Cellular Physiology and Biochemistry

Cell Physiol Biochem 2014;34:346-355
DOI: 10.1159/000363004
Published online: July 09, 2014
Accepted: May 15, 2014

Karger Open access

1421-9778/14/0342-0346$39.50/0

Original Paper

CD49d$^-$ Treg Cells with High Suppressive Capacity are Remarkably Less Efficient on Activated CD45RA$^-$ than on Naive CD45RA$^+$ Teff Cells

Britta Kraczyk Ralph Remus Cornelia Hardt

Institute of Immunology, University Hospital, Essen, Germany

Key Words
Treg cell capacity • Teff cell resistance • CD49d • CD45RA • Suppression

Abstract
Background: Impairment of regulatory T cells (Tregs) in common autoimmune diseases seems likely. However, the extent of Treg deficiency (number, function) or differential susceptibility of T effector cells (Teffs) to suppression is not completely understood. We hypothesize that even in healthy individuals both cell populations are heterogeneous and differ in their suppressive capability and their susceptibility to suppression. ***Material and Methods:*** Lymphocytes were enriched by MACS for CD4$^+$CD25$^+$ Tregs or CD4$^+$CD25$^-$ Teffs. After multicolour staining (anti-CD25, anti-CD127, anti-CD49d or anti-CD45RA) highly purified Treg and Teff subpopulations were collected by FACS. Functional capacity of Tregs or suppressive susceptibility of Teffs was analyzed in an in vitro assay. ***Results:*** When CD4$^+$CD25^{high}CD127$^{-/low}$ CD49d$^-$ Tregs were tested on naive CD4$^+$CD127$^+$CD25$^-$CD45RA$^+$ Teffs (93.8 %) suppression was almost complete, while the suppressive capacity of CD4$^+$CD25^{high}CD127$^{-/low}$ CD49d$^+$ Tregs was significantly less (71.8 %). Suppressive activity was low when CD4$^+$CD25^{high}CD127$^{-/low}$ CD49d$^+$ Tregs were analyzed on CD4$^+$CD127$^+$CD25$^-$CD45RA$^-$ Teffs (48.7%). ***Conclusion:*** Although CD49d$^+$ Tregs are functional, the suppressive capacity is significantly lower compared to CD49d$^-$ Tregs. CD45RA$^+$ Teffs can be completely suppressed, while CD45RA$^-$ Teffs display relative resistance. Phenotypic and functional heterogeneity of Tregs as well as Teffs has to be considered when analyzing deficiencies in immune regulation.

Cornelia Hardt

Institute of Immunology, University Hospital Essen,
Hufelandstrasse 55, 45147 Essen (Germany)
Tel. +49 201 7234202, Fax +49 201 7236837, E-Mail cornelia.hardt@uk-essen.de

Introduction

Several observations highlight the importance of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) in peripheral tolerance and immune homeostasis. First, Sakaguchi et al. [1] described the role of $CD4^+CD25^+$ Tregs in immunologic self-tolerance in the mouse. Second, the transcription factor FoxP3 is required for the development of $CD4^+CD25^+$ Tregs [2, 3]. Third, mutations in the FoxP3 gene are linked to Treg deficiency and cause autoimmune syndromes in mice and humans [4, 5]. Impairment of Tregs may also contribute to common autoimmune diseases but in humans, the analysis is hampered by the fact that a specific surface marker, which unequivocally defines Tregs, is missing. FoxP3 is localized intracellular and not suitable to select Tregs for functional analysis. Whereas in mice expression of CD25 is restricted to $CD4^+CD25^+FoxP3^+$ Tregs, in humans CD25 is also present on recently activated conventional $CD4^+$ T cells [6], yet FoxP3 expression correlates with the intensity of CD25 surface staining [7]. The suppressive activity was confined to the $CD4^+CD25^{high}$ T cell population [8]. Only 1 - 2 % of $CD4^+$ T cells express constitutively high levels of CD25 while the expression is intermediate (interm) to low on 30 % of $CD4^+$ T cells [6]. However, the purity and recovery of Tregs depends on the gating strategy of $CD4^+CD25^{high}$ *versus* $CD4^+CD25^{interm}$ T cells. Therefore additional marker such as CD39 [9, 10] or CD127 [11, 12] have been introduced to separate Tregs from conventional activated T cells. CD39 is not only expressed on CD4+$CD25^{high}$ T cells but also present on $CD4^+CD25^{interm/low}$ T cells [9] and expression correlate but does not completely overlap with the $CD4^+CD25^{high}$ T cell population [10]. Expression of CD127 inversely correlates with FoxP3 and up to 93.6 % of the $CD4^+CD25^+CD127^{-/low}$ T cells are FoxP3 positive [12]. This correlation is not absolute because some $CD127^{-/low}$ T cells are FoxP3 negative while some $CD127^{+/high}$ T cells are FoxP3 positive. $CD4^+CD25^{high}$ Tregs express no or very low CD127 while $CD4^+CD25^{interm/low}$ T cells show high expression of CD127 [12]. Comparing different Treg populations including $CD4^+CD25^+$, $CD4^+CD39^+$, $CD4^+CD73^+$, Yu et al. [13] confirmed that the $CD4^+CD25^+CD127^{-/low}$ T cells displayed the strongest correlation with FoxP3 expression. The combination of CD4, CD25 and CD127 antibodies are now commonly used to select Tregs by MACS or FACS sorting. Functional properties of Tregs are verified in an *in vitro* suppression assay. Functionally Tregs are distinguished from conventional T effector cells (Teffs) by anergy towards antigenic stimuli and their ability to abrogate proliferation of activated $CD4^+$ Teffs [14]. Numerical and / or functional deficiencies of Tregs have been described in a variety of autoimmune diseases [15-22]. Most studies describe reduced Treg cell numbers in patients compared to healthy individuals but Treg cell numbers were also reported to be normal depending on activity, severity or course of the disease [15-23]. Functional deficiency of Tregs was described in multiple sclerosis [15-19], systemic lupus erythematosus [20], rheumatoid arthritis [21], and diabetes type 1 [22, 23].

Functional analysis is usually performed with highly selected Tregs and autologous $CD4^+CD25^-$ Teffs in the presence of antigen specific or polyclonal T cell activation. Assuming that only numerical but not functional alterations of Tregs occur in autoimmune diseases, highly selected Tregs from healthy individuals as well as patients should display equivalent functional properties in polyclonal activated T cell suppression assays. This is however not the case which indeed implicates functional impairment of Tregs but heterogeneity of Tregs and / or T effector cells (Teffs) is still another option. Kleinewietfeld et al. [24] used CD49d antibodies to obtain negatively selected untouched Tregs for in vitro expansion but low suppressive activity was also seen in the $CD49d^+$ T cell control. Thus we considered $CD4^+CD25^{high}CD127^{-/low}CD49d^+$ T cells a likely candidate to contribute to the heterogeneity of Tregs. Since Tregs should prevent the induction of autoreactive T cells, activated $CD45RA^-$ Teffs may be less susceptible to suppression than naive $CD45RA^+$ Teffs. Here we show that $CD49d^+$ and $CD49d^-$ Tregs differ in their suppressive capacity while $CD45RA^+$ and $CD45RA^-$ Teffs differ in their susceptibility to suppression.

Material and Methods

Antibodies

The following antibodies were used for magnetic activated cell separation (MACS): "$CD4^+CD25^+$ Regulatory T Cell Isolation Kit II, human" (Miltenyi Biotec, Bergisch-Gladbach, Germany). The kit contains a $CD4^+$ T Cell Biotin-Antibody Cocktail (biotin-conjugated monoclonal anti-human antibodies against CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCRγ/δ and CD235a); anti-Biotin Microbeads and CD25 Microbeads (microbeads conjugated with monoclonal anti-CD25 antibodies). The following anti-human monoclonal antibodies were used for fluorescence activated cell analysis or sorting (FACS): "Human Regulatory T Cell Cocktail" (anti-CD4-FITC, anti-CD25-PE-Cy7, anti-CD127-Alexa Fluor 647), anti-CD49d-PE-Cy5 (both BD Pharmingen), anti-CD45RA-V450 (BD Horizon). Monoclonal antibodies of equivalent isotype were used to control unspecific staining.

Cell separation

Buffy coats from anonymous healthy volunteers were kindly provided by the Institute for Transfusion Medicine. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with Ficoll-Hypaque (Biochrom AG, Berlin, Germany). Lymphocytes were separated by MACS utilizing the "$CD4^+CD25^+$ Regulatory T Cell Isolation Kit II" according to the manufacturers recommendations (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD4^+$ T cells are negatively selected first and than separated into $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells. MACS isolated $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were stained with the "Human Regulatory T Cell Cocktail" (anti-CD4-FITC, anti-CD25-PE-Cy7, anti-CD127-Alexa Fluor 647) as indicated in the technical data sheet (BD Pharmingen, Heidelberg, Germany). Where indicated $CD4^+CD25^+$ T cells were stained in addition with anti-CD49d antibodies (anti-CD49d-PE-Cy5, BD Pharmingen, Heidelberg, Germany) and $CD4^+CD25^-$ T cells with anti-CD45RA antibodies (anti-CD45RA-V450, BD Horizon, Heidelberg, Germany). Cells were than analyzed and sorted utilizing the FACSAria I and FACAria III cell sorter and the FACSDiva software (BD Biosciences, Heidelberg, Germany). Cells were first gated using a FSC vs. SSC plot followed by doublet discrimination. Singlet cells were then gated for $CD4^+$ expression. Selection of Tregs: $CD4^+$ T cells were gated on $CD25^{high}CD127^{-/low}$ expression to identify Tregs. Staining with anti-CD49d antibodies prior analysis enabled the collection of $CD4+CD25^{high}CD127^{-/low}CD49d^-$ Tregs as well as $CD4+CD25^{high}CD127^{-/low}CD49d^+$ Tregs. Selection of Teffs: $CD4^+$ T cells were gated on $CD25^-CD127^+$ expression to define Teffs. Staining with anti-CD45RA antibodies prior analysis enabled the collection of $CD4^+CD25^-CD127^+RA^+$ and $CD4^+CD25^-CD127^+RA^-$ Teffs.

Suppression assay

In vitro suppression assays were carried out in RPMI 1640 culture medium supplemented with 10 % human serum. $5x10^4$ Teffs together with $1x10^5$ irradiated (30 Gy) autologous PBMCs were stimulated with monoclonal anti-CD3 antibodies (OKT3, 0.5 µg/ml; BD Biosciences, Heidelberg, Germany) in a final volume of 200 µl culture medium in 96-well round-bottom plates. The suppressive capacity of Tregs was measured by the addition of Tregs at Treg : Teff ratios of 0.125:1, 0.25:1, 0.5:1, 1:1. Each sample was tested in triplicates or at least duplicates, the deviation from the mean was in the majority of cases <10 %, deviations up to 15 % were only seen at low Treg : Teff ratios (0.5:1 or 0.125:1). After culture for 72 hours at 37°C and 5% CO_2 in a humified atmosphere, ^{3}[H]-thymidine (0.037 Mbq/well) was added and cells were cultured for additional 16 hours. ^{3}[H]-thymidine uptake was measured by scintillation counting as cpm using a beta-plate reader (Microbeta Trilux, Wallac, Turku, Finland). The capacity of Tregs to inhibit the proliferation of Teffs is indicated as percent [%] suppression. The percentage of suppression was calculated by the formula: [1 – (cpm of Treg with Teff – cpm of Treg w/o Teff) / (cpm of Teffs w/o Tregs)] x 100.

Statistical analysis

Each result was derived from at least three individual experiments. The mean and standard deviation (SD) is given. The SD is more appropriate than the standard error of the mean (SEM) [25], which is always smaller than the SD [SEM=SD/(square root of sample size)]. Statistical significance between experimental groups was analyzed using the two-tailed unpaired t-test and p-values <0.05 were considered statistically significant (*p <0.05, **p <0.01, (***p <0.001).

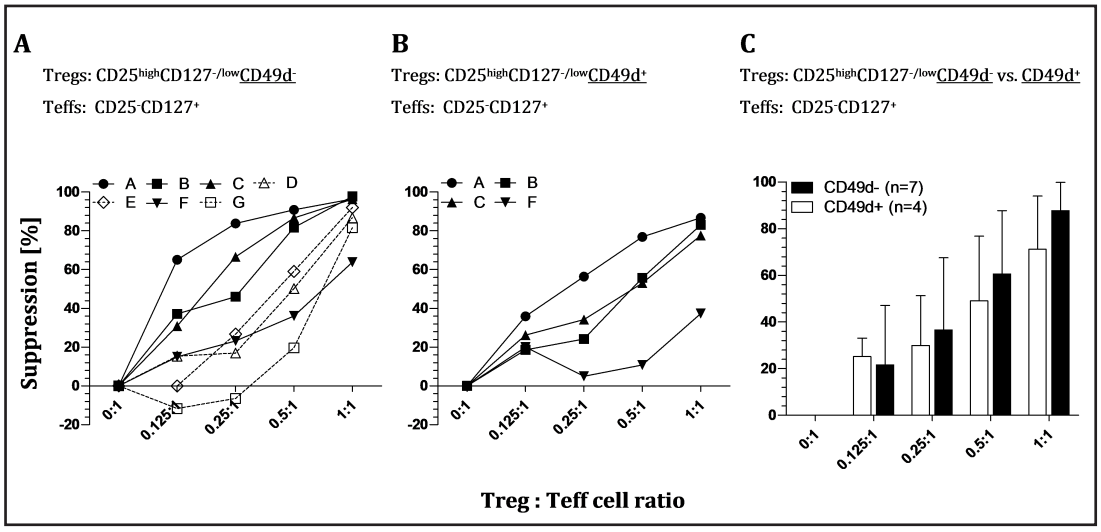

Fig. 1. Heterogeneity of Treg cells. $CD4^+CD25^{high}CD127^{-/low}CD49d^-$ Tregs as well as $CD4^+CD25^{high}CD127^{-/low}CD49d^+$ Tregs obtained from healthy individuals were analyzed on autologous $CD4^+CD25^-CD127^+$ Teffs at Treg : Teff ratios as indicated on the x-coordinate. Functional activity of Tregs is expressed as percent [%] suppression and given on the y-coordinate. A: Dose dependent suppressive activity of $CD49d^-$ Tregs from seven individuals [A-G]. B: Dose dependent suppressive activity of $CD49d^+$ Tregs from the same individuals [A-C, F]. C: Suppressive capacity of $CD49d^-$ Tregs (black column, mean of n=7) compared to $CD49d^+$ Tregs (white column, mean of n=4). Error bars indicate interindividual variability (SD). Differences between both groups were not statistical significant.

Results

Heterogeneity of Treg cells

$CD4^+CD25^+$ T cells of healthy individuals were enriched by MACS, stained as described in material and methods and sorted by FACS into $CD4^+CD25^{high}CD127^{-/low}CD49d^-$ and $CD4^+CD25^{high}CD127^{-/low}CD49d^+$ Tregs. $CD4^+CD25^-$ T cells, negatively selected by MACS, were stained (see material and methods) and $CD4^+CD25^-CD127^+$ Teffs were sorted by FACS to obtain Teffs devoid of $CD25^{+/low}$ T cells. The $CD49d^-$ and $CD49d^+$ Treg cell subpopulations were than compared for their suppressive activity on $CD25^-$ Teffs (Fig. 1). Only in three (open symbols, Fig. 1A) out of seven individuals the number of $CD49d^+$ Tregs was not sufficient to compare $CD49d^-$ and $CD49d^+$ Tregs from the same individual. Dose dependent suppressive capacity was observed in both, the $CD49d^-$ Treg (Fig. 1A) and the $CD49d^+$ Treg (Fig. 1B) subpopulations. Within individuals the suppressive capacity of $CD49d^-$ Tregs was always higher compared to their $CD49d^+$ Treg counterparts. On average the suppressive activity of $CD49d^-$ Tregs was higher (Treg : Teff cell ratio 1:1, 87.7 % ± 12.1 %) than the suppressive activity of $CD49d^+$ Tregs (71.1 % ± 22.9 %). The difference between both subpopulations was reproducible but not yet significant. This might be due to the high interindividual variability of $CD49d^-$ as well as $CD49d^+$ Treg cell activity (Fig. 1A and B). This high interindividual variability in healthy volunteers was unexpected and thus we hypothesized that not only the Treg cell population but although the Teff cell population might be heterogeneous. Naïve Teffs could be more susceptible to suppression than already activated Teffs.

Heterogeneity of Teff cells

$CD4^+CD25^{high}CD127^{-/low}$ Tregs were isolated essentially as in Fig. 1 except that separation into $CD49d^-$ and $CD49d^+$ Tregs was omitted. $CD4^+CD25^-CD127^+$ Teffs were isolated as in Fig. 1 but this time Teffs were further separated into $CD45RA^+$ and $CD45RA^-$ Teffs (see materials and methods). As shown in Fig. 2A, $CD4^+CD25^-CD127^+CD45RA^+$ Teffs are more sensitive to suppression than $CD4^+CD25^-CD127^+CD45RA^-$ Teffs.

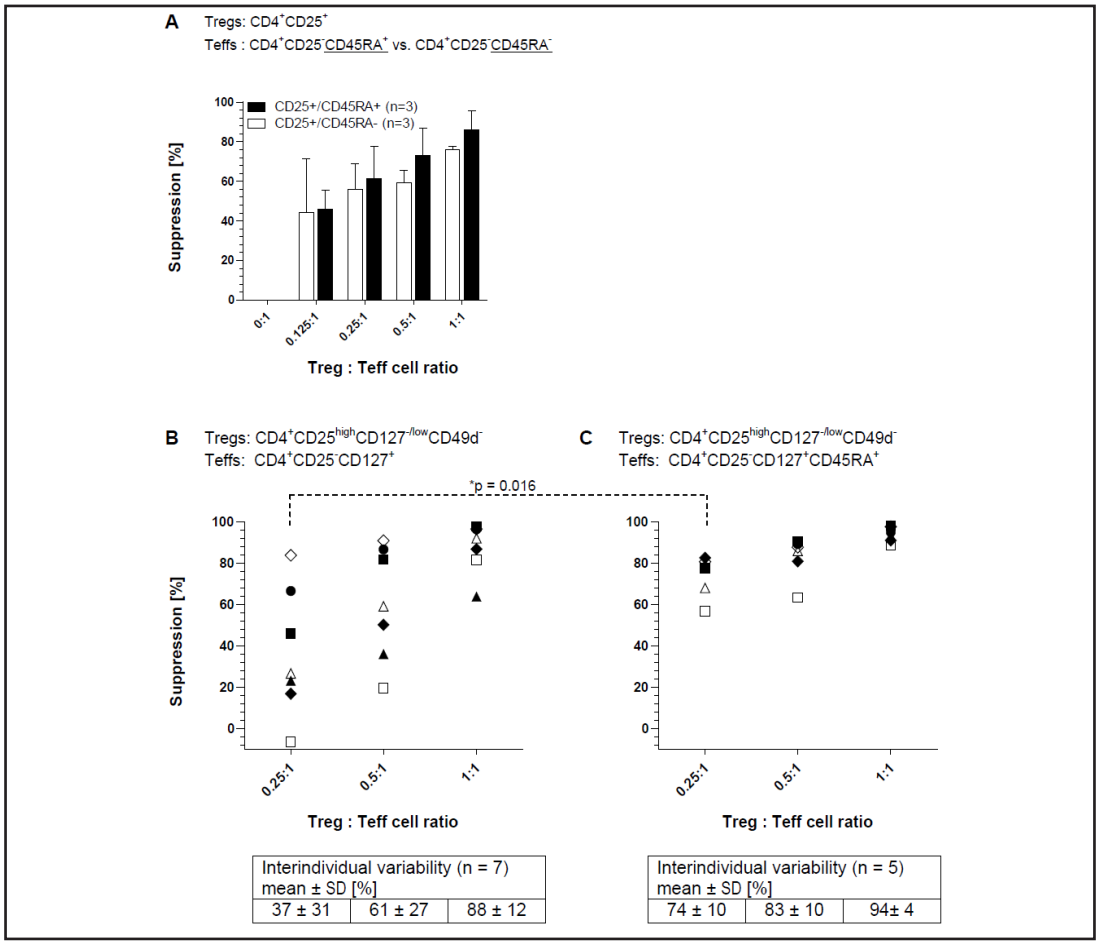

Fig. 2. Heterogeneity of Teff cells. A: Positively selected (MACS) $CD4^+CD25^+$ Tregs obtained from healthy individuals [n=3] were analyzed on autologous highly purified (FACS) $CD4^+CD25^-CD127^+CD45RA^+$ Teffs or $CD4^+CD25^-CD127^+CD45RA^-$ Teffs at Treg : Teff ratios as indicated on the x-coordinate. Functional activity of Tregs is expressed as percent [%] suppression and given on the y-coordinate. Susceptibility to suppression of $CD45RA^+$ Teffs (black column) compared to $CD45RA^-$ Teffs (white column). Error bars indicate interindividual variability (SD). Differences between both groups were not statistical significant. B and C: $CD4^+CD25^{high}CD127^{-/low}CD49d^-$ Tregs analyzed on autologous $CD4^+CD25^-CD127^+$ Teffs (n=7) or $CD4^+CD25^-CD127^+CD45RA^+$ Teffs (n=5). Each symbol represents the suppressive susceptibility of less purified $CD127^+$ Teffs (B) or highly purified $CD127^+CD45RA^+$ Teffs (C). Treg : Teff ratios are indicated on the x-coordinate and percent [%] suppression on the y-coordinate. The mean and interindividual variability (SD) at different Treg : Teff ratios are given below the x-coordinate. At a Treg : Teff ratio of 1:1 susceptibility to suppression is significantly lower in (B) compared to (C); p=0.016.

We than isolated again $CD4^+CD25^{high}CD127^{-/low}CD49d^-$ Tregs and compared their activity either on heterogeneous $CD4^+CD25^-CD127^+$ Teffs (Fig. 2B) or on $CD4^+CD25^-CD127^+CD45RA^+$ Teffs (Fig 2C). High interindividual variability is observed when $CD49d^-$ Treg activity is analyzed on heterogeneous Teff cell populations containing $CD45RA^+$ as well as RA^- Teffs (see mean and SD, Fig. 2B). In contrast interindividual variability is low when $CD49d^-$ Treg activity is analyzed on $CD45RA^+$ Teffs (see mean and SD, Fig. 2C). Thus $CD4^+CD25^-CD127^+$ Teffs contain cells with differential susceptibility to suppression and this heterogeneity is already indicated by high interindividual variability. The question to which extent $CD45RA^-$ Teffs display relative resistance can only be answered when $CD45RA^-$ and $CD45RA^+$ Teffs are compared.

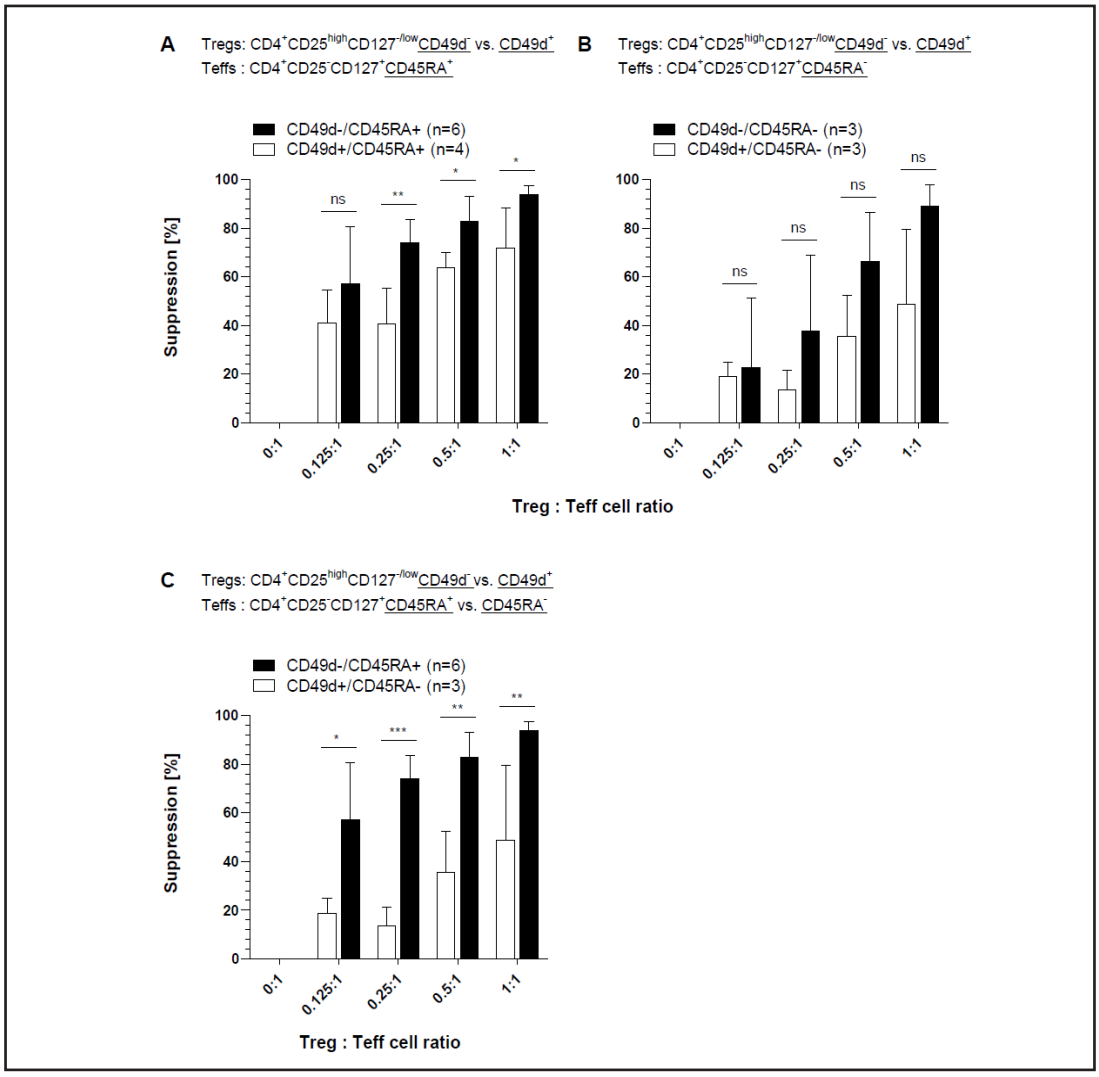

Fig. 3. Suppressive capacity of CD49d⁻ versus CD49d⁺ Tregs and susceptibility to suppression of CD45RA⁺ versus CD45RA⁻ Teffs. *In vitro* suppression assay. A: CD49d⁻ Tregs (n = 6) or CD49d⁺ Tregs (n = 4) co-cultured with CD45RA⁺ Teffs. B: CD49d⁻ Tregs (n = 3) or CD49d⁺ Tregs (n = 3) co-cultured with CD45RA⁻ Teffs. C) CD49d⁻ Tregs (n = 6) co-cultured with CD45RA⁺ Teffs or CD49d⁺ Tregs (n = 3) co-cultured with CD45RA⁻ Teffs. The Treg : Teff ratios are indicated on the x-coordinate and functional activity of Tregs is expressed as percent [%] suppression and given on the y-coordinate. Error bars indicate interindividual variability (SD). Differences between experimental groups were analyzed using the two-tailed unpaired t-test (ns = not significant, p-values = *p <0.05, **p <0.01, *** p< 0.001).

CD49d– Tregs display significantly higher suppressive capacity than CD49d+ Tregs, while CD45RA+ Teffs are significantly more susceptible to suppression than CD45RA- Teffs

In the next set of experiments, we compared the suppressive capacity of CD49d⁻ or CD49d⁺ Tregs either on CD45RA⁺ or CD45RA⁻ Teffs. CD49d⁻ Tregs show significantly higher suppressive capacity than CD49d⁺ Tregs when tested on CD45RA⁺ Teffs (Fig 3A). At a Treg : Teff ratio of 1:1 the suppressive activity of CD49d⁻ Tregs (93.8 %, SD 3.7 %) was significantly different compared to CD49d⁺ Tregs (71.8 %, SD 16.5 %), p = 0.0117. At a Treg : Teff ratio of 0.5:1, the suppressive capacity of CD49d⁻ Tregs was (82.9 %, SD 10.2 %), while the activity of CD49d⁺ Tregs was (63.6 %, SD 6.3 %), p = 0.0101 and at a Treg : Teff ratio of 0.25:1 the suppressive activity of CD49d⁻ Tregs was higher (73.9 %, SD 9.8 %) than the one of CD49d⁺ Tregs (40.6 %, SD 14.7 %), p = 0.0025.

When $CD49d^-$ Tregs or $CD49d^+$ Tregs were tested on $CD45RA^-$ Teffs, the suppressive capacity of $CD49d^-$ Tregs was higher than the one of $CD49d^+$ Tregs (Fig 3B). At a Treg : Teff ratio of 1:1 the suppressive activity of $CD49d^-$ Tregs was 89.2 % (SD 8.7 %) compared to $CD49d^+$ Tregs 48.7 % (SD 30.9 %). At a Treg : Teff ratio of 0.5:1, the suppressive capacity of $CD49d^-$ Tregs was 66.1 % (SD 20.3 %) while the activity of $CD49d^+$ Tregs was 35.5 % (SD 16.7 %). and at a Treg : Teff ratio of 0.25:1 the suppressive activity of $CD49d^-$ Tregs was 37.7 % (SD 31.1 %) and of $CD49d^+$ Tregs 13.6 % (SD 7.8 %). When the data of Treg subpopulations were compared, they did not reach statistical significance. This is obviously due to high interindividual variability observed when Tregs are tested on $CD45RA^-$ Teffs.

Taken together, $CD49d^-$ Tregs display high suppressive capacity on $CD45RA^+$ Teffs while $CD49d^+$ Tregs tested on $CD45RA^-$ Teffs are remarkably less efficient. The suppressive activity of $CD49d^-$ Tregs on $CD45RA^+$ Teffs at a Treg : Teff ratio of 1:1 was 93.8 % (SD 3.7 %), at a ratio of 0.5:1, 82.9 % (SD 10.2 %) and at a ratio of 0.25:1, 73.9 % (SD 9.8 %). In contrast the suppressive activity of $CD49d^+$ Tregs on $CD45RA^-$ Teffs at a Treg : Teff ratio of 1:1 was 48.7 % (SD 30.9 %), at a ratio of 0.5:1, 35.5 % (SD 16.7 %) and at a ratio of 0.25:1, 13.6 % (SD 7.8 %). The difference between both experimental groups was siginificant with p-values at a Treg : Teff ratio of 1:1 ($p = 0.0067$), 0.5:1 ($p = 0.0010$) and 0.25 ($p = 0.0001$). Thus $CD49d^-$ and $CD49d^+$ Tregs display differential suppressive capacity while $CD45RA^+$ Teffs and $CD45RA^-$ Teffs display differential susceptibility to suppression.

Discussion

The T cell marker $CD4^+$, $CD25^+$, $CD127^{-/low}$ have been used in numerous studies to characterize Treg cells and address the question whether or not patients with autoimmune diseases have deficiencies in their number or function of Tregs. While most studies focused on highly selected $CD4^+CD25^{high}CD127^{-/low}$ or $CD4^+CD25^{high/interm}CD127^{-/low}$ Tregs obtained by FACS or MACS respectively, less attention was paid to the selection of Teffs which were mainly characterized as $CD4^+CD25^-$ T cells and obtained by MACS.

In the present study, we defined two Treg cell populations by elimination of $CD127^+$ T-cells [11,12], selection of $CD4^+CD25^{high}$ T-cells [8] and subdivision into $CD49d^-$ and $CD49d^+$ Tregs. The functional activity at a Treg : Teff ratio of 1:1 was higher in $CD25^{high}CD49d^-$ Tregs (87.7 %) compared to $CD25^{high}CD49d^+$ Tregs (71.1 %). In a previous study [24] the suppressive activity of $CD49d^-$ Tregs (76.9%) was shown to be less while the suppressive activity of $CD49d^+$ Tregs was remarkably low (28 %) compared to our study. The difference can be easily explained by the selection procedure. While we used positively selected $CD4^+CD25^{high}$ Tregs, these authors used negatively selected $CD4^+CD25^+$ Tregs. The $CD49d^-$ Tregs contain some $CD25^{low}$ T-cells while $CD49d^+$ Tregs contain substantial numbers with low suppressive capacity [8]. This underscores, that the purity of the Treg cell population has substantial impact on the suppressive activity observed in an *in vitro* suppression assay.

Whether Tregs achieve high suppression is finally determined by the Teff cell population. First, negatively selected $CD4^+CD25^-$ Teffs obtained by MACS isolation still contain $CD25^{low}$ T cells [26] which are remarkably less susceptible to suppression (~30%) compared to $CD25^-$ Teffs (~70%). This observation is confirmed in our study (data not shown), $CD25^-$ Teffs (76.8 %) are sufficiently suppressed while reasonable suppression of $CD25^{low}$ T-cells (22.6 %) was only observed at Treg : Teff ratios of 1:1. Therefore in the present study we exclusively used FACS isolated $CD4^+CD25^-CD127^+$ Teffs. We defined two Teff cell populations by subdivision into $CD45RA^+$ Teffs and $CD45RA^-$ Teffs. We show here that $CD4^+CD127^+CD25^-CD45RA^+$ Teffs are highly susceptible to suppression while $CD4^+CD127^+CD25^-CD45RA^-$ Teffs are suppressed less efficiently. This could reflect low sensitivity to suppression of all $CD45RA^-$ Teffs but may be also due to heterogeneity of this cell population. Thus, the question whether truly resistant Teffs exist will be a matter of debate, but if so we would expect these cells within the $CD4^+CD127^+CD25^-CD45RA^-$ T cell population.

Our findings have implications on previous crossover experiments, comparing Tregs or Teffs from healthy donors and patients with systemic lupus erythematosus [27], type 1 diabetes [28, 29], rheumatoid arthritis [30] or multiple sclerosis [31]. The authors provide evidence that Teffs from patients compared to those from healthy donors are less susceptible or even resistant to the suppressive activity of Tregs obtained from patients or healthy donors. Based on our data it seems more likely that these observations are not due to an overall resistance of Teffs from patients but merely reflects heterogeneity of $CD4^+CD25^-$ Teffs ($CD45RA^-$ *versus* $CD45RA^+$ Teffs) or even contamination with $CD4^+CD25^{low}$ T-cells.

The fact that one has to deal with heterogeneity even within FACS sorted $CD4^+CD25^{--}$ Teffs is exemplified in our study by high interindividual variability observed in $CD4^+CD25^-CD127^-$ Teffs compared to $CD4^+CD25^-CD127^-CD45RA^+$ Teffs. High interindividual variability not only in patients but also in healthy volunteers was also obvious in previous studies [16, 17, 31] but not discussed by these authors. This observation however led us to analyze the Treg as well as Teff cell populations in more detail. As shown here $CD4^+CD25^{high}CD127^{-/low}CD49d^-$ Tregs tested on $CD4^+CD25^-CD127^-CD45RA^+$ Teffs display suppressive activity (Treg : Teff ratio 1:1) close to 100% (93.8 %, SD 3.7 %). These data implicate the following: when $CD4^+CD25^{high}CD127^{-/low}$ Tregs are analyzed on highly selected naïve $CD4^+CD25^-CD127^-CD45RA^+$ Teffs at a Treg : Teff ratio of 1:1 and suppressive activity falls below 90% (-1SD) or 86% (-2SD) this could indicate heterogeneity of Tregs. On the other hand, when highly selected $CD4^+CD25^{high}CD127^{-/low}CD49d^-$ Tregs are used to analyze the susceptibility of Teffs at a Treg : Teff ratio of 1:1 and suppressive activity falls below 90% (-1SD) or 86% (-2SD) this could indicate heterogeneity of Teffs. The threshold set here is an example and not a final rule since the data are from 6 individuals only. Larger and independent studies are needed to confirm these results. Nevertheless we believe that utilization of highly purified $CD4^+CD25^{high}CD127^{-/low}CD49d^-$ Tregs and $CD4^+CD25^-CD127^-CD45RA^+$ Teffs from healthy volunteers as test cells are a good option to define whether patients have a deficiency in Tregs, Teffs or both.

In conclusion four times more $CD49d^+$ Tregs [suppression at a Treg:Teff ratio of 1:1, 71.8 % (SD 16.5 %)] are needed compared to $CD49d^-$ Tregs (suppression at a Treg:Teff ratio of 0.25:1, 73.9 % (SD 9.8 %)] to suppress naïve Teff cells ($CD45RA^+$) to the same extent. On the other hand roughly two times more $CD49d^-$ Tregs are needed to suppress $CD45RA^-$ Teffs [suppression at a Treg:Teff ratio of 1:1, 89.2% (SD 8.7%)] as efficiently as $CD45RA^+$ Teffs [suppression at a Treg:Teff ratio of 0.5:1, 82.9% (SD 10.2%)]. Phenotypic and functional heterogeneity of Tregs as well as Teffs has to be considered when patients are analyzed for Treg cell deficiencies. Already activated Teffs, $CD25^{low}$ as well as $CD25^-CD45RA^-$ T cells, display relative resistance to suppression which poses the question to which extent already activated autoreactive T cells will be controlled *in vivo* after substitution of Tregs.

Abbreviations

Teffs (T effector cells); Tregs (T regulatory cells).

Acknowledgements

The authors thank Peter A. Horn for kindly providing buffy coats of anonymous healthy volunteers and Monika Lindemann for pooled human sera from male volunteers; Institute for Transfusion Medicine, University Hospital, Essen, Germany. The authors thank Namir Shaabani for fruitful discussion.

Disclosure Statement

The authors declare no financial and commercial conflicts of interest.

References

1 Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M: Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol 1995;155:1151-1164.
2 Hori S, Nomura T, Sakaguchi S: Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-1061.
3 Fontenot JD, Gavin MA, Rudensky AY: Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4:330-336.
4 Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F: Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet 2001;27:68-73.
5 Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD: The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet 2001;27:20-21.
6 Baecher-Allan C, Viglietta V, Hafler DA. Human CD4+CD25+ regulatory T cells. Semin Immunol 2004;6:89-98.
7 Yagi H, Nomura T, Nakamura K, Yamazaki S, Kitawaki T, Hori S, Maeda M, Onodera M, Uchiyama T, Fujii S, Sakaguchi S: Crucial role of FOXP3 in the development and function of human CD25+CD4+ regulatory T cells. Int Immunol 2004;16:1643-1656.
8 Baecher-Allan C, Wolf E, Hafler DA: Functional analysis of highly defined, FACS-isolated populations of human regulatory CD4+ CD25+ T cells. Clin Immunol 2005;115:10-18.
9 Dwyer KM, Deaglio S, Gao W, Friedman D, Strom TB, Robson SC: CD39 and control of cellular immune responses. Purinergic Signal 2007;3:171-180.
10 Mandapathil M, Lang S, Gorelik E, Whiteside TL: Isolation of functional human regulatory T cells (Treg) from the peripheral blood based on the CD39 expression. J Immunol Methods 2009;346:55-63.
11 Seddiki N, Santner-Nanan B, Martinson J, Zaunders J, Sasson S, Landay A, Solomon M, Selby W, Alexander SI, Nanan R, Kelleher A, Fazekas de St Groth B: Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med 2006;203:1693-1700.
12 Liu W, Putnam AL, Xu-Yu Z, Szot GL, Lee MR, Zhu S, Gottlieb PA, Kapranov P, Gingeras TR, Fazekas de St Groth B, Clayberger C, Soper DM, Ziegler SF, Bluestone JA: CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. J Exp Med 2006;203:1701-1711.
13 Yu N, Li X, Song W, Li D, Yu D, Zeng X, Li M, Leng X, Li X: CD4(+)CD25(+)CD127 (low/-) T cells: a more specific Treg population in human peripheral blood. Inflammation 2012;35:1773-1780.
14 Jonuleit H, Schmitt E, Stassen M, Tuettenberg A, Knop J, Enk AH: Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. J Exp Med 2001;193:1285-1294.
15 Viglietta V, Baecher-Allan C, Weiner HL, Hafler DA: Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med 2004;199:971-979.
16 Haas J, Hug A, Viehöver A, Fritzsching B, Falk CS, Filser A, Vetter T, Milkova L, Korporal M, Fritz B, Storch-Hagenlocher B, Krammer PH, Suri-Payer E, Wildemann B: Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. Eur J Immunol 2005;35:3343-3352.
17 Kumar M, Putzki N, Limmroth V, Remus R, Lindemann M, Knop D, Mueller N, Hardt C, Kreuzfelder E, Grosse-Wilde H: CD4+CD25+FoxP3+ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis. J Neuroimmunol 2006;180:178-184.
18 Venken K, Hellings N, Broekmans T, Hensen K, Rummens JL, Stinissen P: Natural naive CD4+CD25+CD127low regulatory T cell (Treg) development and function are disturbed in multiple sclerosis patients: recovery of memory Treg homeostasis during disease progression. J Immunol 2008;180:6411-6420.
19 Fletcher JM, Lonergan R, Costelloe L, Kinsella K, Moran B, O'Farrelly C, Tubridy N, Mills KH: CD39+Foxp3+ regulatory T Cells suppress pathogenic Th17 cells and are impaired in multiple sclerosis. J Immunol 2009;183:7602-7610.

20 Liu MF, Wang CR, Fung LL, Wu CR: Decreased CD4+CD25+ T cells in peripheral blood of patients with systemic lupus erythematosus. Scand J Immunol 2004;59:198-202.
21 Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, Mauri C: Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J Exp Med 2004;200:277-285.
22 Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI: Defective suppressor function in CD4(+) CD25(+) T-cells from patients with type 1 diabetes. Diabetes 2005;54:92-99.
23 Brusko T, Wasserfall C, McGrail K, Schatz R, Viener HL, Schatz D, Haller M, Rockell J, Gottlieb P, Clare-Salzler M, Atkinson M: No alterations in the frequency of FOXP3+ regulatory T-cells in type 1 diabetes. Diabetes 2007;56:604-612.
24 Kleinewietfeld M, Starke M, Di Mitri D, Borsellino G, Battistini L, Rötzschke O, Falk K: CD49d provides access to "untouched" human Foxp3+ Treg free of contaminating effector cells. Blood 2009;113:827-836.
25 Barde MP, Barde PJ: What to use to express the variability of data: Standard deviation or standard error of mean? Perspect Clin Res 2012;3:113-116.
26 Jana S, Campbell H, Woodliff J, Waukau J, Jailwala P, Ghorai J, Ghosh S, Glisic S: The type of responder T-cell has a significant impact in a human in vitro suppression assay. PLoS One 2010;DOI: 10.1371/0015154.
27 Venigalla RK, Tretter T, Krienke S, Max R, Eckstein V, Blank N, Fiehn C, Ho AD, Lorenz HM: Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25high,CD127 -/low regulatory T cells in patients with active systemic lupus erythematosus. Arthritis Rheum 2008;58:2120-2130.
28 Lawson JM, Tremble J, Dayan C, Beyan H, Leslie RD, Peakman M, Tree TI: Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes. Clin Exp Immunol 2008;154:353-359.
29 Schneider A, Rieck M, Sanda S, Pihoker C, Greenbaum C, Buckner JH: The effector T cells of diabetic subjects are resistant to regulation via CD4+ FOXP3+ regulatory T cells. J Immunol 2008;181:7350-7355.
30 Xiao H, Wang S, Miao R, Kan W: TRAIL is associated with impaired regulation of CD4+CD25- T cells by regulatory T cells in patients with rheumatoid arthritis. J Clin Immunol 2011;31:1112-1119.
31 Schneider A, Long SA, Cerosaletti K, Ni CT, Samuels P, Kita M, Buckner JH: In active relapsing-remitting multiple sclerosis, effector T cell resistance to adaptive T(regs) involves IL-6-mediated signaling. Sci Transl Med 2013;DOI: 10.1126/3004970.