

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

Jan Broder Engler,¹ Reinmar Undeutsch,¹ Lutz Kloke,¹ Stefan Rosenberger,¹ Marina Backhaus,¹ Udo Schneider,¹ Karl Egerer,¹ Duska Dragun,² Jörg Hofmann,³ Dörte Huscher,^{1, 4} Gerd-Rüdiger Burmester,¹ Jens Y Humrich,¹ Philipp Enghard,² Gabriela Riemekasten¹

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¹Department of Rheumatology and Clinical Immunology, University Hospital Charité, Berlin, Germany

²Clinic for Nephrology and Intensive Care Medicine, University Hospital Charité, Berlin, Germany

³Institute of Virology, University Hospital Charité, Berlin, Germany

⁴Department of Epidemiology, German Rheumatism Research Center, a Leibniz Institute, Berlin, Germany

Correspondence to

Professor Gabriela Riemekasten, Klinik für Rheumatologie und Klinische Immunologie, Charité Campus Mitte, Charitéplatz 1, 10117 Berlin, Germany; gabriela.riemekasten@charite.de

P. E- and G- R- contributed equally to the study.

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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 peripheral blood. The authors assess autoreactive CD4 T cells in a representative SLE collective (n=38) 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 6-h 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, CD25 Treg were depleted by magnetic activated cell sorting before 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 the SLE disease activity index ($p=0.005^*$, $r=0.779$).

Conclusions These 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.

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 approximately one-third of the SLE patients reveal CD4 T-cell 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, emphasising their involvement in the pathogenesis of SLE.⁵

However, direct access to these cells is restricted due to their low frequency in peripheral blood, causing difficulties in their reliable detection.⁶ The measurement of [³H]thymidine incorporation is a classic approach to determine cell proliferation after antigenic stimulation. Its specificity is limited, though. As 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 peripheral blood mononuclear cells (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 detection is restricted to T-cell subsets with defined cytokine profiles.¹⁰ Finally, the use of peptide major histocompatibility complex (MHC) multimers, currently the most specific method, is strongly limited because only a few peptide MHC constructs are available so far.¹¹⁻¹⁴

To address this problem, Frentsch *et al*¹⁰ introduced CD154, also known as CD40L, as a marker molecule of CD4 T cells that have recently been activated upon antigen-specific short-term in-vitro stimulation. 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 hypothesised 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 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 before antigenic stimulation.

METHODS

Patients

Collections of human PBMC were approved by the Human Research Ethic Committee. We obtained 41 blood samples of 38 consecutive SLE patients (table 1 and supplementary table 1, available online only) 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 were collected in vacuum collection tubes (Becton Dickinson, Franklin Lakes, New Jersey, 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

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 phosphate-buffered saline (PBS) containing 0.5% bovine serum

albumin (BSA). In selected experiments a depletion of CD25 Treg was performed before 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 Roswell Park Memorial Institute 1640 medium (Invitrogen, Paisley, UK) in a cell density of 1–2×10⁶/ml. Stimulations were performed in the presence of 10 µg/ml CD28-specific antibody (BD Biosciences, San Jose, California, USA) for 6 h at 37°C using one of the following antigens: 1 µg/ml staphylococcal enterotoxin B (SEB; Sigma-Aldrich, St Louis, Missouri, USA), 20 µl/ml cytomegalovirus peptide mix pp65 (Miltenyi Biotec) or 20 µg/ml SmD1(83-119) (VEPKVKSKKREAVAGRGRGRGRGRGRGRGGPRR), synthesised 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 4 h of stimulation. After completing the stimulation time, PBMC were fixed for 15 min at 21°C in PBS/BSA containing 0.05% sodium azide and 2% paraformaldehyde, washed twice in PBS/BSA/sodium azide and stored at 4°C.

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/days | 24 | 63.1% | | |
| | Corticosteroids > 10 mg/days | 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.

CNS, central nervous system; SLE, systemic lupus erythematosus; SLEDAI, systemic lupus erythematosus disease activity index.

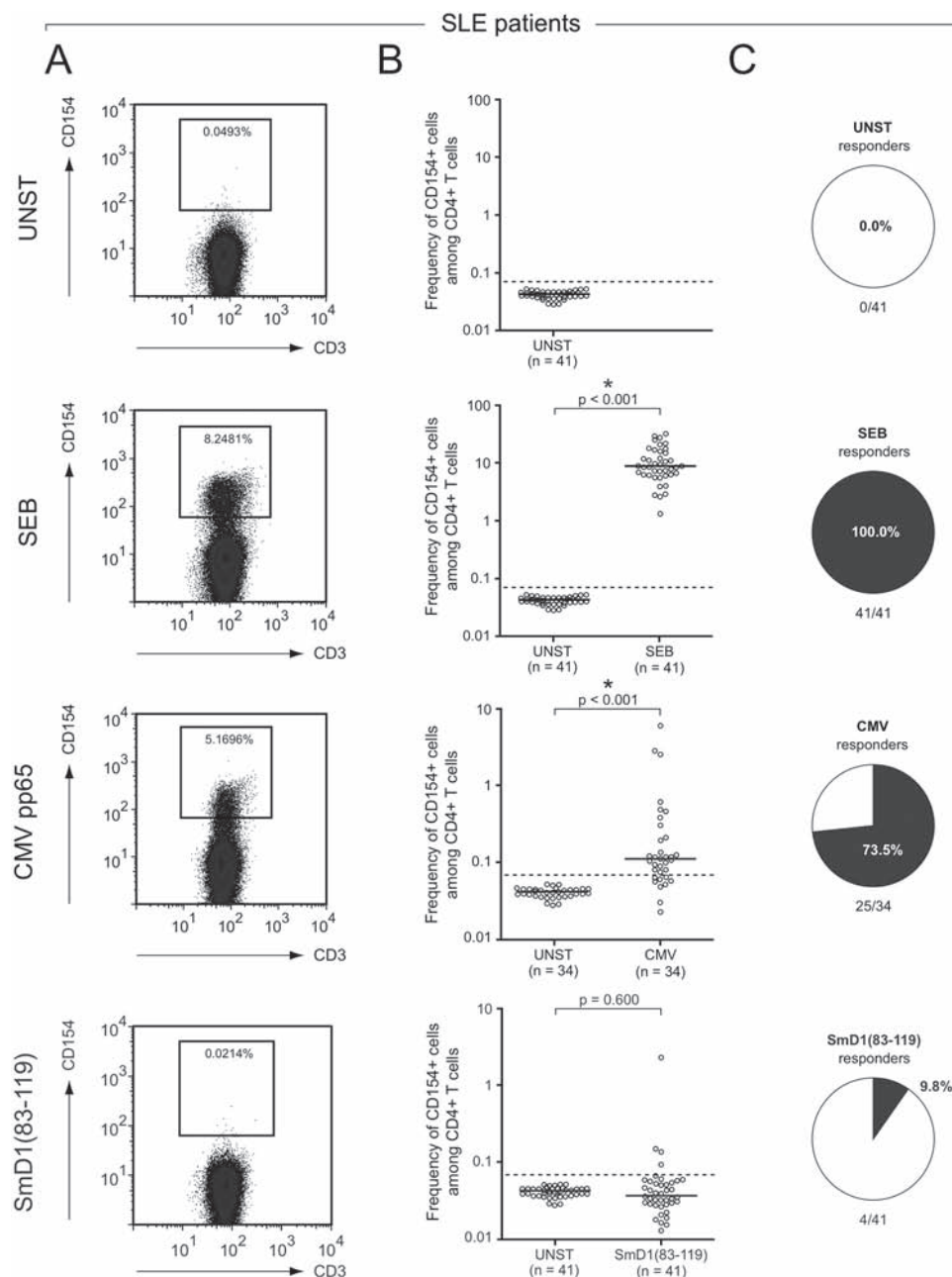


Figure 1 Antigen-specific CD4 T-cell activation measured by CD154 expression. Peripheral blood mononuclear cells isolated from 41 systemic lupus erythematosus (SLE) blood samples were stimulated for 6 h with one of the following antigens: staphylococcal superantigen B (SEB), cytomegalovirus pp65 peptide 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 CD3CD4 T cells after stimulation with the indicated antigens (SLE patient no 5 is shown exemplarily). (B) The 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 + 4 SD). (C) The percentage of samples showing a positive CD4 T-cell response to the indicated antigen is shown.

Flow cytometry analysis

Fixed cells were permeabilised in PBS/BSA/sodium azide containing 0.5% saponin (Sigma-Aldrich) and stained for 15 min at 4°C using a selection of the following specific antibodies: fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (UCHT-1), phycoerythrin-conjugated anti-CD3 (UCHT-1), phycoerythrin-conjugated anti-CD4 (TT1), biotin-conjugated anti-CD4 (TT1), FITC-conjugated anti-CD25 (BC96), allophycocyanin-conjugated anti-CD25 (4E3), allophycocyanin-conjugated anti-CD154 (5C8) and peridinin chlorophyll protein complex-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 Flowjo 7.2.5 software (Tree Star, Ashland, Oregon, USA). The frequency of antigen-specific CD3CD4 T cells was determined by antigen-induced intracellular CD154 expression. Mean background activity in unstimulated controls

was 0.0429% CD154 cells among CD3CD4 T cells with a SD of 0.062%. Samples exceeding this mean by 4 SD or more were considered positive (threshold 0.07%).

Statistical analysis

Statistical analysis was conducted using the software SPSS 18. 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 Pearson's correlation coefficient was used.

RESULTS

CD154 expression reliably detects infectious CD4 T-cell recall responses but not autoreactive CD4 T-cell activation

Forty-one PBMC samples of 38 SLE patients were stimulated with either the superantigen SEB, the cytomegalovirus peptide mix pp65, 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 CD3CD4 T cells. Sample results exceeding the unstimulated mean by 4 SD (equalling 0.07% or higher) were considered positive.

As expected, the stimulation with SEB resulted in a high frequency of CD154 cells among CD3CD4 T cells (median 8.5991%). Compared with the unstimulated controls (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 cytomegalovirus recall responses also significantly exceeded the unstimulated controls ($p < 0.001$). A positive response was observed in 25 of 34 cytomegalovirus-stimulated samples (73.5%) (figure 1).

In contrast, stimulation with the autoantigen SmD1(83-119) elicited no specific CD4 T-cell response compared with the unstimulated controls in this experimental setting (median 0.0428 vs 0.0372%, $p = 0.600$). Only four of 41 samples (9.8%) stimulated with SmD1(83-119) reached the threshold level of 0.07% CD154 cells among CD3CD4 T cells (figure 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 magnetic-activated cell sorting (MACS) before 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 (figure 2A–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 cytomegalovirus-specific CD4 T cells by 24.9%, however, was not significant because of the small sample number ($n = 3$, $p = 0.593$) (figure 2D,E). In order to guarantee a constant cell density in SEB, UNST and SmD1(83-119) stimulations, we had to omit cytomegalovirus 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. In addition, the percentage of SLE patients who showed a positive CD4 T-cell response to SmD1(83-119) increased from 18.2% to 63.6% (figure 3).

To determine whether this unmasked SmD1(83-119)-specific CD4 T-cell response may also be present in healthy individuals, we tested eight 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 one of eight 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 (figure 4A and table 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.

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 with 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 cytomegalovirus was highly significant and led to a responder rate of 73.5%, which matches the reported cytomegalovirus 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 hypothesised that the presence of Treg may impair autoreactive CD4 T-cell responses during short-term stimulation with autoantigens. We therefore eliminated CD25 Treg by MACS before antigenic stimulation in 11 PBMC samples and compared the achieved stimulation results in the presence and absence of Treg. We addressed CD25, the α -chain of the interleukin (IL)-2 receptor, because there are still no convincing alternatives available for depleting human Treg by MACS. Despite the great 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 as Foxp3⁺ T cells that lack suppressive capacity have been reported in humans.²² In any case, intracellular Foxp3 is not applicable for surface staining. Consequently, we focused on CD25 to remove Treg, accepting the potential loss of preactivated CD25 effector T cells.

Extended report

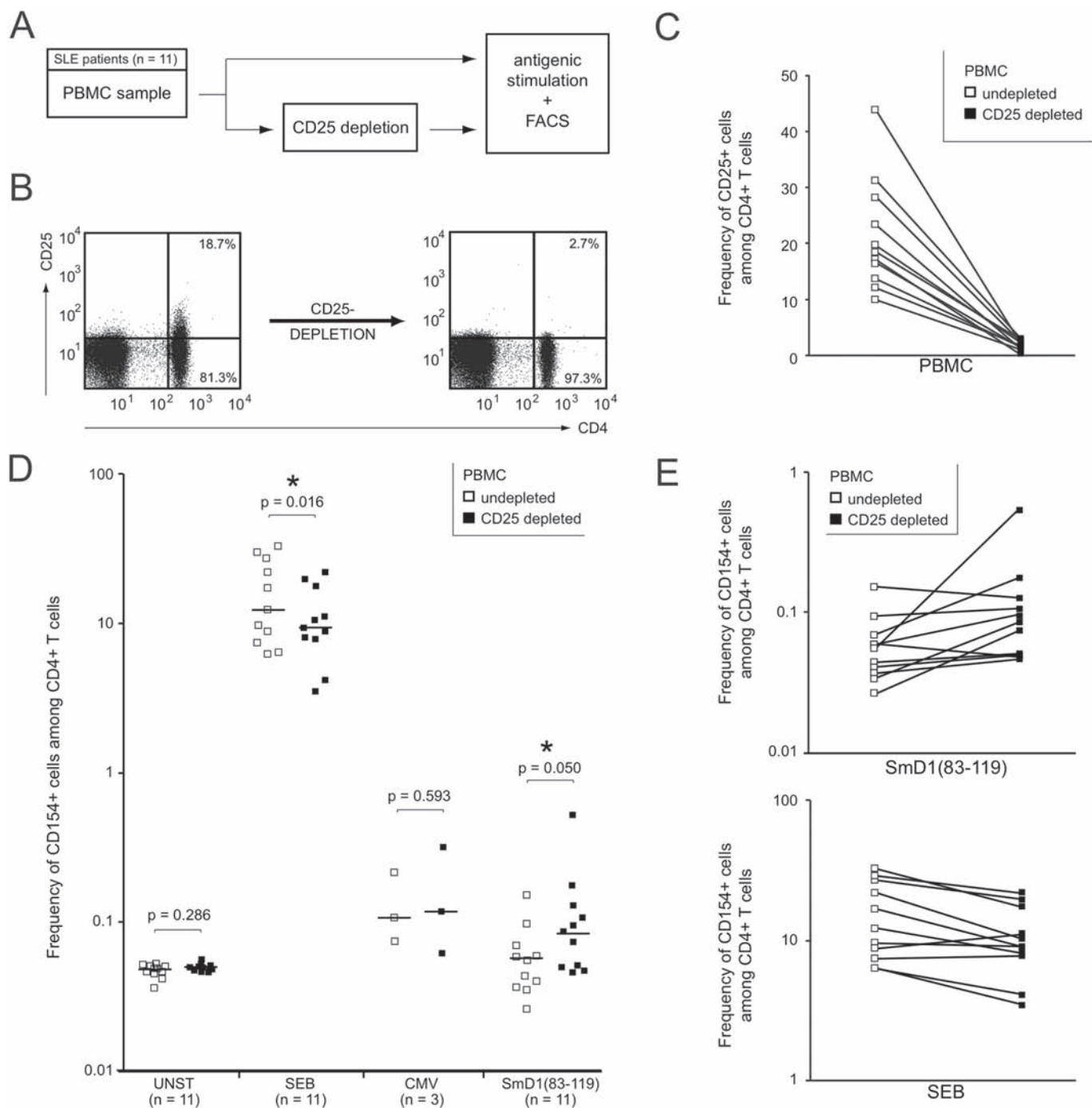


Figure 2 Influence of CD25 regulatory T cells (Treg) on antigen-specific CD4 T-cell responses. (A) Peripheral blood mononuclear cells (PBMC) were isolated from 11 systemic lupus erythematosus blood samples and each PBMC sample was subdivided. In one PBMC subset CD25 Treg were removed by magnetic-activated cell sorting before antigen-specific stimulation, while a second control subset remained undepleted. After this, antigenic stimulation and fluorescence-activated cell sorting (FACS) analysis were performed in both subsets. (B) The depletion effectiveness was confirmed by measuring the reduction in CD25 cells among CD4 cells. Numeric values in the two exemplary dot plots indicate the frequency of CD25⁺ and CD25⁻ cells among CD4 cells. (C) The reduction in CD25 cells among CD4 cells is shown for every individual sample. (D) Antigen-specific stimulation was performed over 6 h 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 CD3CD4 T cells after stimulation with the indicated antigens. The 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. SLE, systemic lupus erythematosus; UNST, unstimulated control samples.

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 non-depleted controls. The increase in the cytomegalovirus 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 Epstein-Barr virus in lupus pathogenesis.^{24 25} In contrast, SEB responses decreased by

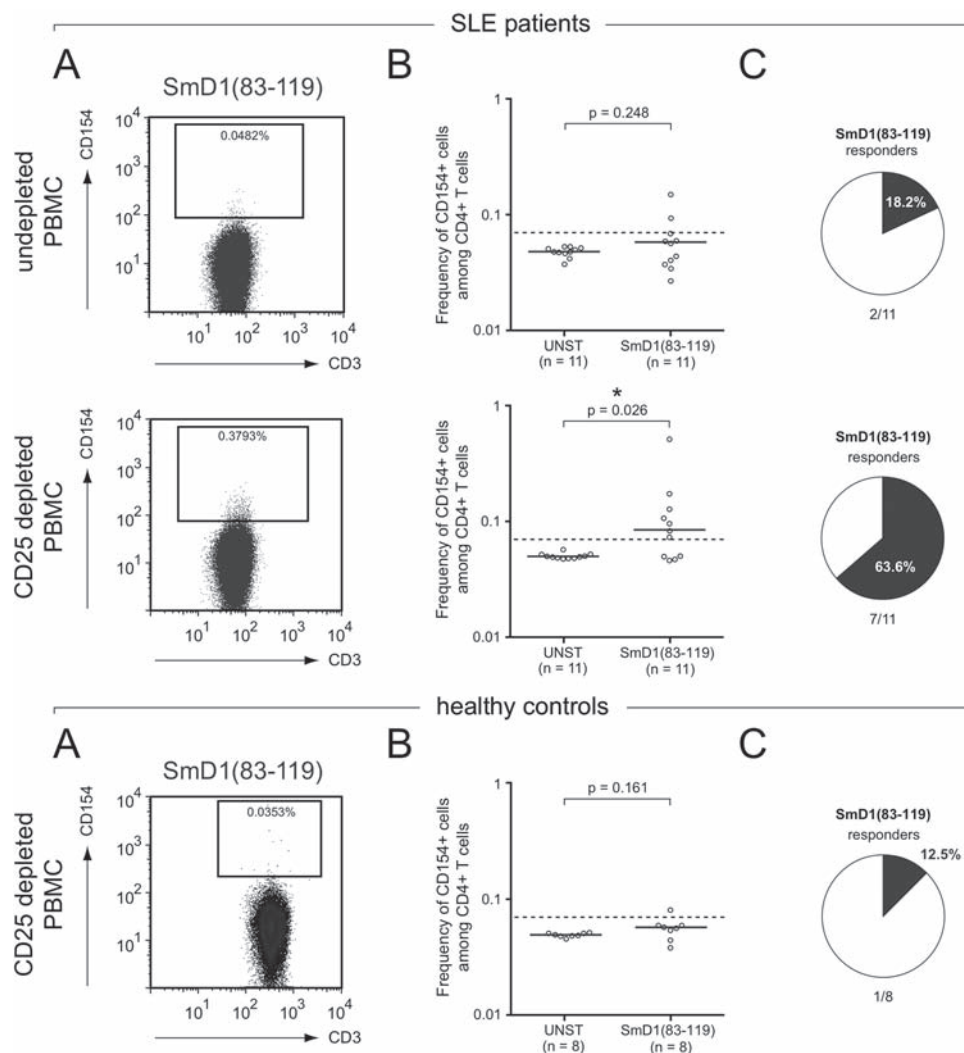


Figure 3 Unmasking of autoreactive SmD1(83-119)-specific CD4 T-cell response by depletion of CD25 regulatory T cells (Treg). Peripheral blood mononuclear cells (PBMC) were isolated from 11 systemic lupus erythematosus (SLE) blood samples and each PBMC sample was subdivided. In one PBMC subset CD25 Treg were removed before antigen-specific stimulation, while a second control subset remained undepleted. After this, a stimulation with SmD1(83-119) was performed over 6 h. 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 CD3CD4 T cells (SLE patient no 108 and healthy control no 905 are shown exemplarily). (B) The Wilcoxon signed-rank test was performed to test for significant differences between the SmD1(83-119)-stimulated samples and UNST. Dotted lines indicate the threshold for the discrimination between responders and non-responders defined at 0.07% (unstimulated mean + 4 SD). (C) The percentage of samples showing a positive CD4 T-cell response to SmD1(83-119) is shown.

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 figure 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) increased 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.

Unquestionably, there are some limitations of this study. First, the detected frequencies of SmD1(83-119)-specific CD4 T cells are still very low. To detect these small differences reliably we measured three probes per stimulation antigen and calculated the means. While setting the CD154 gate we tolerated approximately 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 did not exactly match 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 eliminate the majority of Treg effectively CD25 depletion is

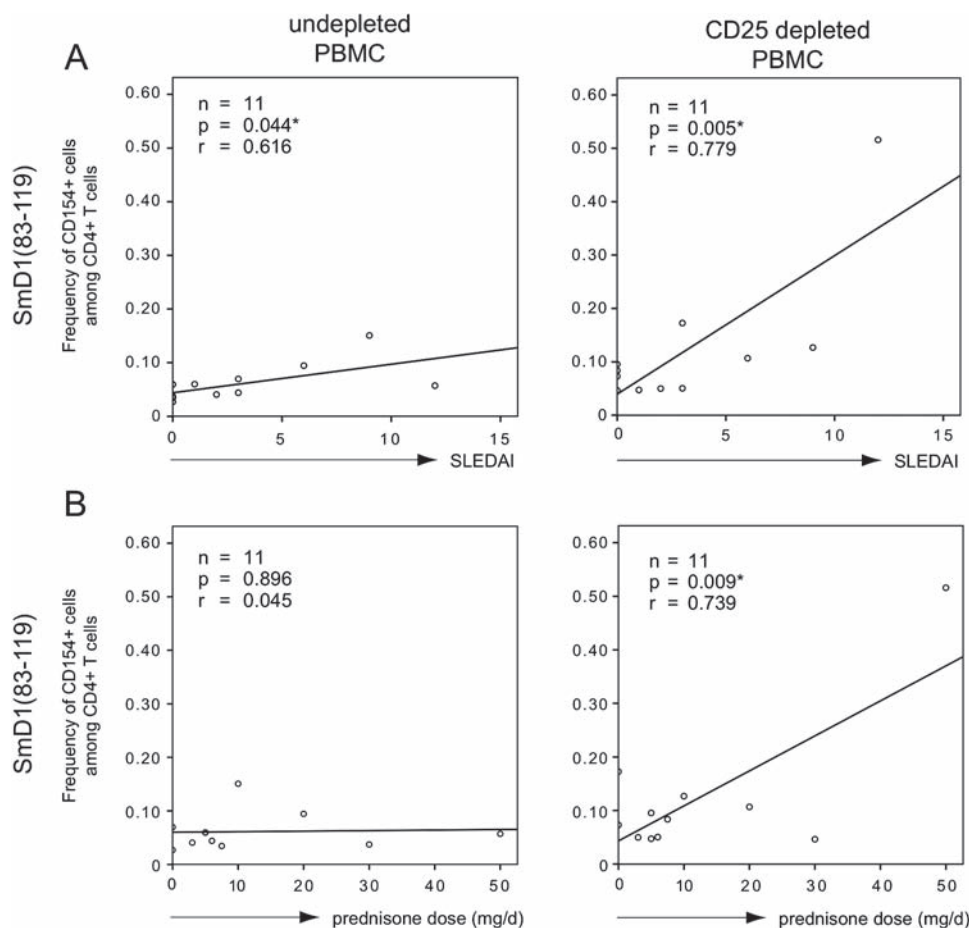


Figure 4 Correlations with the Smd1(83-119)-specific CD4 T-cell response. (A) Linear correlations of the disease activity as assessed by the systemic lupus erythematosus disease activity index (SLEDAI) and the detected autoreactive CD4 T-cell response to Smd1(83-119) in undepleted peripheral blood mononuclear cells (PBMC) and CD25 regulatory T cells (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.

still a practical approach.^{26 27} This is further underlined by our recent studies showing that CD25 depletion effectively aggravates disease progression in 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 large blood probes needed from SLE patients with predominantly low lymphocyte counts. 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 (figure 4B). However, this observation might simply be caused by the fact that active patients are characterised by both elevated prednisone doses and increased unmasked autoreactive Smd1(83-119) responses (table 2). Beyond that, no further connections between administered medication and Smd1(83-119) response were observed (see supplementary table 1, available online only).

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 suppress these autoreactive cells effectively in our in-vitro stimulations but not in patients with SLE. One possible explanation could be a predominance of Treg inhibiting mediators such as IL-6, which is detectable at high levels in the sera of SLE patients and was recently shown to impede Treg differentiation and promote autoreactive T-helper 17 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 30-32} 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 unmask efficiently 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

Table 2 Correlations

| SLEDAI | n | p Value | r |
|----------------------------------------------------|----|----------|--------|
| Anti-SmD1 antibodies | 40 | 0.674 | -0.069 |
| Anti-nucleosome antibodies | 40 | 0.003* | 0.452 |
| Anti-nuclear antibodies | 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 | 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 | 5 | 0.077 | -0.837 |
| Anti-dsDNA antibodies | 4 | 0.820 | -0.180 |
| Daily steroid dose | 11 | 0.009* | 0.739 |

PBMC, peripheral blood mononuclear cell; SLEDAI, systemic lupus erythematosus disease activity index.

presented in this work, provides a promising approach to auto-reactive CD4 T cells that may also be valuable in other autoimmune diseases.

Contributors The authors PE and GR contributed equally to this work.

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Patient consent We obtained informed consent regarding the blood collection.

Ethics approval This study was conducted with the approval of the Human Research Ethics Committee (EA1/098/07).

Provenance and peer review Not commissioned; externally peer reviewed.

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

Jan Broder Engler, Reinmar Undeutsch, Lutz Kloke, et al.

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