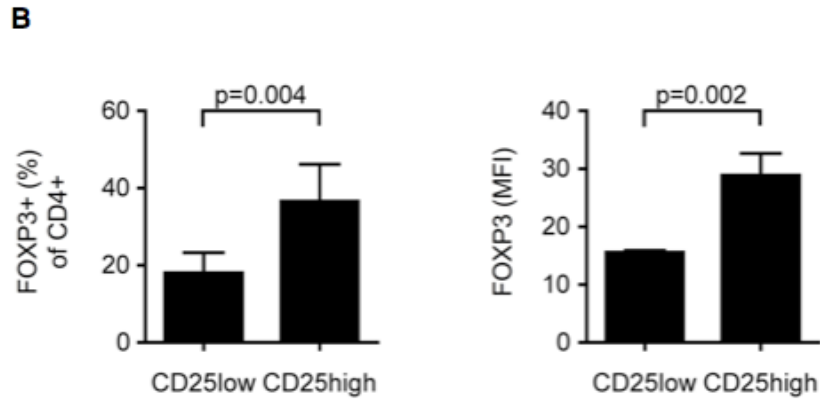


Figure 5B. CD25high Treg Cells Express More Intense FOXP3 than CD25low T Cells.

FOXP3+ (%) of CD4+ cells (left) and mean fluorescence intensity (MFI) of intracellular FOXP3 staining among gated CD25low and CD25high T-cell populations. Data are mean \pm S.D. of five independent experiments (t-test, two-tailed).

FOXP3 intensity and frequency was always higher in CD25high than in CD25low cells (FOXP3 [MFI] mean \pm S.D.: 28.9 ± 3.74 compared to 15.6 ± 0.75 , $n=5$, $p=0.002$ t-test, two-tailed) (Figure 5B); FOXP3+ [%] mean \pm S.D.: 36.6 ± 9.56 % compared to 18.1 ± 11.63 %, $n=5$, $p=0.004$ t-test, two-tailed), and the same was true for FOXP3 mRNA expression (Figure 5C) (FOXP3 mRNA [units/HPRT] mean \pm S.D.: 0.84 ± 0.27 compared to 0.17 ± 0.06 , $n=3$, $p=0.071$ t-test, two-tailed).

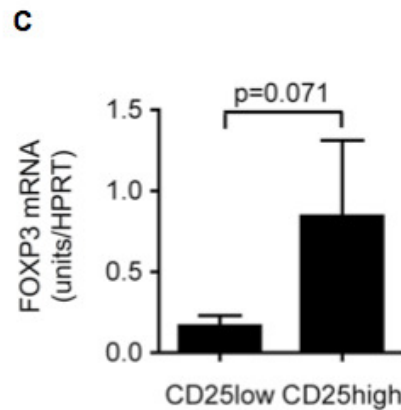


Figure 5C. CD25^{high} Treg Cells Express More FOXP3 mRNA Than CD25^{low} T Cells.

FOXP3 mRNA expression of sorted CD25^{low} and CD25^{high} T-cell populations was analyzed by real-time PCR and normalized to HPRT (house keeping gene). Data are mean \pm S.D. of three independent experiments (t-test, tow-tailed).

These findings prompted us to test whether intermediate expression level of FOXP3 in CD25^{high} T cells during CMV peptide-specific expansion might simply represent a transient induction of FOXP3. To clarify this hypothesis we took advantage of a recent study in mice that has proven that a stable *Foxp3* expression, as committed in the *ex vivo* isolated *Foxp3*⁺*CD4*⁺*CD25*⁺ Treg lineage must be epigenetically modified by complete demethylation of CpG motifs within an evolutionary conserved region on the *foxp3* locus (Floess, et al., 2007).

On the basis of this evaluation, we analyzed the methylation status of the homologous conserved CpG-rich region (Amplicon 5) on the human *foxp3* locus (Baron, et al., 2007) in sorted CD25^{high} iTreg cells and CD25^{low} T cells on day 14 of expansion by bisulfite sequencing. Interestingly, no difference in the degree of methylation between CD25^{high} and CD25^{low} cells was observed. Both populations displayed incomplete *foxp3*-demethylation as illustrated for CD25^{high} and CD25^{low} samples from two independent expanded CD4⁺ T cell lines specific for the epitopes IE-1_{<269-288>} and pp65_{<245-263>} (Figure 5D).

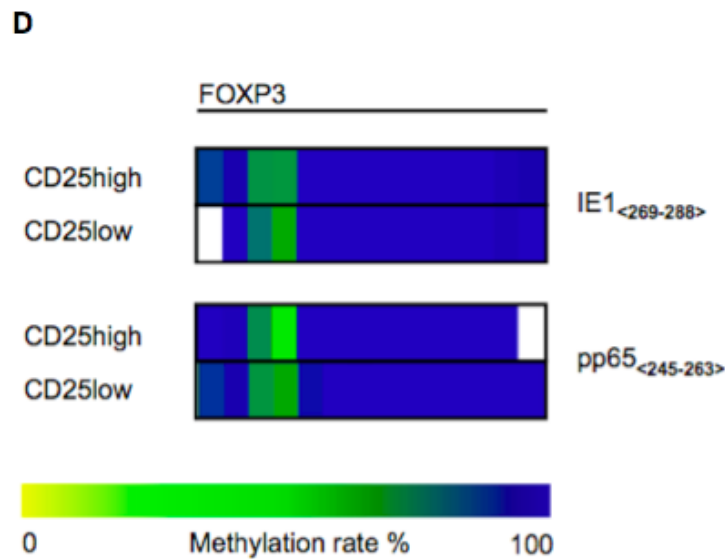


Figure 5D. Incomplete Demethylation of FOXP3-DNA Locus in Expanded CD25high iTreg and CD25low Teff Cells.

DNA-methylation status of the human *foxp3* amplicon 5 (Amp 5) is depicted. Data of two independent expanded epitope-specific (IE-1_{<269-288>} or pp65_{<245-263>}) CD4⁺ T cells from a female donor are shown. Data are representative for five individual donors (female and man). The upper panel depicts CD25^{high} T cells and the lower CD25^{low} T cells, separated by FACS Sort and tested for antigen-specificity and in vitro function, respectively. The selected amplicon is subdivided by horizontal boxes; each box represents methylation rate of a single CpG motif according to the colour code (yellow = 0% methylation, blue = 100% methylation).

This result corresponds to a recent finding that *in vitro* TGF- β induced Tregs in mice and also in humans display incomplete demethylation despite of suppressive activity and high Foxp3 expression (Floess, et al., 2007) (Baron, et al., 2007). Upon withdraw of TGF- β during re-stimulation these *in vitro* induced Tregs lost Foxp3 expression and correspondingly their suppressive activity. In contrast, human *ex vivo* freshly isolated polyclonal CD4+CD25^{high} Treg cells but not naïve CD4+CD25⁻ T cells comprise complete demethylation of CpG motifs as described previously (Baron, et al., 2007).

In summary, our experiments verify that human *in vitro* expanded CMV-specific CD4+CD25^{high} Treg cells are suppressive and contain a proportion of FOXP3 high expressing T cells. Furthermore, incomplete *foxp3*-demethylation status contributes to an antigen-induced transient FOXP3 upregulation in this CD25^{high} iTreg population.

2.6 Treg AND TEFF CELLS ARE CLONALLY IDENTICAL *IN VITRO*

To analyze the clonal TCR-repertoire of iTreg and non-Treg cells, which respond to the same foreign epitope, we took advantage of a recently described approach to type TCR-clones by highly sensitive analysis of V β -chain rearrangements and sequencing of CDR3 hypermutation region (Wlodarski, et al., 2006) (Wlodarski, et al., 2006).

When we assessed TCR repertoire within purified CD25^{high} and CD25^{low} T cell populations responding to the same CMV peptide, it became apparent that in all three independently expanded specific CD4⁺ T-cell populations single TCR V β -CDR3 clones were dominantly expanded, as reflected by their frequency; 25-50 % in CD25^{high} and 5-63 % in CD25^{low} T cells, respectively (Table 2). Importantly, both CD25^{high} and CD25^{low} populations shared the dominantly expanded TCR clone (Figure 6A), indicating that human CMV-peptide specific CD25^{high} and CD25^{low} cells are clonally identical.

Tab. 2: Frequency of Shared Dominant TCR Clones.

Epitope	Dominant TCR V β -CDR3 Clone			Frequency	
	V β	CDR3	J β	CD25 ^{low}	CD25 ^{high}
IE1 _{<269-288>}	V β 22	CASRGTGDQPQHF	J β 5.1	35 %	31 %
pp65 _{<25-39>}	V β 14	CASSTTGETAFF	J β 1.4	5 %	50 %
pp65 _{<245-263>}	V β 12	CAISESGMTVATNEKLFF	J β 1.1	63 %	25 %

V β : variable region of the T-cell receptor (TCR) beta-chain; CDR3: complementary determining region 3 of the TCR beta-chain; J β : joining region of the TCR beta-chain

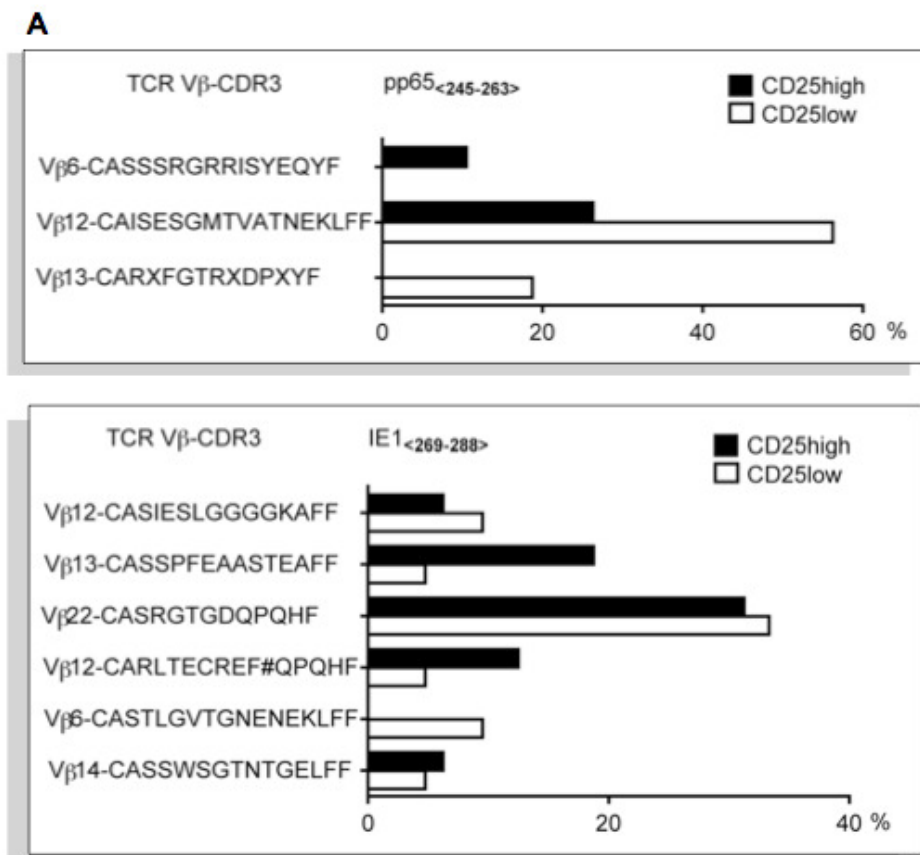


Figure 6A. Epitope-Specific CD25^{high} Treg and CD25^{low} Teff Cells Share Same Dominant Expanded TCR Clone.

Sequence-overlap of dominant TCR in regulatory CD25^{high} and non-regulatory CD25^{low} T cells expanded antigen-specifically in vitro. Dominant TCR clones were calculated from the number of expanded TCR sequences (≥ 2) within all sequenced clones ($16 \geq 21$) of a given population. Shown amino acid sequences represent complementary determining regions 3 (CDR3) within the observed variable region of the TCR beta-chain (TCR V β). Observed frequency of most dominant TCR in CD25^{high} and CD25^{low} T cell subsets were 25% and 63% of V β 12-CDR3 in pp65_{<245-263>} specific cells (Figure 6A, top) and 31% and 35% of V β 22-CDR3 in IE-1_{<269-288>} specific cells (Figure 6A, bottom), respectively. TCR clono-typing by V β -CDR3 sequencing was performed as described previously (Wlodarski, et al., 2006). Two exemplary data of three independent experiments are shown.

To more directly assess a possible clonal identity between iTreg and non-Treg cells, expanded T cells specific for pp65_{<245-263>} were sorted by flow cytometry for the dominant encountered V β 12⁺ clone to high purity (Figure 6B). Subsequently, V β 12⁺ cells were separated according to their CD4⁺CD25^{high} or CD4⁺CD25^{low} phenotype (Figure 6C, top).

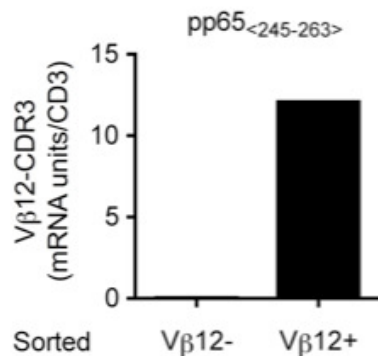
B

Figure 6B. Single V β 12-CDR3 T-cell clone is highly expressed in sorted pp65_{<245-263>} specific V β 12⁺ T cells.

The single V β 12-CDR3 T-cell clone was in V β 12⁺ T cells 12-fold higher expressed compared to V β 12⁻ T cells. Expression was verified by V β 12-CAISESGMTVATNEKLFF-J β 1.1 clone-specific real time PCR (Figure 6D) and normalized to CD3zeta (CD3). Data are representative of two experiments with independent specificities.

In fact, V β 12⁺CD25^{high} T-cell clones suppressed the CMV peptide-dependent IL-2 secretion of clonally identical V β 12⁺CD25^{low} T cells (Figure 6C, bottom) upon *in vitro* co-cultivation. In other words, the same “mother” clone can differentiate into CMV peptide-specific cells expressing regulatory or non-regulatory functions.

C

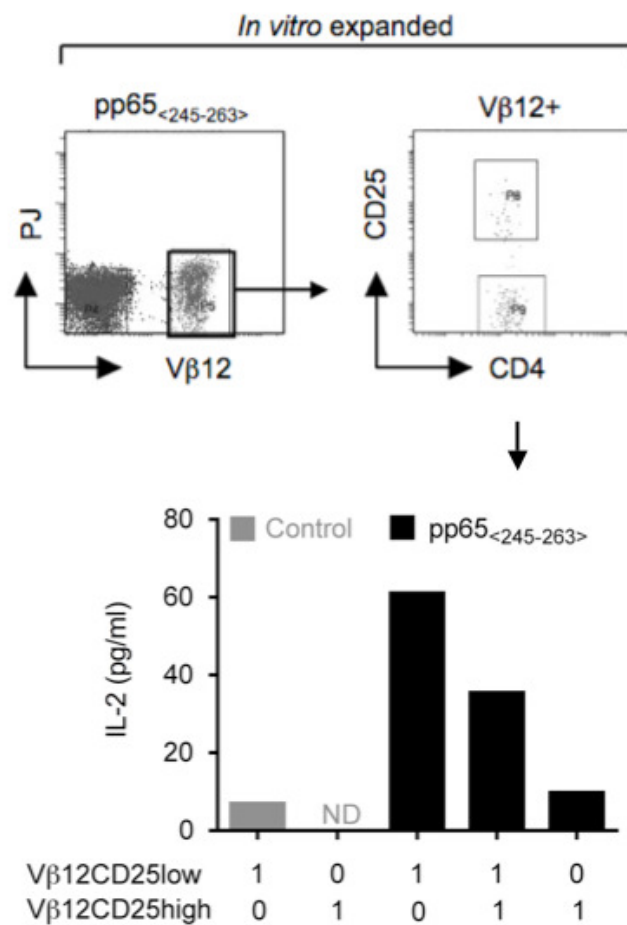


Figure 6C. Epitope-Specific Expanded T Cells of the Same Clonality Are Capable of Regulatory and Effector Function.

Top, Vβ12 expressing cells were sorted from in vitro expanded pp65_{<245-263>} specific T cells by flow cytometry and subdivided into Vβ12CD25^{low} and Vβ12CD25^{high} CD4⁺ T cells. Sorted populations were subsequently co-cultured at a ratio of 1:1 for 24 h in the presence of APC and the indicated specific peptides.

Bottom, Vβ12CD25^{low} secreted IL-2 in the presence of pp65_{<245-263>}, whereas in absence (Control) IL-2 secretion was near the limit of detection (ND) and cultured together with Vβ12CD25^{high} cells IL-2 secretion was suppressed. Data are representative of two experiments with independent T-cell clones of different specificity.

2.7 FRESHLY ISOLATED CD25^{HIGH} AND CD25^{LOW} CELLS SHARE SAME TCR CLONE

The observed incomplete FOXP3-demethylation might suggest that the CMV-triggered CD25^{high} iTreg isolated after expansion, are generated from CD25^{low} non-Treg cells as seen for TGF- β induced Treg. To address this point, we screened freshly isolated CD4⁺CD25^{high} and low subsets for the presence of the same T-cell clones detected in both subsets after CMV-specific *in vitro* expansion (see above). On the basis of the identified dominantly expanded TCR-V β CDR3 clones within the CMV-epitope specific CD25^{high} population, we designed a TCR clonotype specific PCR (Figure 6D).

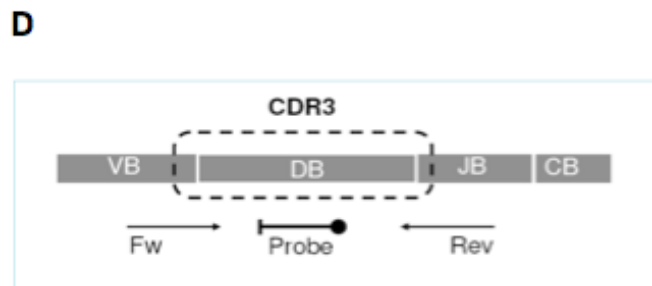


Figure 6D. Design of TCR Clonotype Specific PCR.

Real-time PCR is designed to employ CDR3-specific Taq-probe (Probe), V β -family specific forward primer (Fw) and J β -specific reverse primer (Rev). To increase the fidelity, each primer covered the flanking nucleotides of each CDR3 sequence. TCR: T-cell receptor; VB: variable region of the TCR β -chain; CDR3: complementary determining region 3 of the TCR β -chain; JB: joining region of the TCR β -chain

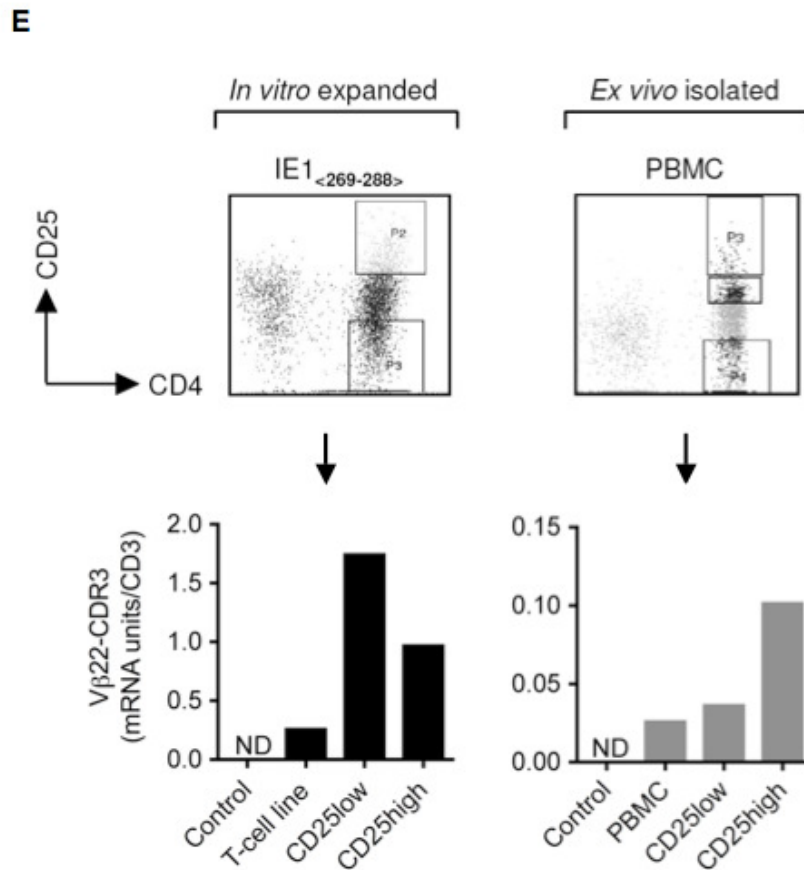


Figure 6E. Freshly Isolated CD25^{low} and CD25^{high} CD4⁺ Cells Contain The Same CMV-Epitope Specific T-cell Clone.

Dominant expanded epitope-specific CD25^{high} Treg clone was detectable in *ex vivo* isolated autologous peripheral CD4⁺CD25^{low} and CD4⁺CD25^{high} T-cell population by single V β -CDR3 specific PCR. Top, *in vitro* expanded IE1_{<269-288>} specific T cells (top left) and *ex vivo* isolated autologous PBMC (top right) were sorted according to CD25 expression. Bottom, TCR V β 22-CDR3 clonotype specific real-time PCR. Relative expression of the V β 22-CDR3 T-cell clone in IE1_{<269-288>} specific expanded CD25^{low}, CD25^{high} and the total T-cell line (bottom left) was found to be tenfold higher compared to directly *ex vivo* isolated CD25^{low}, CD25^{high} subsets and total PBMC (bottom right). In allogeneic PBMC of a CMV seronegative donor (Control) and also in expanded pp65-epitope specific T cells (Control) the V β 22-CDR3 clone was not detectable (ND). Data are representative of two independent experiments.

First, we tested the established V β 22-CDR3 PCR for specificity by using *in vitro* expanded IE1_{<269-288>} T cells (Figure 6E, left). As expected we detected high expression of the dominantly expanded T-cell clone in the purified CD25^{high} as well as CD25^{low} T cells, and also in the total non-purified T-cell line pool. By contrast, in autologous expanded T cells of a different CMV-specificity (Control), the IE1_{<269-288>} specific T-cell clone was not detectable. Moreover, PCR was sensitive enough to detect the V β 22-CDR3 T-cell clone in the freshly isolated non-cultured autologous PBMC. As an additional negative control allogeneic PBMC from a CMV seronegative donor (Control) were used (Figure 6E, right).

Most notably, the CMV IE1_{<269-288>} specific T-cell clone was present in the both freshly isolated non-cultured CD4⁺CD25^{high} and CD4⁺CD25^{low} subsets of the autologous PBMC, implying that the *in vitro* confirmed clonal identity of Treg and non-Treg populations reflects the *in vivo* situation. Additionally, this experiment was also performed with a V β 12-CDR3 clonotype-specific PCR with equal results (data not shown).

These data underline that the generation of CMV-specific iTreg does not arise by an *in vitro* culture artefact (conversion from CD25^{low}) but occurs *in vivo*, likely as a result of chronic exposition to CMV.

2.8 HIGH Treg FREQUENCY IN PATIENTS WITH RECURRING CMV REACTIVATIONS

If iTreg cells are generated from effector or memory CD4⁺ T cells continuously throughout life, persistent infections with viruses, such as CMV that induce large-scale differentiation of human memory T cells (Fletcher, et al., 2005) (Appay, et al., 2002) (Khan, et al., 2002), would be expected to also induce a disproportionate representation of CMV-specific iTreg in the total Treg cell pool.

Such an imbalance might contribute to the CMV-related hyporesponsiveness as reported in some solid organ transplant (SOT) patients undergoing chronic immunosuppression (Reinke, et al., 1999).

Figure 7

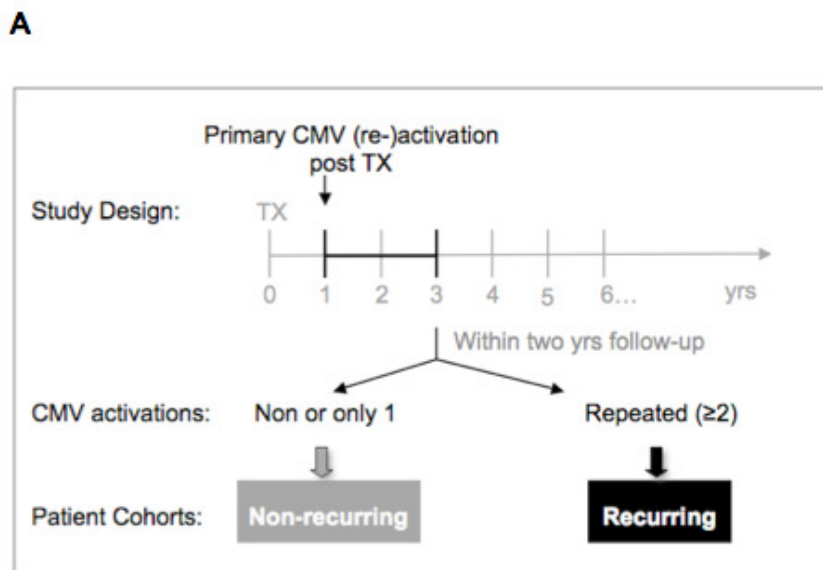


Figure 7A. Study Design.

CMV seropositive transplant patients with Non-recurring or Recurring (Patient Cohorts) CMV-activations are monitored for frequency and impact of Treg cells. CMV: Cytomegalovirus; Tx: Transplantation; yrs: years

To investigate this issue, we monitored kidney transplant patients with or without recurring CMV outbreaks (Recurring vs. Non-recurring) (Figure 7A) not only for frequencies of CD25⁺FOXP3⁺ Treg cells in peripheral blood but also for the putative negative impact of CD25^{high} Treg cells on CMV-antigen specific immune response. Characteristics of both patient cohorts are summarized in Table 3 (Methods).

In fact, patients with recurring CMV outbreaks displayed significantly higher CD25+FOXP3+ Treg frequencies compared to Non-recurring patients (CD25+FOXP3+ [of CD4+ %]: mean \pm S.D., Recurring [n=11] and Non-recurring [n=8] 3.7 ± 1.06 and 2.4 ± 0.94 , respectively, $p=0.016$ *t*-test, two-tailed) (Figure 7B).

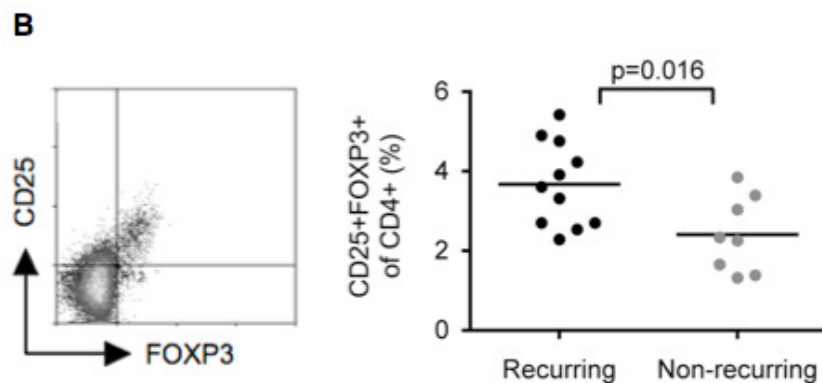


Figure 7B. Patients With Recurring CMV Outbreaks Display Higher Treg Frequency.

Representative dot plot for staining characteristics and gating strategies of CD25+FOXP3+ Treg cells from CD4+ T cells in the peripheral blood is shown (Figure 7B, left). Isotype control staining for anti-FOXP3 showed a background of 0.5% or less.

Frequency of CD25+FOXP3+ cells among CD4+ T cells is plotted (Figure 7B, right). Patients undergoing recurring CMV outbreaks (Recurring) show significant higher Treg frequency than patients with single CMV outbreak (Non-recurring). Data are mean \pm S.D.: of Recurring [n=11], 3.7 ± 1.06 versus Non-recurring [n=8], 2.4 ± 0.94 , $p=0.016$ (*t*-test, two-tailed).

Additionally, CD4+CD25high T cells displayed significantly higher FOXP3 MFI in Recurring-CMV patients ($p=0.010$ *t*-test, two-tailed) whereas the MFI of CD4+CD25intermediate T cells was comparable in the Recurring and Non-recurring cohort (not significant) (Figure 7C).

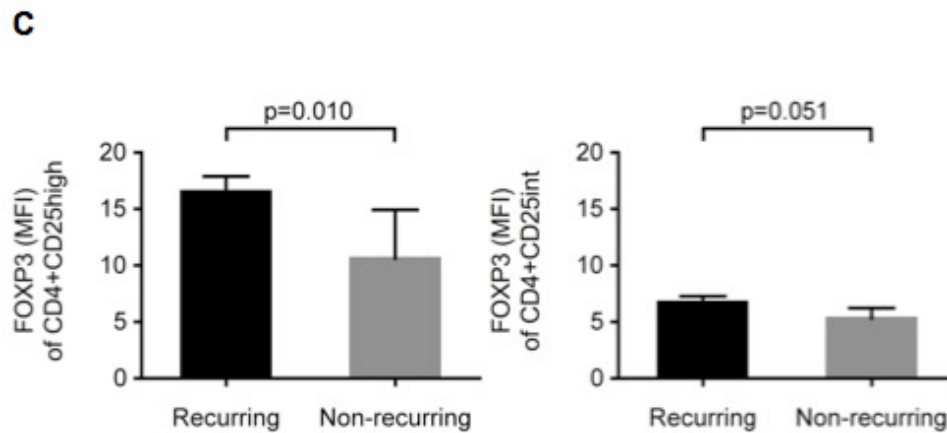


Figure 7C. CD4+CD25^{high} but Not CD4+CD25^{int} T Cells Show Significant Increased FOXP3 Intensity Within Recurring Patient Cohort.

Mean Fluorescence Intensity (MFI) of FOXP3⁺ among CD4+CD25^{high} T cells (Treg) and CD4+CD25^{intermediate} (activated Teff) is plotted. Patients undergoing recurring CMV outbreaks (Recurring) show significant higher FOXP3 intensity of CD4+CD25^{high} Treg cells than patients with single CMV outbreak (Non-recurring). Data are mean \pm S.D.: of Recurring [n=11], versus Non-recurring [n=8], (t-test, two-tailed).

2.9 HIGH SUPPRESSIVE IMPACT OF CD25^{HIGH} Treg IN CHRONICALLY CMV-INFECTED PATIENTS

To examine a possible involvement of peripheral iTreg cells on CMV-specific T cell response in these patients, we depleted CD25^{high} T cells from patient's PBMC *in vitro* before stimulation and functional analysis of CMV-specific non-Treg. We focused on CD25^{high} T cells with regard to our previous observation that these cells comprise the majority of FOXP3 high cells and, moreover, because CMV-specific iTreg clones are present in freshly isolated CD25^{high} T-cell populations. For selective CD25^{high} Treg but not CD25^{intermediate} depletion anti-CD25 magnetic beads were down titrated (Figure 8A).

Subsequently, PBMC and CD25^{high}-depleted cells were incubated with CMV pp65 and IE1 protein-spanning overlapping peptide pools which encode all possible epitope sequences and thereby provide HLA-type independent CMV-specific stimulation.

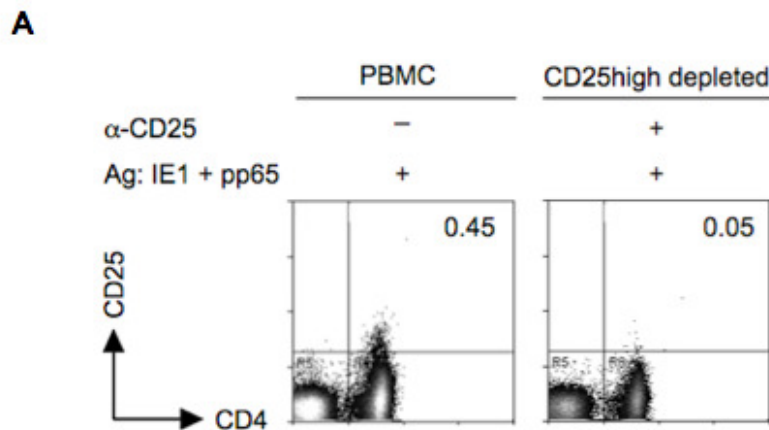


Figure 8A. In-Vitro Depletion of CD25^{high} Cells from PBMC of Recurring and Non-Recurring CMV Patients.

PBMC were isolated from peripheral blood samples donated from patients with Recurring or Non-recurring CMV reactivations, respectively. Selective depletion of CD25^{high} cells by magnetic separation (α -CD25 beads 3 μ g/1x 10⁷ cells) was performed. Numbers in dot plots indicate percentages of the gated CD4⁺CD25^{high} cells. Resulting cell-subsets (PBMC and CD25^{high} depleted, respectively) were subsequently stimulated with peptide pools of the CMV antigens (Ag) IE1 and pp65 for 24 h.

Analysis showed that peptide-specific IL-2 and IFN γ cytokine release was considerably increased in Recurring-CMV patients after iTreg depletion (mean \pm S.D.: n=6, IL-2 [fold-change]: 6.6 \pm 10.5; IFN γ [fold change]: 65.0 \pm 129.2) (Figure 8B). Furthermore, Treg related cytokine suppression was correlated with the clinical status of infection history. Treg depletion led to six fold higher IL-2 and eight fold higher IFN γ increase in Recurring-CMV patients compared to Non-recurring patients (Data are mean \pm S.D. of six and five experiments per cohort) (Figure 8B).

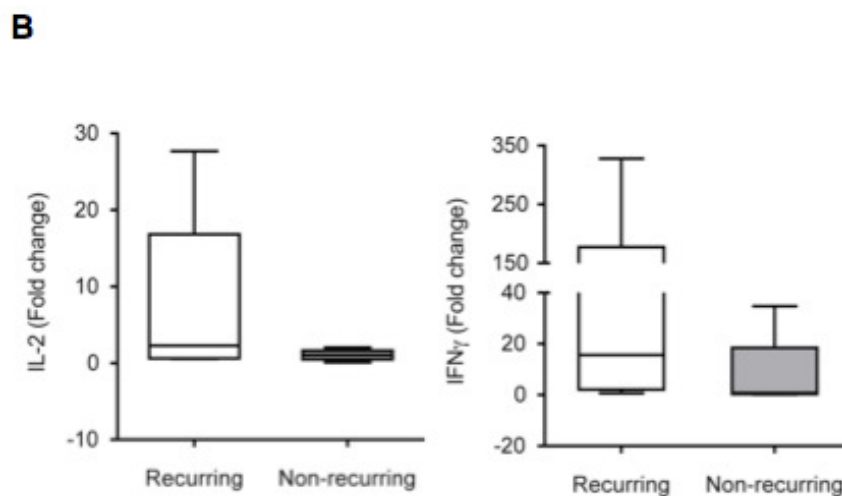


Figure 8B. Recurring CMV Patients Show Higher Regulatory Impact by CD25^{high} Treg Cells On Antigen-Specific T-cell Response.

Recurring-CMV patients displayed higher and more often increased CMV-antigen specific IL-2 and IFN γ response after CD25^{high} Treg depletion compared CMV Non-recurring patients. Cytokine release after 24h of stimulation with CMV IE1 and pp65 peptide pools was analyzed by flow-cytometric bead array. Fold change of IL-2 and IFN γ after CD25^{high} depletion are plotted. Data are mean \pm S.D. of six and five experiments per cohort (Recurring, Non-recurring), respectively.

These data demonstrate a higher frequency and a higher negative regulatory impact of CD4⁺CD25^{high} Treg in patients who suffered from history of recurring CMV outbreaks and strengthen the hypothesis of a CMV-driven process of iTreg generation *in vivo*.

3 DISCUSSION

Our results show that human CMV-specific T-cell lines contain CD4⁺CD25^{high} Treg cells that express IL-2^{low} but IL-10^{high} and exhibit incomplete *foxp3* DNA demethylation. The CMV-specific Treg cells possess a phenotype of induced regulatory T cells with similarity to the recently described Tr-1 cells. Importantly, these CD25^{high} Treg cells suppress CD25^{low} Teff cells in an antigen specific fashion; and both subsets share the same dominantly expanded TCR clones. Moreover, the identical TCR clone could also be identified in freshly isolated CD4⁺CD25^{high} and CD4⁺CD25^{low} T-cell subsets suggesting the generation of CMV-specific Treg occurred already *in vivo*. Finally, the impact of iTreg on the CMV-specific T-cell response increases with recurring outbreaks of CMV compared to patients with non-recurring CMV activation.

3.1 FOREIGN-ANTIGEN SPECIFIC Treg IN HUMAN CMV MODEL

The delicate balance between effector and regulatory T cells *in vivo* is critical in order to generate controlled host immune-response to pathogens, and an imbalance has been identified in many pathological situations (Belkaid and Rouse, 2005) (Vukmanovic-Stejic, et al., 2006) (Suvas, et al., 2003) (Suffia, et al., 2006) (Boettler, et al., 2005) (Rushbrook, et al., 2005), particularly in chronic infections. As human Treg are difficult to clone only a few cognate epitopes of peripheral blood nTreg and iTreg cells have been described up to date (Walker, et al., 2005) (Ebinuma, et al., 2008). In addition, although peripheral Treg induced by foreign antigens, such as viruses resemble phenotypical and functional aspects of thymic-derived nTreg, studies for their suppressive stability, including epigenetic modification of the *foxp3* locus and clonal TCR diversity remained to be elucidated (Sakaguchi and Powrie, 2007).

To address these questions we used the model of human CMV infection as the life-long persistence of the virus should support the clonal expansion not only of effector T cells but also of iTreg that might generate a clonal size allowing the analysis on clonal level. After specific stimulation of CD4⁺ T cells recognizing epitopes within the major CMV target antigens, pp65 and IE-1, our data have shown that in parallel to the memory effector T cells (expressing CD25^{low} - intermediate and CD127^{high}, and the phenotype IL-2^{high} IFN γ ^{high} IL-10^{low}) iTreg (expressing CD25^{high} CD127^{low}, and the phenotype IL-2^{low} IFN γ ^{high} IL-10^{high}) could be expanded. CMV-specific CD4⁺CD25^{high} T cells clonally expanded in parallel to CD4⁺ effector T cells *in vitro*, gain suppressive activity on their CD4⁺CD25^{low} non-Treg counterparts as consequence of specific recognition by their respective target antigen. In contrast to nTreg, iTreg express an incomplete FOXP3-demethylation pattern that is reflected by an intermediate FOXP3 protein and mRNA expression.

3.2 HUMAN VIRAL-SPECIFIC TEFF AND Treg ARE CLONALLY IDENTICAL

Remarkably, we showed that *in vitro* expanded CD4⁺CD25^{high} iTreg and CD4⁺CD25^{low} non-Treg responding to the same foreign CMV epitope, share the dominantly expanded TCR defined by its unique V β -CDR3 sequence. Furthermore, we noted that this immuno-dominant TCR clone is capable to possess the phenotype and function of iTreg and non-Treg cells after specific expansion and TCR activation *in vitro*. Moreover, *ex vivo* CD4⁺CD25^{high} Treg and CD4⁺CD25⁻ conventional non-Treg freshly isolated from autologous peripheral blood comprised T-cell clones expressing the respective TCR with high affinity to the same CMV-epitope.

Thus, T-cell clones that recognize a specific foreign epitope dominantly expanded in Treg as non-Treg effector populations after specific triggering *in vitro* are already present in both peripheral CD4+CD25high and CD4+CD25low T-cell pools *in vivo* and do not result of an *in vitro* artefact.

In summary, for our knowledge we could directly demonstrate for the first time that human virus-specific Treg and Teff induced by foreign antigens *in vivo* and *in vitro* are clonally identical. Additionally, our data suggest that iTreg gain higher influence on the response of conventional non-Treg following chronically repetitive antigen exposition *in vivo*, as shown by the dramatically enhanced cytokine secretion of conventional CMV-specific non-Treg from immuno-suppressed transplant patients with chronic active CMV infection after *in-vitro* depletion of CD25high iTreg.

3.3 IMPLICATION FOR ADOPTIVE ANTI-VIRAL THERAPY

The ability to analyze the clonal repertoire of immunosuppressive Treg clones with specificity for defined antigens, as shown in this study, represents an improvement over published experiments, relying on TCR-analysis of polyclonal peripheral Treg cells with unknown antigen-specificity (Fujishima, et al., 2005) (Kasow, et al., 2004) (Scheinberg, et al., 2007). Future comparative characterization of the specifically activated effector and regulatory T-cell clones promise to enable a 'fine-tuning' of antigen-specific T-cell transfer in various settings, such as adoptive therapy in patients suffering from Adenovirus, Hepatitis C virus, Epstein-Barr Virus or Cytomegalovirus related diseases, and thus is of considerable clinical importance.

3.4 POSSIBLE CO-EVOLUTION OF TEFF AND Treg *IN VITRO* AND *IN VIVO*?

Our observations suggest that peripheral iTreg cells can be generated not only *in vitro* but also *in vivo*, to exert suppressive function when exposed to relevant antigens and thus are suited for controlling inflammatory response to chronic infections, such as caused by CMV.

These data do not answer the question whether the antigen-specific CD4⁺CD25^{high} iTreg result by conversion from CD4⁺CD25^{low} effector or memory T cells (Figure 9A, Sequential Model) as suggested in *in vivo* studies in TCR transgenic mice (Knoechel, et al., 2005) and *in vitro* studies in humans (Walker, et al., 2005). Alternatively, it is possible that the shared specificity and clonal identity of peripheral Treg with Teff cells is due to parallel development from naïve peripheral blood T cells exposed to the foreign antigen after infection (Figure 9A, Parallel Model) or rely on inherent cross-reactivity of naturally occurring Treg cells.

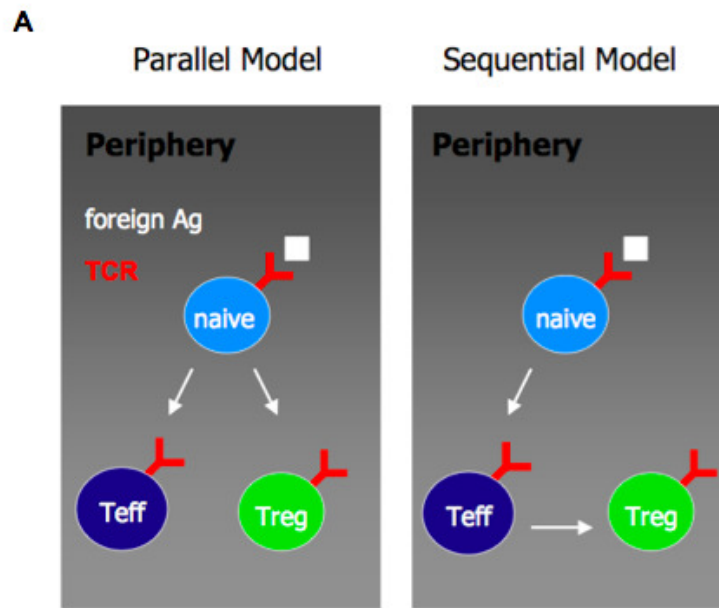


Figure 9A. Parallel Model and Sequential Model.

Putative peripheral Treg-development models from the same naive “mother-clone” are illustrated as comic. Ag: Antigen; TCR: T-cell Receptor; Teff: effector T-cell clone; Treg: regulatory T-cell clone.

Indeed, in humans it is not yet established whether foreign-antigen iTreg display similar TCR repertoires to those of nTreg. The total repertoire of nTreg cells is apparently more skewed to recognizing self-antigens (Hsieh, et al., 2004) (Figure 9B, Separate Model), although it has been described that they are able to perceive a broad repertoire of self- and nonself-antigens including pathogens (Belkaid and Rouse, 2005). Interestingly, a recently published report in mice has shown that thymically derived Foxp3⁺ Treg recognize foreign antigenic peptides as often as non-regulatory T cells (Pacholczyk, et al., 2007).

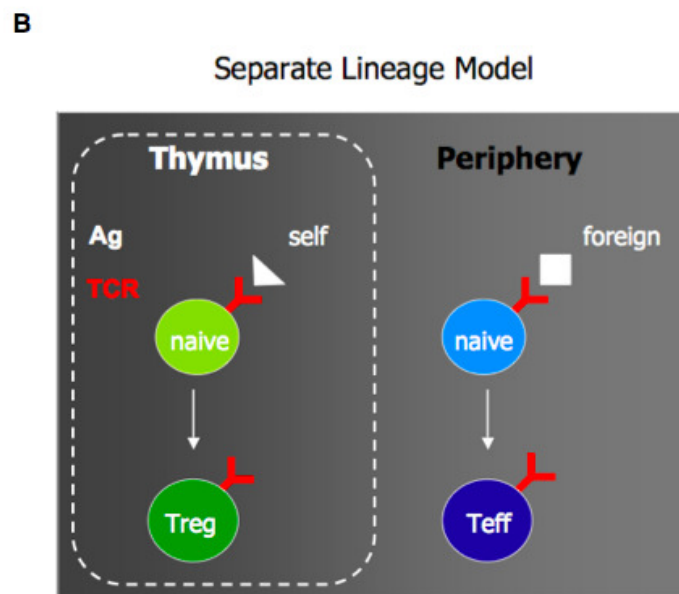


Figure 9B. Separate Lineage Model.

Treg-development model in the thymus, resulting in self-antigen specific Treg lineage with clonal TCR-repertoire separate from foreign-antigen T-effector lineage induced in the periphery. Foreign-antigen recognition by Treg cells would rely on inherent TCR cross-reactivity. Ag: Antigen; TCR: T-cell Receptor; Teff: effector T-cell clone; Treg: regulatory T-cell clone.

3.5 FIRST DIRECT VERIFICATION OF ANTIGEN-SPECIFIC SINGLE Treg CLONE IN HUMAN INFECTION

So far, only a few antigen-specific Treg populations have been directly pictured in humans. These elegant approaches correlated suppressive activation in expanded CD25+ T cells (Walker, et al., 2005) or FOXP3 expression (Ebinuma, et al., 2008) with MHC-class II tetramer staining. Although, tetramer-positive CD4+ T cells discern the same antigen, they still constitute an oligoclonal population. Hence, by using TCR-V β CDR3 clonotype-specific

PCR our data provide the first direct verification of single antigen-specific Treg clones in human infection to our knowledge.

The CDR3 of the TCR-V β -chain is, due to the NDN modification the most diverse region of the $\alpha\beta$ -TCR (Davis and Bjorkman, 1988); furthermore it is highly unique for each T-cell clone and most directly engages the antigenic peptide presented within the HLA groove of an antigen presenting cell (Borg, et al., 2005). Importantly, the TCR α -chain is not suitable for detection of $\alpha\beta$ -T cell clonality as up to 30% of rearranged human peripheral blood T cells (Padovan, et al., 1993) and most human thymic and peripheral-blood CD4⁺CD25^{high}+ Treg (Tuovinen, et al., 2006) can express a dual TCR with two distinct α -chains but only one β -chain. Again, clonal TCR-V β CDR3 identity of CD25^{high} Treg and CD25^{low} non-Treg expressing the same TCR specific for a foreign-antigen strongly supports the idea of antigen-related co-evolution between Tregs and effector or memory T cells in humans.

3.6 CORELLATION OF Treg FREQUENCY AND SPECIFIC SUPPRESSION WITH CMV-INFECTION STATUS *IN VIVO*

If that assumption is correct, then persistent infections such as CMV that are mainly controlled by T-cell immune response, would be expected to also induce a high proportion of CMV-specific regulatory T cells in the total Treg-cell pool.

To address this hypothesis in biologically relevant conditions we monitored the involvement of Treg cells in transplanted patients undergoing recurrent CMV-reactivations, in comparison to patients with no or only one episode of CMV antigenemia. The latter situation is associated with increasing frequencies of conventional CMV-specific Th1 cells over time (Bunde, et al., 2005). In fact, patients with recurrent active CMV infections compared to control groups showed higher frequency of CD25⁺FOXP3⁺ Treg within the peripheral CD4⁺ T cells and a dramatic increase of Teff function after depletion of the former. These findings support our hypothesis of antigen-exposure driven iTreg expansion.

We envisage an imbalance of iTreg and effector T cells containing clonally identical T cells that can be activated by the same CMV epitope in acute phase of viral infection, as demonstrated in this study, may interfere with specific CMV response and clearance and thereby permit repeated viral outbreaks.

3.7 FOXP3 STATUS IN SUPPRESSIVE ACTIVE FOREIGN-ANTIGEN INDUCED HUMAN Treg

Here we asked if the phenotype of the studied iTreg is stable? As we could easily reproduce the generation of iTreg recognizing the same epitope specificity from the same donor/patient even >1 year after the initial analysis, the phenomenon seems to be quite robust. Along with, the cells could be sorted at different time points of the expansion culture without significant functional differences. The durable absence of CD127 expression (in contrast to the CD25^{low} and intermediate subsets) further supports the Treg stability.

Published reports have shown that stable Foxp3 expression, going along with demethylation of a CpG-rich region on the *foxp3* locus is critical for the lineage stability and suppressive function in nTreg (Floess, et al., 2007). To examine if foreign-antigen specific iTreg also require epigenetic modification of *foxp3*, we analyzed in CD4⁺CD25^{high} and CD4⁺CD25^{low} T cells expanded *in vitro* after induction by their cognate epitope. Interestingly, both populations the CD25^{high} Treg cells and the CD25^{low} effector T cells of five separate donors exhibit almost no demethylation in this CpG-rich *foxp3* region, although the suppressive CD25^{high} cells comprised significantly more FOXP3 high T cells compared to CD25^{low} cells. Consequently, the partial FOXP3^{high} expression concurrently with methylated *foxp3* status depicted in suppressive antigen-specific CD25^{high} population *in vitro* suggests a transient FOXP3 upregulation.

This argues along with possibility of Treg conversion (Figure 9A, Sequential Model) from antigen-induced effector T cells *in vitro* or *in vivo*. Additionally, human Tr1-like Treg cells display a similar FOXP3 pattern (Roncarolo and Gregori, 2008).

The mechanism of action by iTreg is less clear. We could demonstrate the need for prior antigen-specific stimulation of iTreg to exhibit their suppressive activity. Once activated, however, the iTreg are able to suppress not only effector T cells of the same but also of distinct specificity. Regarding their phenotype (CD4⁺CD25^{high} IL-2^{low} IFN γ ^{high} IL-10^{high}) and their FOXP3 regulation (FOXP3 intermediate, incomplete FOXP3-demethylation) the studied iTreg population express similarities to the recently described Tr1 cell subset (Levings, et al., 2005) (Roncarolo and Gregori, 2008). As recent reports suggest that FOXP3 does not correlate with suppressor function in human Tr1; furthermore FOXP3-independent mechanisms, mediated by IL-10, contribute to the induction and suppressor function of these iTreg (Roncarolo and Gregori, 2008), we wondered whether IL-10 might mediate the observed suppressive effects. Preliminary data, however, suggest that IL-10 neutralization does not prevent the suppressive function of CMV-specific iTreg.

3.8 CONCLUSIONS AND OUTLOOK

Taken together, our data support the impact of iTreg on the quality of virus-specific T-cell response and the effective control of viral infection. Moreover, we suggest that exploring the proliferative capacity and suppressive activity as well as differential expressed molecules on these CMV antigen-specific Treg clones, could impart strategies to expand antigen-specific effector T cells while inhibiting the activation and expansion of regulatory T cells. This might strengthen or re-establish the immune response in immuno-compromised patients, such as organ-transplanted patients often suffering from chronic viral disease, by highly specific adoptive T-cell transfer.

4 MATERIALS AND METHODS

4.1 SUBJECTS

After informed consent, blood samples were collected from randomly selected kidney transplant patients and healthy subjects on the basis of CMV-IgG seropositivity, clinical course of CMV infection and HLA-class II type. The institutional review board of the Charité University Medicine, Berlin, approved the study. Transplant patients (>1 yr post-Tx) without actual signs of active CMV infection, and mostly on triple drug maintenance immunosuppression were recruited from the transplant outpatients unit.

They were separated into two subsets: those patients with recurring CMV activations (Recurring) showed at least 2 positive CMV-antigenemia tests during >2 years follow-up before the study, despite adequate antiviral therapy; and those patients who had no or only one CMV-antigenemia (Non-recurring) during 2-6 years prior follow-up. Mean age of "Recurring" and "Non-recurring" was 51.1 and 54.4 years, respectively. Patients showed a current stable graft function and no other clinical complications. Patient characteristics are shown in the following Table 3.

Tab. 3: Characteristics of Monitored CMV Seropositive Patients.

Patient	Gender	Age	Maintenance Immunosuppression Post TX	Highest CMV-Ag (x/10,000)	History of CMV-antigenemia (Times*)
R1	Male	43	Triple therapy	8.95	2
R2	Male	57	CNI, steroid	2.18	2
R3	Female	56	Triple therapy	10.80	3
R4	Male	69	CNI, steroid	9.60	3
R5	Male	48	CNI, steroid	5.45	4
R6	Male	52	Triple therapy	6.29	4
R7	Female	51	CNI, steroid	3.33	3
R8	Male	40	CNI, steroid	32.00	2
R9	Male	42	CNI, steroid	62.30	5
R10	Male	64	CNI, steroid	3.5	5
R11	Female	40	CNI, steroid	7.64	4
N1	Male	73	Triple therapy	1.87	1
N2	Female	47	Triple therapy	1.29	1
N3	Male	33	Triple therapy	7.00	1
N4	Female	43	Triple therapy	0.91	0
N5	Male	40	Triple therapy	0.00	0
N6	Male	57	CNI, steroid	0.54	0
N7	Female	69	Triple therapy	0.00	0
N8	Male	73	Triple therapy	0.00	0

CMV: Cytomegalovirus; R: Recurring CMV activations (≥ 2 within > 2 years follow up); N: Non-recurring CMV activation (≤ 1); TX: kidney transplantation; CNI: calcineurin inhibitors; Triple therapy: application of CNI, mycophenolatmofetil and steroid; CMV-Ag positive: immunocytological CMV-antigenemia test with $\geq 1/10,000$ positive cells; Times*: per year one positive test was enumerated.

4.2 EXPANSION OF CMV-ANTIGEN SPECIFIC CD4+ T CELLS

4.2.1 CD4+ T-CELL EPITOPE MAPPING

Epitope mapping was conducted in accordance to the protocol published by our group previously (Kern, et al., 1998) and several MHC-class II restricted CMV epitopes were identified. Sequences of CD4-epitopes and considered presenting HLA-DR molecules are listed in the Table 1 (Results).

In brief, CD4+ T-cell epitope mapping was based on the previously described *ex vivo* IFN γ induction assay (Hammer, et al., 2005) (Kern, et al., 1998) using crossover pools of pp65 and IE1 peptides. Single peptides consist of 15-amino acid (AA) each. Peptide pools comprise 15-AA peptides (11 overlaps), spanning the total CMV pp65 or IE1 protein, respectively. Quality control included mass spectroscopy and HPLC (purity >70 %). Purified peptides were purchased from JPT Peptide Technologies, Berlin, Germany.

Firstly, PBMC (2×10^6) of CMV seropositive donors were stimulated for 6 h with crossover peptide pools (final concentration of each peptide 1 $\mu\text{g/mL}$). Un-stimulated control sample was run in parallel (DMSO). Then, Brefeldin A (4 $\mu\text{g/mL}$) was added after 2h of culture and at 6 h, cells were washed twice with ice-cold PBS. Next, cells were stained for surface markers (CD3+, CD4+ and CD8+) and finally intracellular FACS-stained for IFN γ production. Per sample, 150,000-250,000 events in the FSC/SSC lymphocyte gate were acquired on a FACS Calibur cytometer (Becton Dickinson). For data analysis CD3+ events were displayed in a CD8+ or CD4+ versus IFN γ + dot plot. CD8+IFN γ + or CD4+IFN γ + cells were expressed as percent of the respective reference population. Un-specifically positive events in the corresponding regions in control samples were subtracted. All identified epitopes were confirmed using individual peptides for stimulation.

In this study, peptides coding for identified CMV CD4-epitopes pp65_{<25-39>}, pp65_{<45-59>}, pp65_{<245-263>}, IE1_{<89-107>} and IE1_{<269-288>} were used for specific stimulation, subsequent isolation and CD4+ T-cell expansion.

4.2.2 ISOLATION AND EXPANSION OF SPECIFIC CD4⁺ CELLS

Human PBMC were stimulated for 6 hours with CMV peptides (1 µg/mL), which were identified as CD4-epitopes on pp65 and IE1 by epitope mapping as described above.

IFN γ secreting cells, isolated by magnetic cell sorting (IFN γ secretion assay; Miltenyi Biotech) were expanded for 14 days with irradiated peptide-loaded autologous PBMC and rIL-2 (100 U/ml) (Chiron Behring). During expansion T cells were cultured in complete medium (RPMI 1640-stable glutamine, 10% FCS, 100 U/ml penicillin and 100 µg/mL streptomycin).

4.3 FLOW-CYTOMETRY ANALYSIS

Monoclonal antibodies to CD3 (UCHT1), CD4 (RPA-T4), CD25 (M-A251), CD127 (eBRDR5), IL-10 (JES3-19F1) and IFN γ (B27) and were purchased by Becton Dickinson and monoclonal antibodies to TCR-V β 22 (IMU-546) and TCR-V β 12 (VER2.32.1) by Beckman Coulter. FACS staining was performed according to the manufacturer's protocols. CytomationSummit (MoFlow) and FlowJo (Tree Star) software were used for analysis.

4.3.1 INTRACELLULAR FACS STAINING

For intracellular cytokine staining, cells were stimulated with CMV or control peptide for 6 hours (IFN γ analysis) or 16 hours (IL-10 analysis), respectively. Brefeldin A (4 µg/ml; Sigma-Aldrich) was added after 2 hours. Subsequent surface staining, the cells were then

fixed and permeabilized (Becton Dickinson) before staining for the cytokines IFN γ or IL-10. Short-term re-stimulation and subsequent intracellular cytokine staining were performed as described previously (Hammer, et al., 2005).

For analysis of FOXP3 expression at single cell level, cells were first stained for the respective surface molecules and, after fixation and permeabilization (eBioscience), were incubated with FITC-conjugated monoclonal antibody PCH101 (anti-human FOXP3; eBioscience) or the IgG2a isotype control (eBR2a) based on the manufacturer's recommendations.

4.3.2 FACS-SORTING

T-cell lines and peripheral blood mononuclear cells (PBMC) were stained with appropriate antibodies. CD4⁺CD25^{low} (CD25^{low}), CD4⁺CD25^{high} (CD25^{high}) cells, as well as V β 12⁺, V β 12⁻, V β 22⁺, V β 22⁻, V β 12CD25^{low}, V β 12CD25^{high}, V β 22CD25^{low} and V β 22CD25^{high} CD4⁺ cells were sorted to high purity (92-99%) with DIVA cell sorter (Becton Dickinson). The sorting strategy included each time gating for CD3⁺ cells and excluded dead cells by gating of forward and side scatter.

4.4 FUNCTIONAL IN-VITRO ASSAYS

4.4.1 ANTIGEN PRESENTATION AND CO-CULTURE

For re-stimulation cells were incubated for 24 h with 5×10^4 or 2×10^4 irradiated autologous lymphoblastoid B cells (Amrolia, et al., 2003), serving as antigen-presenting cells (APC), which were loaded with the respective specific CMV peptides (1 $\mu\text{g}/\text{mL}$) or control peptides (unspecific CMV-epitope or HCV-NS3_{<1027-1041>}).

FACS sorted CD25^{low} and CD25^{high} (5×10^4) T cells from independent expanded T-cell lines were specifically re-stimulated and co-cultivated for 24 hours or 5 days at different ratios (1:0, 1:1, 1:0.5, 1:0.1 and 0:1), for analysis of cytokine release or quantification of proliferating cells, respectively. V β 12CD25^{low} and V β 12CD25^{high} (2×10^4) T cells were purified from pp65_{<245-263>} antigen-specifically expanded T cells by additional staining with the clonotypic TCR-V β 12 antibody. IE1_{<269-288>} specific T-cell lines were additionally stained with TCR-V β 22 antibody to sort for dominant expanded T-cell clones. Cell culture was performed in complete RPMI medium at 37°C.

4.4.2 CYTOKINE RELEASE AND PROLIFERATION

Supernatants were collected for quantification of IL-2, IL-10 and IFN γ release by cytometric bead array (CBA Kit; Becton Dickinson) based on the manufacturer's protocol. For assessment of proliferation, co-cultivation was extended to 5 days with added rIL-2 (100 U/ml, Proleukin). Cells dividing within the last 24 h were detected by [^3H] thymidine incorporation.

4.5 FOXP3-DNA METHYLATION ANALYSIS

4.5.1 DNA PREPARATION AND BISULFITE CONVERSION

DNA methylation analysis was performed by bisulfite sequencing as described previously (Baron, et al., 2007). Genomic DNA was isolated using the DNeasy tissue kit (Qiagen) following the manufacturer's protocol for cultured human cells. Bisulfite treatment of genomic DNA was performed according to (Olek, et al., 1996), with minor modifications, resulting in the deamination of unmethylated cytosines to uracil, whereas methylated cytosines remain unchanged. In a subsequent PCR amplification, uracils were replicated as thymidines. Thus detection of a "C" in sequencing reactions reflects methylation of the genomic DNA at that site. Detection of a "T" at the same site reflects instead the absence of a methyl modification of the genomic cytosine CpG Motifs and transcription factor binding sites within differentially methylated, conserved element of the *foxp3* locus.

4.5.2 FOXP3 PCR AND SEQUENCING

PCR was performed in a final volume of 25 μ L containing 1x PCR Buffer, 1 U Taq DNA polymerase (Qiagen), 200 μ M dNTP, 12.5 pmol each of forward and reverse primers, and 7 ng bisulfite-treated genomic DNA at 95°C for 15 min, and 40 cycles of 95°C for 1 min, 55°C for 45 s and 72°C for 1 min, and a final extension step of 10 min at 72°C. PCR products were purified using ExoSAP-IT (USB Corp.) and sequenced applying the PCR primers and the ABI Big Dye Terminator v1.1-chemistry (Applied Biosystems) followed by capillary electrophoresis on an ABI 3100 genetic analyzer. Sequences of primers used for bisulfite-specific PCR and sequence reactions were previously described (Baron, et al., 2007).

4.6 TCR V β -CDR3 TYPING

TCR V β -CDR3 typing was performed by sequencing as described previously (Wlodarski, et al., 2006). In brief, CDR3 sequences of TCR V β -chain were amplified on cDNA samples using a multiplex PCR (BIOMED2) that covers all V β TCR gene rearrangements (van Dongen, et al., 2003). PCR product was ligated into TA cloning vector pCR2.1 (Invitrogen), heat-shock transformed into TOP10F *E.coli* and plated on X-gal-covered agarose. Colony PCR and subsequent sequencing of positive colonies were performed as described previously (Wlodarski, et al., 2006). The frequency of an expanded clonotype was calculated as percentage of the particular unique clonotype out of the total number of sequenced V β -CDR3 regions. Sequences were analyzed using the ImMunoGeneTics information system TCR alignment tool (<http://imgt.cines.fr>).

4.7 QUANTITATIVE REAL-TIME PCR

4.7.1 RNA ISOLATION AND cDNA AMPLIFICATION

Total RNA from sorted cells was purified (Miniprep Kit; Stratagene) and a reverse transcription Kit (Ambion) was used for transcription into cDNA. The mRNA expression of human FOXP3, HPRT and CD3zeta were analyzed by quantitative RT-PCR. Sequences of primers (Metabion) and probes (Eurogentec) employed for quantitative RT-PCR are summarized under Table 4. Quantitative RT-PCR was performed as described previously (Kotsch, et al., 2004) in an Applied Biosystems 7500 using the following thermal protocol: 2 min at 50 °C, 10 min at 95 °C, 15 s at 95 °C, 60 s at 60 °C.

4.7.2 DESIGN OF TCR CLONOTYPE-SPECIFIC PCR

Sequence derived from dominantly expanded clones in antigen-specific CD25^{high} and CD25^{low} T-cell subsets were used to design a TCR clonotype-specific PCR, employing CDR3-specific Taq-probe, V β -family specific forward primer and J β -specific reverse primer. To increase the fidelity, each primer covered the flanking nucleic acids of each CDR3 sequence. In parallel, CD3zeta gene expression was analyzed. Quantitative RT PCR was performed as described (Kotsch, et al., 2004). Sequences of primers and probes (Eurogentec) are shown in the Table 4.

4.7.3 PRIMERS AND PROBES

Probes	Sequence
HPRT	TTTCACCAGCTTGCGACCTTGA
CD3zeta	TGAGAGTGAAGTTCAGCAGGAGCGCA
FOXP3	ACTTCCTCAAGCACTGCCAGGCGG
IE1 _{<269-288>} Vβ22-CDR3	AGGACTCCATTCTGTCCAGC
pp65 _{<245-263>} Vβ12-CDR3	ACTGTCATCCCCTCTCACTGATGGCAC
Primers	Sequence
HPRT	FW: AGTCTGGCTTATATCCAATTCG RW: GACTTTGCTTTCCTTGGTCAGG
CD3zeta	FW: TGCTGGATGGAATCCTCTTCAT RW: GGTTCTGGCCCTGCTGGTA
FOXP3	FW: AAGTGGCCCGGATGTGAGA RW: CATTGTGCCCTGCCCTTCT
IE1 _{<269-288>} Vβ22-CDR3	FW: GAAAGGCCTGATGGATCAAA RW: TCGAGTCCCATCCAAAAT
pp65 _{<245-263>} Vβ12-CDR3	FW: GACAGAGAGCTGGGTTCCACT RW: TCACTCTGGAGTCCGCTACCA

Table 4. Primers and Probes.

FW: forward primer; RW: reverse primer. Sequences are shown in the 5' - 3' direction. HPRT: Hypoxanthine-guanine phosphoribosyltransferase; FOXP3: human Fork head box 3; IE1: Immediate early protein 1; pp65: Phospho-protein 1; Vβ: T-cell receptor variable β chain, CDR3: complementary determining region 3.

4.8 IN-VITRO CD25^{HIGH}-Treg DEPLETION

To deplete selectively CD25^{high} but not CD25^{intermediate} cells from PBMC, anti-CD25 beads (Miltenyi Biotech) were down titrated to 3 μ L per 1×10^7 cells for magnetic separation. PBMC and CD25^{high}-depleted cells were subsequently cultured in complete RPMI medium and stimulated for 24 h HLA-type independently with protein-spanning peptide pools (15-amino acid peptides with 11mer overlaps) of CMV pp65 and IE1 (JPT Peptide Technologies).

4.9 STATISTICAL ANALYSIS

Mean values, S.D. values and Student's *t*-tests (paired and unpaired) were calculated with GraphPad Prism (GraphPad Software).

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SELBSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde.

Der Inhalt der Promotionsordnung der Mathematischen Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin vom 19.06.2002 ist mir bekannt.

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