Unmasking of autoreactive $CD4^+$ T cells by depletion of $CD25^+$ regulatory T cells in systemic lupus erythematosus

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ABSTRACT

Objective

Autoreactive $CD4^+$ T cells specific for nuclear peptide antigens play an important role in tolerance breakdown during the course of systemic lupus erythematosus (SLE). However, reliable detection of these cells is limited due to their low frequency in the peripheral blood. Here, we assess autoreactive $CD4^+$ T cells in a representative SLE collective (n=41) by flow cytometry and study the influence of regulatory T cells (Treg) on their antigenic challenge.

Methods

$CD4^+$ T cell responses were determined according to intracellular CD154 expression induced after 6h short-term *in vitro* stimulation with the SLE-associated autoantigen SmD1(83-119). To clarify the influence of Treg on the activation of autoreactive $CD4^+$ T cells, we depleted $CD25^+$ Treg by MACS prior to antigen-specific stimulation in selected experiments.

Results

In the presence of Treg, autoreactive $CD4^+$ T cell responses to SmD1(83-119) were hardly observable. However, Treg removal significantly increased the frequency of detectable SmD1(83-119)-specific $CD4^+$ T cells in SLE patients but not in healthy individuals. Consequently, by depleting Treg the percentage of SmD1(83-119)-reactive SLE patients increased from 18.2% to 63.6%. This unmasked autoreactivity of $CD4^+$ T cells correlated with the disease activity as determined by SLEDAI ($p=0.005^*$, $r=0.779$).

Conclusions

Our data highlight the pivotal role of the balance between autoreactive $CD4^+$ T cells and $CD25^+$ Treg in the dynamic course of human SLE. Analysing CD154 expression in combination with a depletion of $CD25^+$ Treg, as shown here, may be of further use in approaching autoantigen-specific $CD4^+$ T cells in SLE and other autoimmune diseases.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease with tissue damage driven by the activation of autoreactive T and B cells. In this context, autoreactive $CD4^+$ T cells specific for nuclear peptide antigens play an important role in tolerance breakdown.[1, 2] Previously our group identified the SmD1(83-119) peptide, a C-terminal peptide fragment of the intranuclear SmD1 protein, as one of the key autoantigens in lupus.[3] SmD1(83-119)-specific autoantibodies have a high sensitivity (70%) and specificity (93.7%) for SLE and about one third of the SLE patients reveal $CD4^+$ T cells responses to this particular peptide as detected by [3H]thymidine incorporation.[3, 4] Furthermore, SmD1(83-119)-specific $CD4^+$ T cells have been shown to trigger the production of autoantibodies against double stranded DNA, emphasizing their involvement in the pathogeneses of SLE.[5]

However, direct access to these cells is restricted due to their low frequency in the peripheral blood, causing difficulties in their reliable detection.[6] The measurement of [3H]thymidine incorporation is a classical approach to determine cell proliferation after antigenic stimulation. Its specificity is limited, though. Since several days of stimulation time are required to achieve detectable proliferation rates, this method allows for unspecific effects during cultivation.[7] In addition, proliferation rates represent the entirety of PBMC and cannot be assigned to a specific cell type. Beyond that, weakly proliferating populations can get lost in the background proliferation. The measurement of T cell activation by cytokine production represents another common approach.[8, 9] However, T cells that do not produce cytokines after activation can be missed and the detection is restricted to T cell subsets with defined cytokine profiles.[10] Finally, the use of peptide MHC multimers, currently the most specific method, is strongly limited since only a few peptide MHC constructs are available so far.[11, 12, 13, 14]

To address this problem, Frentsch *et al.* introduced CD154, also known as CD40L, as a marker molecule of $CD4^+$ T cells, that have been recently activated upon antigen-specific short-term *in vitro* stimulation.[10] CD154 is stored in secretory lysosomes of effector and memory $CD4^+$ T cells and is quickly expressed on the cell surface in an antigen-specific manner.[15] Therefore this method enables a direct access to all $CD4^+$ T cells with a defined specificity and can be used for their enrichment by magnetic- or fluorescence-activated cell sorting.[10]

Recently, our group focused on the role of regulatory T cells (Treg) in the dynamic course of murine lupus. We found strong evidence that Treg effectively counteract lupus autoreactivity and ameliorate disease progression.[16] We therefore hypothesized that Treg, known to control the activation and expansion of autoreactive T cell clones,[17] may also effectively counteract the activation of SmD1(83-119)-specific $CD4^+$ T cells, impeding their reliable detection. In this work, we aimed to detect and quantify autoantigen-specific $CD4^+$ T cells in a representative SLE collective according to the CD154 expression after SmD1(83-119) stimulation. Subsequently, we determined the influence of $CD25^+$ Treg on autoreactive $CD4^+$ T cell responses by removing $CD25^+$ Treg prior to antigenic stimulation.

METHODS

Patients

Collections of human peripheral blood mononuclear cells (PBMC) were approved by Human Research Ethic Committee. We obtained 41 blood samples of 38 consecutive SLE patients (Tab. 1 and Suppl.Tab. 1) attending ambulant or inpatient care in the Department of Rheumatology and Clinical Immunology of the University Hospital Charité, Berlin, Germany. After informed consent was provided, 30-50 ml of heparinised whole blood was collected in vacuum collection tubes (Becton Dickinson, Franklin Lakes, NJ, USA). All patients fulfilled the American College of Rheumatology classification criteria for SLE.[18] Medical records were reviewed to determine clinical characteristics, immunosuppressive treatment, age, disease duration and disease activity measured by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI).[19]

Table 1: Patient statistics

SLE patients (n=38)		n	percentage	mean	range
sex	female	32	84.2%		
	male	6	15.8%		
age (years)				42	23-76
care unit	inpatients	13	34.2%		
	outpatients	25	65.8%		
disease activity (SLEDAI)				5	0-20
disease duration (years)				11	0-26
organ manifestations	kidney	15	39.5%		
	lung	8	21.1%		
	CNS	8	21.1%		
	heart	3	7.9%		
immunosuppressive medication	none	1	2.6%		
	corticosteroids	36	94.7%		
	corticosteroids ≤ 10mg/d	24	63.1%		
	corticosteroids > 10mg/d	12	31.6%		
	chloroquine	14	36.8%		
	mycophenolate mofetil	11	28.9%		
	azathioprine	11	28.9%		
	cyclophosphamide	5	13.2%		
	cyclosporine A	1	2.6%		
	methotrexate	1	2.6%		
	leflunomide	1	2.6%		

In patients with multiple samples the most active episode is shown.

Cell preparation

PBMC were separated from heparinised whole blood with a Ficoll-Hypaque gradient (PAA Laboratories GmbH, Pasching, Austria) and erythrocytes were lysed with an erythrocyte-lysis-buffer (DRFZ, Berlin, Germany). PBMC were washed twice in PBS buffer containing 0.5% BSA (PBS/BSA). In selected experiments a depletion of $CD25^+$ Treg was performed prior to the antigen-specific stimulation.

Depletion of $CD25^+$ cells

PBMC were stained with anti-CD25-antibodies conjugated to ferromagnetic microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and directed through a cell separation column containing a magnetic field (Miltenyi Biotec). $CD25^-$ cells were collected for further examination.

In vitro antigen-specific stimulation

Undepleted and CD25-depleted PBMC were cultured in 96 well plates (Greiner Bio-One AG, Kremsmünster, Austria) containing 200 µl/well RPMI 1640 medium (Invitrogen, Paisley, UK) in a cell density of 1-2 x 10^6/ml. Stimulations were performed in the presence of 10µg/ml CD28-specific antibody (BD Biosciences, San Jose, CA, USA) for 6h at 37°C using one of the following antigens: 1µg/ml SEB (Sigma-Aldrich, St. Louis, MO, USA), 20 µl/ml CMV peptide mix pp65 (Miltenyi Biotec) or 20 µg/ml SmD1(83-119) (VEPKVKSKKREAVAGRGRGRGRGRGRGRGRGGPRR), synthesized as previously described.[3]

In order to improve the reliability, all stimulations were set up in triplicate cultures and means were calculated from triplicate data. Unstimulated samples were used as controls. For the analysis of

intracellular CD154 expression we added 20 µg/ml Bref A (Sigma-Aldrich) for the last 4h of stimulation. After completing the stimulation time, PBMC were fixed for 15min at 21°C in PBS/BSA containing 0.05% NaN_3 (PBS/BSA/NaN_3) and 2% paraformaldehyde, washed twice in PBS/BSA/NaN_3 and stored at 4°C.

Flow cytometry analysis

Fixed cells were permeabilised in PBS/BSA/NaN_3 containing 0.5% saponin (Sigma-Aldrich) and stained for 15min at 4°C using a selection of the following specific antibodies: FITC-conjugated anti-CD3 (UCHT-1), PE-conjugated anti-CD3 (UCHT-1), PE-conjugated anti-CD4 (TT1), PE-conjugated anti-CD4 (TT1), biotin-conjugated anti-CD4 (TT1), FITC-conjugated anti-CD25 (BC96), APC-conjugated anti-CD25 (4E3), APC-conjugated anti-CD154 (5C8) and PerCP-conjugated streptavidin. To avoid unspecific Fc receptor binding, the cells were stained in the presence of 5 mg/ml Flebogamma (Grifols, Barcelona, Spain).

The stained cells were immediately analysed on a FACSCalibur™ cytometer with Cellquest software (Becton Dickinson). At least 250,000 PBMC were recorded for each sample. Data analysis was performed using the Flowjo 7.2.5 software (Tree Star, Ashland, OR, USA). The frequency of antigen-specific $CD3^+CD4^+$ T cells was determined by antigen-induced intracellular CD154 expression. Mean background activity in unstimulated controls was 0.0429% $CD154^+$ cells among $CD3^+CD4^+$ T cells with a standard deviation (SD) of 0.062%. Samples exceeding this mean by 4SD or more were considered positive (threshold: 0.07%).

Statistical analysis

Statistical analysis was conducted using the software SPSS 18 (IBM SPSS, Chicago, IL, USA). In order to assess whether two non-normal distributed samples have equally large values the non-parametric Wilcoxon signed-rank test was performed. As a measure of linear dependence between two continuous variables the Pearson's correlation coefficient was used.

RESULTS

CD154 expression reliably detects infectious CD4$^+$ T cell recall responses but not autoreactive CD4$^+$ T cell activation.

41 PBMC samples of 38 SLE patients were stimulated with either the superantigen staphylococcal enterotoxin B (SEB), the cytomegalovirus peptide mix pp65 (CMV), the SLE-associated autoantigen SmD1(83-119) or were incubated in the absence of a stimulatory antigen as unstimulated negative controls (UNST). The antigen-specific activation was determined by CD154 expression of CD3$^+$CD4$^+$ T cells. Sample results exceeding the unstimulated mean by 4SD (equalling 0.07% or higher) were considered positive.

As expected, the stimulation with SEB resulted in a high frequency of CD154$^+$ cells among CD3$^+$CD4$^+$ T cells (median: 8.5991%). Compared to the unstimulated samples (UNST) (median: 0.0428%), this difference was highly significant ($p<0.001$). All 41 SEB-stimulated samples (100%) showed a response above the threshold of 0.07%. With a median of 0.1109% the CMV recall responses also significantly exceeded the unstimulated controls ($p<0.001$). A positive response was observed in 25 of 34 CMV-stimulated samples (73.5%) (Fig. 1).

In contrast, the stimulation with the autoantigen SmD1(83-119) elicited no specific CD4$^+$ T cell response compared to the unstimulated controls in this experimental setting (median: 0.0428% vs. 0.0372%, $p=0.600$). Only 4 of 41 samples (9.8%) stimulated with the SmD1(83-119) reached the threshold-level of 0.07% CD154$^+$ cells among CD3$^+$CD4$^+$ T cells (Fig. 1).

***In vitro*-depletion of CD25$^+$ Treg amplifies antigen-specific CD4$^+$ T cell responses.**

In order to investigate whether the CD4$^+$ T cell responses may be under a steady Treg control, we collected PBMC samples of 11 SLE patients and subdivided each sample. In one PBMC subset we eliminated CD25$^+$ Treg by MACS prior to antigenic stimulation while a second control subset remained undepleted. The efficiency of the CD25 depletion was confirmed by the determination of the percentage of CD25$^+$ cells among CD4$^+$ cells before and after the depletion (Fig. 2A, B and C).

The depletion of CD25$^+$ Treg significantly decreased the SEB-provoked response by 23.1% ($p=0.016$) and increased the SmD1(83-119)-specific response by 46.6% ($p=0.050$). The increase of CMV-specific CD4$^+$ T cells by 24.9%, however, was not significant because of the small sample number ($n=3$, $p=0.593$) (Fig. 2D and E).. In order to guarantee a constant cell density in SEB, UNST and SmD1(83-119) stimulations, we had to omit CMV stimulations in patients with pronounced lymphopenia.

***In vitro*-depletion of CD25$^+$ Treg unmasks autoreactive CD4$^+$ T cell response to SmD1(83-119) in SLE patients but not in healthy controls.**

We further investigated the increase in the SmD1(83-119)-specific CD4$^+$ T cell response in more detail. While the SmD1(83-119) response was not statistically different from the unstimulated controls ($p=0.248$) in the presence of CD25$^+$ Treg, it significantly exceeded the unstimulated background level ($p=0.026$) in the absence of CD25$^+$ Treg. Additionally, the percentage of SLE patients that showed a positive CD4$^+$ T cell response to SmD1(83-119) increased from 18.2% to 63.6% (Fig. 3).

To determine whether this unmasked SmD1(83-119)-specific CD4$^+$ T cell response may be present in healthy individuals as well, we tested 8 PBMC samples from healthy blood donors using the same experimental setting. In these healthy controls the removal of CD25$^+$ Treg was not able to unmask a significant CD4$^+$ T cell response to SmD1(83-119) ($p=0.161$). Only 1 of 8 tested samples slightly exceeded the threshold at 0.07%.

Unmasked CD4$^+$ T cell response to SmD1(83-119) correlates with SLE disease activity.

Taking into account the previous data indicating a certain relevance of SmD1(83-119)-specific CD4$^+$ T cells in SLE, we further checked for correlations between the unmasked SmD1(83-119) response and the disease activity index SLEDAI.

We found a significant positive correlation between SmD1(83-119) response and SLEDAI after stimulation of both undepleted and Treg depleted PBMC (Fig. 4A and Tab. 2). However, the depletion of CD25$^+$ Treg clearly strengthens the correlation in terms of significance ($p=0.044$ vs. $p=0.005$) and strength ($r=0.616$ vs. $r=0.779$). Table 2 gives a brief overview of all correlations tested in this study.

Table 2: Correlations

SLEDAI	n	p	r
anti-SmD1 antibodies	40	0.674	- 0.069
anti-nucleosome antibodies	40	0.003*	0.452
anti-nuclear antibodies (ANA)	18	0.001*	0.719
anti-dsDNA antibodies	32	< 0.001*	0.597
daily steroid dose	41	< 0.001*	0.739
SmD1(83-119) response in unmodified PBMC			
SLEDAI	41	0.668	- 0.069
anti-SmD1 antibodies	40	0.738	- 0.054
anti-nucleosome antibodies	40	0.465	- 0.119
anti-nuclear antibodies (ANA)	18	0.303	- 0.257
anti-dsDNA antibodies	32	0.611	- 0.094
daily steroid dose	41	0.641	- 0.075
SmD1(83-119) response in CD25 depleted PBMC			
SLEDAI	11	0.005*	0.779
anti-SmD1 antibodies	11	0.524	- 0.216
anti-nucleosome antibodies	11	0.337	- 0.320
anti-nuclear antibodies (ANA)	5	0.077	- 0.837
anti-dsDNA antibodies	4	0.820	- 0.180
daily steroid dose	11	0.009*	0.739

DISCUSSION

Since the implementation of CD154 as a marker for antigen-induced T cell activation,[10] this method has been corroborated in different contexts. For instance, patients suffering from Morbus Whipple could be identified by their insufficient $CD4^+$ T cell recall response to Tropheryma whipplei, while reactivity to other infectious agents was not affected.[20] However, the assessment of autoreactive $CD4^+$ T cell responses according to this method has not been reported so far.

In our first approach, $CD4^+$ T cell responses to the SLE-associated autoantigen SmD1(83-119) did not statistically differ from the background of unstimulated controls and exhibited a low responder rate. In contrast, the infectious recall response to CMV was highly significant and led to a responder rate of 73.5% which matches the reported CMV seroprevalence.[21] Based on our previous work, showing that Treg control the activation and expansion of both effector and memory $CD4^+$ T cells and are important to impede the progression of disease in murine lupus,[16] we hypothesized that the presence of Treg may impair autoreactive $CD4^+$ T cell responses during the short-term stimulation with autoantigens. We therefore eliminated $CD25^+$ Treg by MACS prior to antigenic-stimulation in 11 PBMC samples and compared the achieved stimulation results in presence and absence of Treg. We addressed CD25, the alpha chain of the IL-2 receptor, because there are still no convincing alternatives available for depleting human Treg by MACS. Despite of the big effort that was undertaken to identify specific Treg marker molecules, many promising candidates disappointed at a second look.[22] Currently, the forkhead family transcription factor (Foxp3) is the most specific Treg marker. However, the specificity of Foxp3 in the human system is still subject to ongoing discussions, especially since $Foxp3^+$ T cells that lack suppressive capacity were reported in human.[22, 23] In any case, intracellular Foxp3 is not applicable for surface staining. Consequently, we focussed on CD25 to remove Treg, accepting the potential loss of pre-activated $CD25^+$ effector T cells.

As shown here, the removal of $CD25^+$ Treg in 11 samples resulted in a significant 46.6% increase in SmD1(83-119)-specific $CD4^+$ T cells compared with the non-depleted controls. The increase of the CMV response, however, was not significant due to the small sample number (n=3). Nevertheless, Treg seem to control autoreactive and viral $CD4^+$ T cell responses likewise while several studies indicate a role of viral infections such as EBV in lupus pathogenesis.[24, 25] In contrast, SEB responses decreased by CD25 depletion, which might have been caused by differences in the T cell regulation and interaction upon bacterial versus autoantigenic stimulation. Concentrating on the autoreactive response to SmD1(83-119), the removal of $CD25^+$ Treg was able to unmask a significant $CD4^+$ T cell response to this particular SLE-associated autoantigen. To our knowledge, this is the first time SLE-associated autoreactive T cells were cytometrically detected after short-term stimulation in human. As shown in Fig. 3 the achieved dot plots do not show a clearly circumscribable population of

CD154 positive $CD4^+$ T cells, however, an increase in predominantly weak CD154 positive cells is observable and the percentage of patients responding to SmD1(83-119) raised from 18.2% to 63.6%. The relevance of these SmD1(83-119)-specific $CD4^+$ T cells is further underlined by the fact that they were only significantly detectable in SLE patients but not in healthy individuals. Furthermore, they also showed a linear correlation with the disease activity index SLEDAI and thereby closely reflect the disease's dynamics.

Unquestionable, there are some limitations of this study. First of all, the detected frequencies of SmD1(83-119)-specific $CD4^+$ T cells are still very low. To reliably detect these small differences we measured 3 probes per stimulation antigen and patient and calculated the means. While setting the $CD154^+$ gate we tolerated about 0.05% $CD154^+$ cells among $CD4^+$ T cells in the unstimulated controls and applied this gate to all the other probes of the sample. Consequently, we used the unstimulated samples as our main reference. Nevertheless, the achieved results come close to the very limits of cytometry and further enhancements of the protocol may be necessary to improve the readout. Another critical point is that the depletion of $CD25^+$ cells not exactly matches the depletion of Treg. As already discussed above, CD25 is also detectable on recently activated effector T cells. In addition, naturally occurring Treg that lack CD25 expression have been reported.[26] However, to effectively eliminate the majority of Treg CD25 depletion is still a practicable approach.[26, 27] This is further underlined by our recent studies showing that CD25 depletion effectively aggravates disease progression lupus mice[16] and that the majority of peripheral $CD25^+$ T cells in our SLE patients is characterised by a Treg-like $FoxP3^+$ $CD127^-$ phenotype (unpublished observations). Furthermore, the low sample numbers in our depletion experiments represent another limitation of this study. These low numbers are due to the comparatively big blood probes needed from SLE patients with predominantly low lymphocyte count. However, most of the results shown here were significant in statistical analysis. Finally, studying autoreactive T cell responses from patients receiving different immunosuppressive therapies including different steroid doses could have confounded the stimulation results. Indeed, we found a significant correlation of the daily prednisone dose and the SmD1(83-119) response in CD25 depleted samples (Fig. 4B). However, this observation might be simply caused by the fact that active patients are characterised by both elevated prednisone doses and increased unmasked autoreactive SmD1(83-119) responses (Tab. 2). Beyond that, no further connections between administered medication and SmD1(83-119) response were observed (Suppl.Tab. 1).

Despite these limitations, the data presented here provide an interesting insight into the role of Treg and autoreactive $CD4^+$ T cells in human SLE and strongly support our observations in the animal model.[16] The necessity of depleting $CD25^+$ Treg to unmask SmD1(83-119)-specific $CD4^+$ T cells, however, raises the question why Treg seem to effectively suppress these autoreactive cells in our *in vitro* stimulations but not in the patients suffering from SLE. One possible explanation could be a predominance of Treg inhibiting mediators such as IL-6 which is detectable in high levels in sera of SLE patients and was recently shown to impede Treg differentiation and promote autoreactive Th17 cells.[28, 29] In our *in vitro* experimental setting IL-6 should have been largely eliminated though, which could have improved Treg functionality. However, further research is needed to clarify the role of Treg and autoreactive $CD4^+$ T cells in lupus and lay open the central pathogenetic mechanisms.

In summary, our findings support the current understanding of Treg in maintaining tolerance to self and thereby preventing autoimmune disorders.[17, 30, 31, 32] The critical balance of autoreactive $CD4^+$ T cells and Treg seems to be a central pathogenetic mechanism in human SLE. By removing Treg from this balance we were able to efficiently unmask a $CD4^+$ T cell reactivity to SmD1(83-119), a major autoantigen in SLE. In contrast, we were not able to unmask a significant SmD1(83-119)-specific $CD4^+$ T cell response in healthy individuals. Furthermore, we found a correlation between the unmasked SmD1(83-119)-specific $CD4^+$ T cell response and the patient's disease activity. This underlines the relevance of autoreactive $CD4^+$ T cells in the dynamic course of the disease. The assessment of autoantigen-specific $CD4^+$ T cells according to antigen-induced CD154 expression in combination with a depletion of $CD25^+$ Treg, as presented in this work, provides a promising approach to autoreactive $CD4^+$ T cells that may also be valuable in other autoimmune diseases.

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DECLARATION

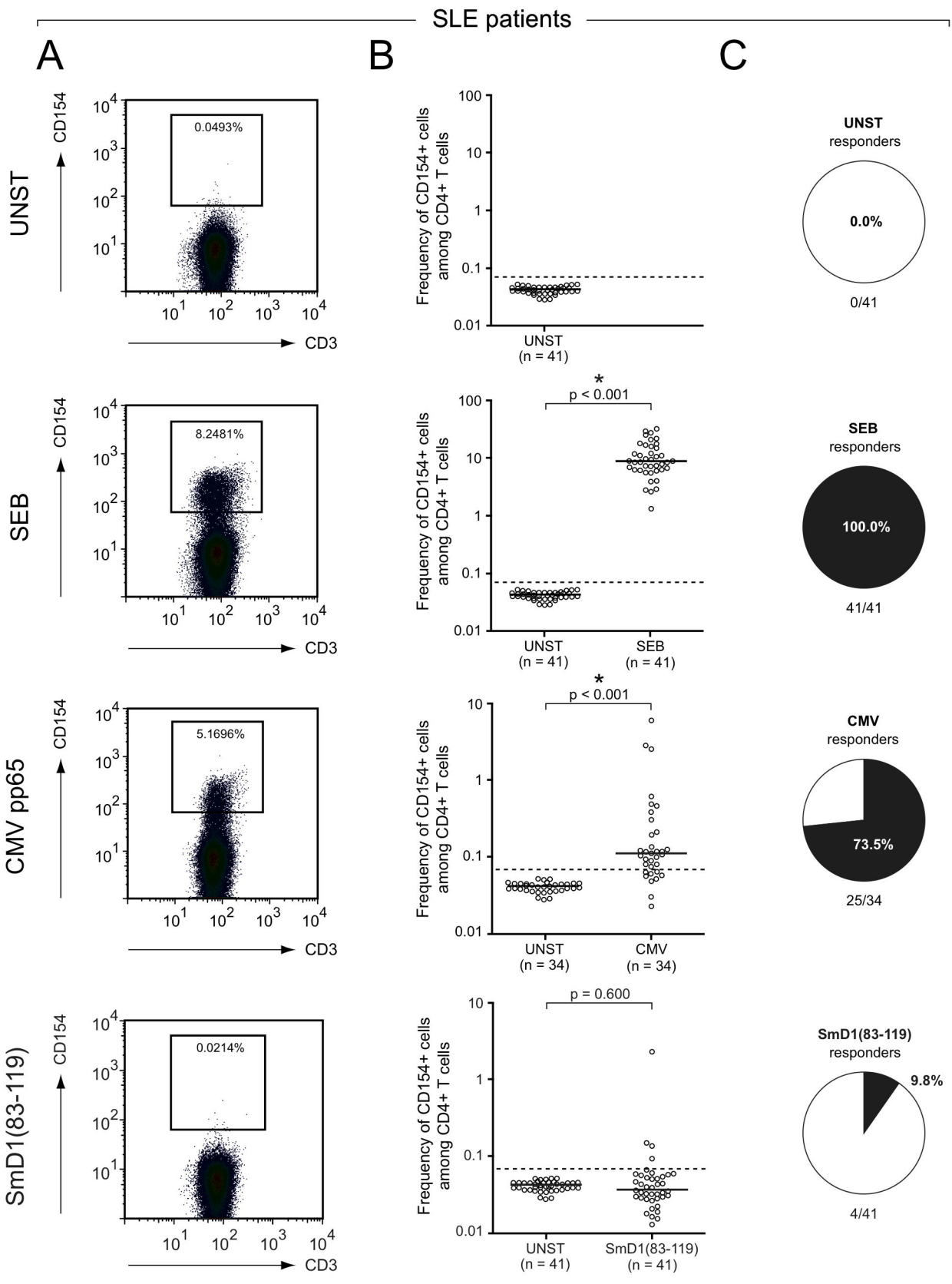
FIGURE LEGENDS

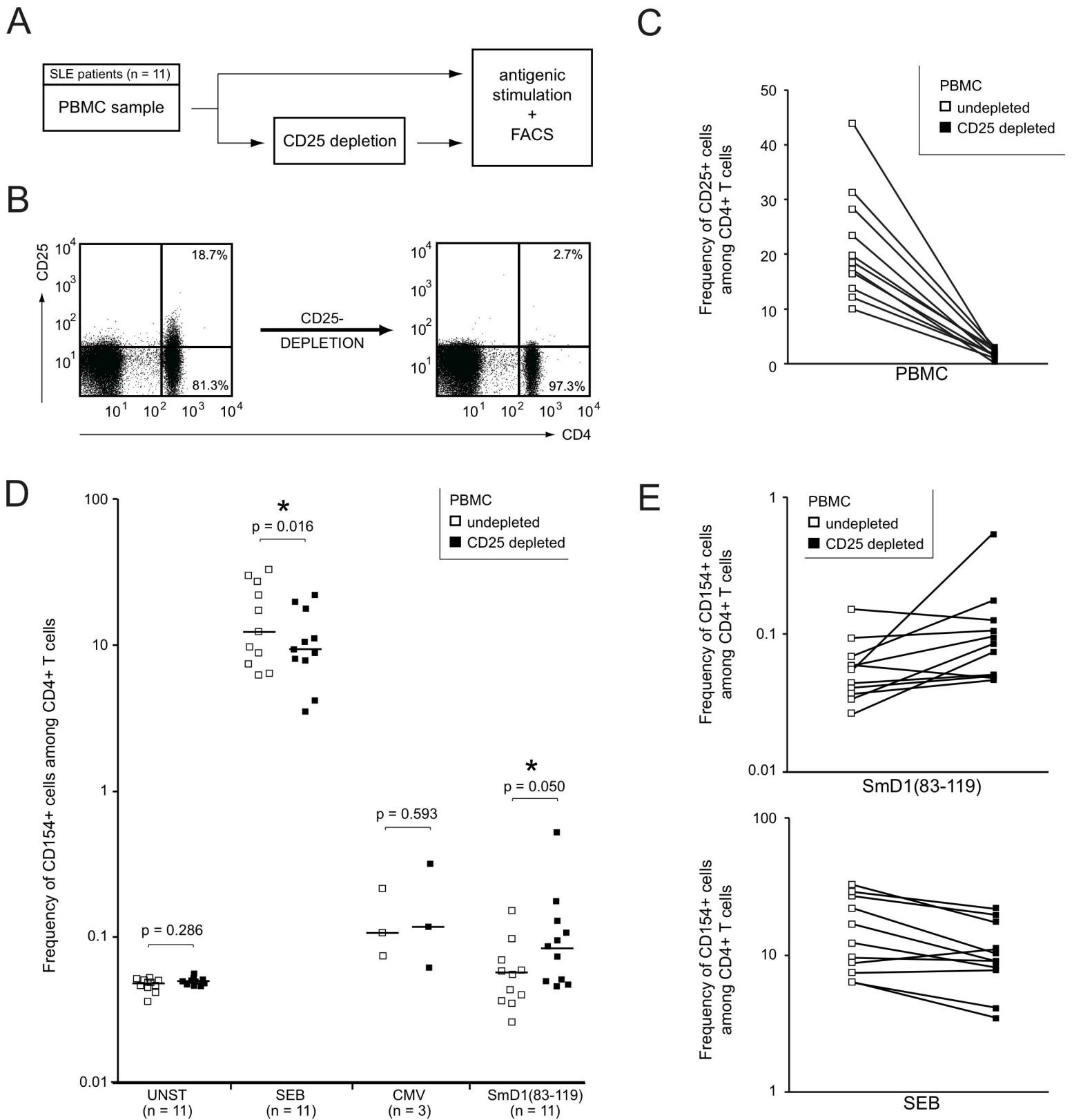
Figure 1: Antigen-specific $CD4^+$ T cell activation measured by CD154 expression.
PBMC isolated from 41 SLE blood samples were stimulated for 6h with one of the following antigens: staphylococcal superantigen B (SEB), cytomegalovirus pp65 peptide mix (CMV) or SmD1(83-119). Unstimulated samples (UNST) were cultivated in the absence of stimulatory antigens as negative controls. (A) The frequency of antigen-specific $CD4^+$ T cells was determined by flow cytometry according to antigen-induced intracellular CD154 expression on $CD3^+CD4^+$ T cells after stimulation with the indicated antigens (SLE patient #5 is shown exemplarily). (B) Wilcoxon signed-rank test was performed to test for significant differences between the antigen-stimulated samples and unstimulated control samples (UNST). Dotted lines indicate the threshold for the discrimination between responders and non-responders defined at 0.07% (unstimulated mean + 4SD). (C) The percentage of samples showing a positive $CD4^+$ T cell response to the indicated antigen is shown.

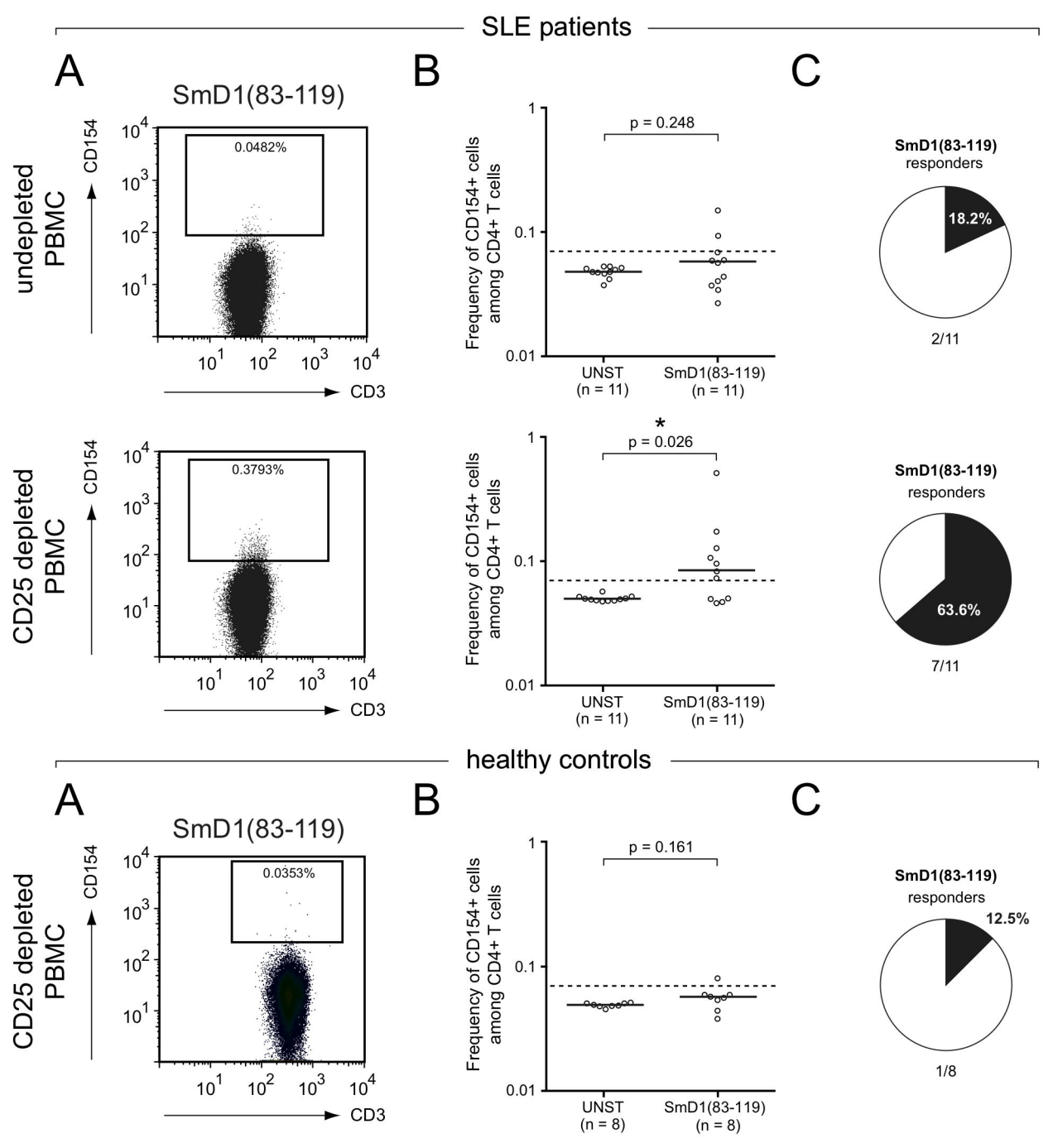
Figure 2: Influence of $CD25^+$ Treg on antigen-specific $CD4^+$ T cell responses.
(A) PBMC were isolated from 11 SLE blood samples and each PBMC sample was subdivided. In one PBMC subset $CD25^+$ Treg were removed by MACS prior to antigen-specific stimulation, while a second control subset remained undepleted. After this, antigenic stimulation and FACS analysis were performed in both subsets. (B) The depletion effectiveness was confirmed by measuring the reduction of $CD25^+$ cells among $CD4^+$ cells. Numeric values in the two exemplary dot plots are indicating the frequency of $CD25^+$ and $CD25^-$ cells among $CD4^+$ cells. (C) The reduction of $CD25^+$ cells among $CD4^+$ cells is shown for every individual sample. (D) Antigen-specific stimulation was performed over 6h with one of the following antigens: staphylococcal superantigen B (SEB), cytomegalovirus pp65 peptide mix (CMV) or SmD1(83-119). The frequency of antigen-specific $CD4^+$ T cells was determined by flow cytometry according to antigen-induced intracellular CD154 expression on $CD3^+CD4^+$ T cells after stimulation with the indicated antigens. Wilcoxon signed-rank test was performed to test for significant differences between the stimulation results of CD25 depleted and undepleted PBMC. (E) The effect of CD25 depletion on the T cell response to SEB and SmD1(83-119) is shown for every individual sample.

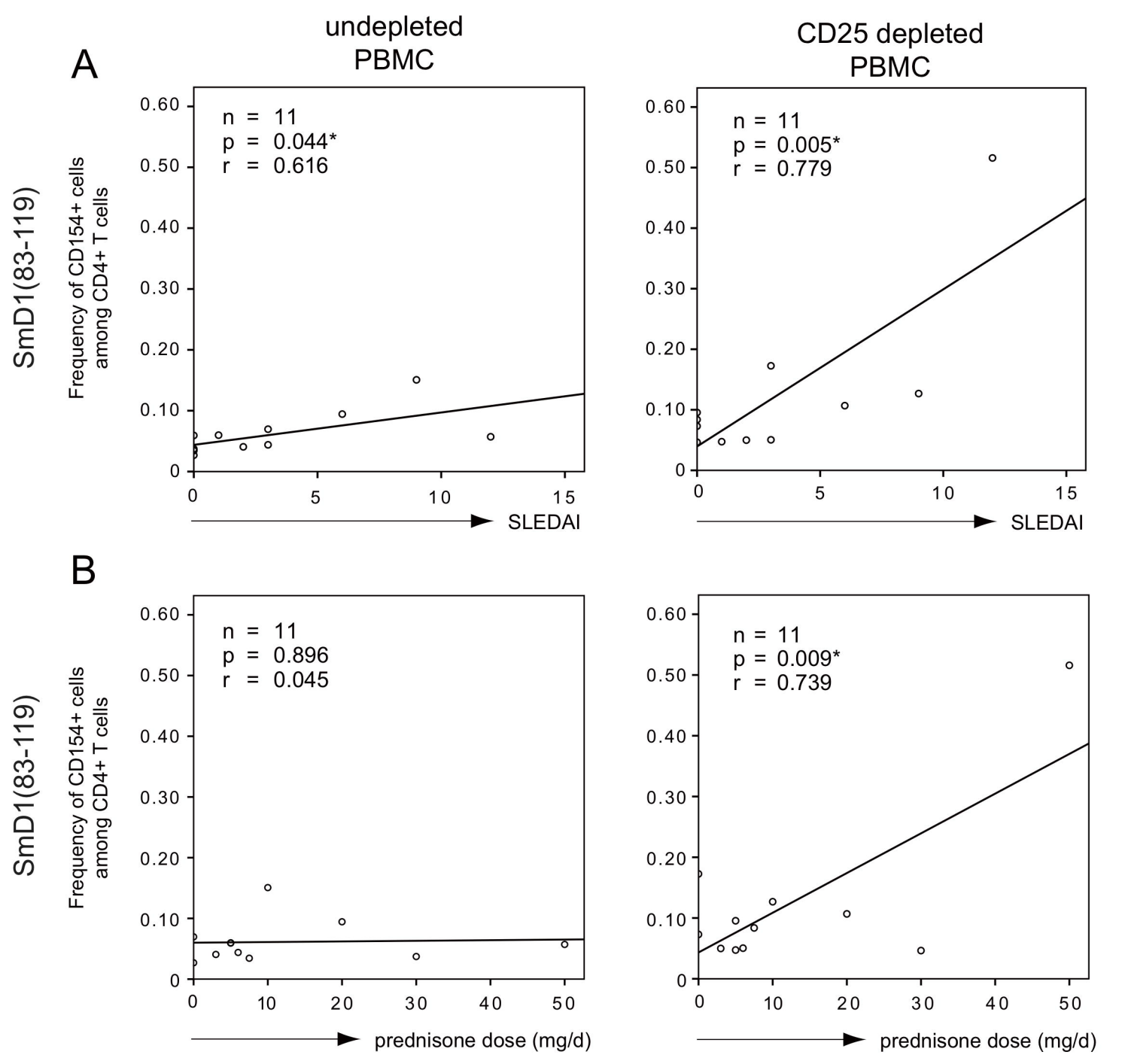
Figure 3: Unmasking of autoreactive SmD1(83-119)-specific $CD4^+$ T cell response by depletion of $CD25^+$ Treg.
PBMC were isolated from 11 SLE blood samples and each PBMC sample was subdivided. In one PBMC subset $CD25^+$ Treg were removed prior to antigen-specific stimulation, while a second control subset remained undepleted. After this, a stimulation with SmD1(83-119) was performed over 6h. Unstimulated samples (UNST) were cultivated in the absence of stimulatory antigens as negative controls. (A) The frequency of SmD1(83-119)-specific $CD4^+$ T cells was determined by flow cytometry according to antigen-induced intracellular CD154 expression on $CD3^+CD4^+$ T cells (SLE patient #108 and healthy control #905 are shown exemplarily). (B) Wilcoxon signed-rank test was performed to test for significant differences between the SmD1(83-119)-stimulated samples and unstimulated control samples (UNST). Dotted lines indicate the threshold for the discrimination between responders and non-responders defined at 0.07% (unstimulated mean + 4SD). (C) The percentage of samples showing a positive $CD4^+$ T cell response to SmD1(83-119) is shown.

Figure 4: Correlations with the SmD1(83-119)-specific $CD4^+$ T cell response.
(A) Linear correlations of the disease activity as assessed by SLEDAI and the detected autoreactive $CD4^+$ T cell response to SmD1(83-119) in undepleted PBMC and $CD25^+$ Treg depleted PBMC. (B) Linear correlations of the daily prednisone dose and the detected autoreactive $CD4^+$ T cell response to SmD1(83-119) in undepleted PBMC and $CD25^+$ Treg depleted PBMC.


SLE patients
A
B
C
UNST
SEB
CMV pp65
SmD1(83-119)
CD154
CD3
0.0493%
8.2481%
5.1696%
0.0214%
Frequency of CD154+ cells among CD4+ T cells
UNST (n = 41)
SEB (n = 41)
CMV (n = 34)
SmD1(83-119) (n = 41)
p < 0.001
p = 0.600
UNST responders
0.0%
0/41
SEB responders
100.0%
41/41
CMV responders
73.5%
25/34
SmD1(83-119) responders
9.8%
4/41


A
SLE patients (n = 11)
PBMC sample
CD25 depletion
antigenic stimulation + FACS
B
CD25
18.7%
81.3%
CD25- DEPLETION
2.7%
97.3%
CD4
C
PBMC
undepleted
CD25 depleted
Frequency of CD25+ cells among CD4+ T cells
PBMC
D
PBMC
undepleted
CD25 depleted
Frequency of CD154+ cells among CD4+ T cells
p = 0.016
p = 0.286
p = 0.593
p = 0.050
UNST (n = 11)
SEB (n = 11)
CMV (n = 3)
SmD1(83-119) (n = 11)
E
PBMC
undepleted
CD25 depleted
Frequency of CD154+ cells among CD4+ T cells
SmD1(83-119)
SEB


SLE patients
A
SmD1(83-119)
undepleted PBMC
CD154
CD3
0.0482%
B
Frequency of CD154+ cells among CD4+ T cells
p = 0.248
UNST (n = 11)
SmD1(83-119) (n = 11)
C
SmD1(83-119) responders
18.2%
2/11
CD25 depleted PBMC
0.3793%
*
p = 0.026
63.6%
7/11
healthy controls
0.0353%
p = 0.161
UNST (n = 8)
SmD1(83-119) (n = 8)
12.5%
1/8


A
undepleted PBMC
CD25 depleted PBMC
SmD1(83-119)
Frequency of CD154+ cells among CD4+ T cells
n = 11
p = 0.044*
r = 0.616
n = 11
p = 0.005*
r = 0.779
SLEDAI
B
SmD1(83-119)
Frequency of CD154+ cells among CD4+ T cells
n = 11
p = 0.896
r = 0.045
n = 11
p = 0.009*
r = 0.739
prednisone dose (mg/d)