Depletion of autoreactive immunologic memory followed by autologous hematopoietic stem cell transplantation in patients with refractory SLE induces long-term remission through de novo generation of a juvenile and tolerant immune system

*Tobias Alexander,1,2 *Andreas Thiel,1,3 Oliver Rosen,4 Gero Massenkeil,4 Arne Sattler,1 Siegfried Kohler,1 Henrik Mei,1,5 Hartmut Radtke,2 Erika Gromnica-Ihle,6 Gerd-Rüdiger Burmester,2 †Renate Arnold,4 †Andreas Radbruch,1 and †Falk Hiepe1,2

1German Rheumatism Research Center, Berlin; 2Department of Rheumatology and Clinical Immunology, 3Regenerative Immunology and Aging, Berlin-Brandenburg Center for Regenerative Therapies, 4Department of Hematology and Oncology and 5Institute for Transfusion Medicine, Charité Universitätsmedizin Berlin, Berlin; and 6Rheuma-Klinik Berlin-Buch, Berlin, Germany

Clinical trials have indicated that immunoablation followed by autologous hematopoietic stem cell transplantation (ASCT) has the potential to induce clinical remission in patients with refractory systemic lupus erythematosus (SLE), but the mechanisms have remained unclear. We now report the results of a single-center prospective study of long-term immune reconstitution after ASCT in 7 patients with SLE. The clinical remissions observed in these patients are accompanied by the depletion of autoreactive immunologic memory, reflected by the disappearance of pathogenic anti–double-stranded DNA (dsDNA) antibodies and protective antibodies in serum and a fundamental resetting of the adaptive immune system. The latter comprises recurrence of CD31+CD45RA+CD4+ T cells (recent thymic emigrants) with a doubling in absolute numbers compared with age-matched healthy controls at the 3-year follow-up (P = .016), the regeneration of thymic-derived FoxP3+ regulatory T cells, and normalization of peripheral T-cell receptor (TCR) repertoire usage. Likewise, responders exhibited normalization of the previously disturbed B-cell homeostasis with numeric recovery of the naive B-cell compartment within 1 year after ASCT. These data are the first to demonstrate that both depletion of the autoreactive immunologic memory and a profound resetting of the adaptive immune system are required to reestablish self-tolerance in SLE. This trial was registered at www.clinicaltrials.gov as NCT00742300. (Blood. 2009;113:214-223)

Introduction

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with heterogeneous clinical manifestations. It is characterized by the generation of pathogenic antibodies directed against a variety of autoantigens, including nuclear and cytoplasmic antigens, such as double-stranded DNA (dsDNA), nucleosomes, and by complement activation. It is thought that, in genetically susceptible persons, an initial breakdown of peripheral tolerance permits the activation of autoreactive lymphocytes, which then propagate autoimmune responses in a self-perpetuating process. We recently demonstrated that autoimmune reactions in susceptible persons, an initial breakdown of peripheral tolerance permits the activation of autoreactive lymphocytes, which then propagate autoimmune responses in a self-perpetuating process. We now report the results of a single-center prospective study of long-term immune reconstitution after ASCT in 7 patients with SLE. The clinical remissions observed in these patients are accompanied by the depletion of autoreactive immunologic memory, reflected by the disappearance of pathogenic anti–double-stranded DNA (dsDNA) antibodies and protective antibodies in serum and a fundamental resetting of the adaptive immune system. The latter comprises recurrence of CD31+CD45RA+CD4+ T cells (recent thymic emigrants) with a doubling in absolute numbers compared with age-matched healthy controls at the 3-year follow-up (P = .016), the regeneration of thymic-derived FoxP3+ regulatory T cells, and normalization of peripheral T-cell receptor (TCR) repertoire usage. Likewise, responders exhibited normalization of the previously disturbed B-cell homeostasis with numeric recovery of the naive B-cell compartment within 1 year after ASCT. These data are the first to demonstrate that both depletion of the autoreactive immunologic memory and a profound resetting of the adaptive immune system are required to reestablish self-tolerance in SLE. This trial was registered at www.clinicaltrials.gov as NCT00742300. (Blood. 2009;113:214-223)

*T.A. and A.T. contributed equally to this work.
†R.A., A.R., and F.H. (all senior authors) contributed equally to this work.
blood stem cells were collected by leukapheresis after infusion of 2.0 g/m² cyclophosphamide infusion. The graft was enriched for CD34

Table 2. Demographic data and clinical features of patients

Patient no. | Sex/age, y | Clinical manifestation | SLEDAI pretransplantation | Therapy pretransplantation | Follow-up, mo | Clinical outcome | Current therapy |
---|---|---|---|---|---|---|---|---|
1 | F/27 | Nephritis, abdominal vasculitis, polyserositis, APS, cytopenia | 25 | CY, AZA, HCQ, CSA, MMF | 96 | Clinical remission | 2 mg prednisolone |
2 | F/48 | Nephritis, peripheral neuropathy, polyserositis, ventricular arrhythmia | 23 | CY, AZA, HCQ, MTX, MMF | 96 | Clinical remission | None |
3 | M/37 | Nephritis WHO IV, ventricular arrhythmia, polyserositis, APS, cytopenia | 30 | CY, AZA, HCQ, CSA, MTX, MMF | 38 | Relapse 18+ mo, exitus letalis 38+ mo | — |
4 | F/24 | Nephritis WHO V, seizures, psychosis, polyserositis, APS, cytopenia | 28 | CY, AZA, CSA | 72 | Clinical remission | None |
5 | F/31 | Nephritis WHO II, seizures, psychosis, APS, cytopenia | 26 | CY, AZA, MTX | 3 | Exitus letalis 3+ mo | — |
6 | F/30 | Nephritis WHO Ila, APS, cytopenia | 23 | CY, AZA, MTX, HQC | 48 | Clinical remission | 4 mg prednisolone |
7 | F/19 | Nephritis, cerebritis, APS cytopenia | 19 | CY, AZA, MTX, HQC | 24 | Clinical remission | 4 mg prednisolone |

Flow cytometry

Absolute CD4+ and CD19+ lymphocyte numbers were calculated based on the total lymphocyte count and the percentage of CD4+ and CD19+ cells, as identified by flow cytometry using the BD Multitest panel (BD Biosciences, San Jose, CA). The following monoclonal antibodies (mAbs) were used for phenotypic analyses: anti-CD19-peridinin chlorophyll protein (Cy5.5 (S123C1)), anti-CD20-phycocerythrin (PE; 2H7), and anti-IgD-fluorescein isothiocyanate (FITC; IA6-2), anti-CD4-peridinin chlorophyll protein Cy5.5 (SK3), anti-CD31-PE (MEC13.3), anti-CD45RA-FITC (L48), and anti-CD45RO-allophycocyanin (APC; UCHL1-1), all obtained from BD Biosciences. Anti-CD27-Cy5 (2E4) was conjugated to Cy5 (GE Healthcare) according to the manufacturer’s instructions. Immunofluorescence staining was performed by incubating PBMCs in the presence of mAbs in 1% bovine serum albumin in phosphate-buffered saline on ice for 10 minutes after blocking with 10 μg of human IgG for 10 minutes. Cells were washed before analysis on a FACScalibur flow cytometer (BD Biosciences).

We performed analysis of T-cell receptor (TCR) Vβ expression on freshly isolated peripheral blood CD4+ T cells by 4-color flow cytometry using 22 TCR Vβ-specific monoclonal antibodies (OText Beta Mark; Beckman Coulter, Fullerton, CA) as described recently.13 TCR designations are according to Arden’s nomenclature.14 At least 2.5 × 10⁶ CD4+ T cells were acquired.

We performed analysis of T-cell receptor (TCR) Vβ expression on freshly isolated peripheral blood CD4+ T cells by 4-color flow cytometry using 22 TCR Vβ-specific monoclonal antibodies (OText Beta Mark; Beckman Coulter, Fullerton, CA) as described recently.13 TCR designations are according to Arden’s nomenclature.14 At least 2.5 × 10⁶ CD4+ T cells were acquired. Normal ranges were established for each TCR member based on confidence intervals (CIs) of 97.5% determined in 20 healthy persons.

Table 2. Demographic data and clinical features of patients

Patient no. | Sex/age, y | Clinical manifestation | SLEDAI pretransplantation | Therapy pretransplantation | Follow-up, mo | Clinical outcome | Current therapy |
---|---|---|---|---|---|---|---|---|
1 | F/27 |— | 25 | CY, AZA, HCQ, CSA, MMF | 96 | Clinical remission | 2 mg prednisolone |
2 | F/48 |— | 23 | CY, AZA, HCQ, MTX, MMF | 96 | Clinical remission | None |
3 | M/37 |— | 30 | CY, AZA, HCQ, CSA, MTX, MMF | 38 | Relapse 18+ mo, exitus letalis 38+ mo | — |
4 | F/24 |— | 28 | CY, AZA, CSA | 72 | Clinical remission | None |
5 | F/31 |— | 26 | CY, AZA, MTX | 3 | Exitus letalis 3+ mo | — |
6 | F/30 |— | 23 | CY, AZA, MTX, HQC | 48 | Clinical remission | 4 mg prednisolone |
7 | F/19 |— | 19 | CY, AZA, MTX, HQC | 24 | Clinical remission | 4 mg prednisolone |

APS indicates antiphospholipid syndrome; CY, cyclophosphamide; AZA, azathioprine; HCQ, hydroxychloroquine; CSA, cyclosporine; MTX, methotrexate; MMF, mycophenolate mofetil; and —, not applicable.
Perturbations of Vβ families were considered to be significant in patients when they were outside of these normal intervals.

Bone marrow mononuclear cells (BM-MNCs) were collected by bone marrow aspiration in 1 patient after ASCT and in 1 healthy volunteer from the femur after joint surgery. BM-MNCs were isolated by Ficoll-Hypaque density gradient centrifugation (GE Healthcare) and stained with anti-CD38-APC (HIT2; BD PharMingen, San Diego, CA) and anti–CD138-PE (B-B4; Chemicon International, Temecula, CA). Cells were washed before acquisition (LSR II flow cytometer; BD Biosciences) and analysis (FlowJo Software; TreeStar, San Carlos, CA).

Stimulation assays

For in vitro lymphocyte stimulation assays, 1 mL freshly collected heparinized peripheral blood was stimulated in the presence of 1 µg/mL CD28 (clone 28.2; BD PharMingen) for 6 hours at 37°C with the following antigens: 1 µg/mL Staphylococcus aureus enterotoxin B as the positive control (Sigma Chemie, Deisenhofen, Germany), 20 µg/mL nucleosomess, as described previously,15 10 µg/mL SmD1 peptide, as described previously,16 varicella zoster virus lysate and cytomegaly virus (CMV) lysate (both Biodesign International, Kennebunk, ME), Brefeldin A (Sigma Chemie) was added for the last 4 hours of stimulation. Erythrocytes were lysed with FACS lysing solution and permeabilized with FACS-Perm2 (both from BD PharMingen) according to the manufacturer’s instructions. Fixed cells were stained for 30 minutes at room temperature with the following antibodies: anti–CD154-PE (TRAP1) or anti–CD69-PE (FN-50), anti–CD4-PerCp Cy5.5 (RPA-T4), anti–CD14-FITC (MSE2) and anti–interferon gamma (IFN-γ)-APC (B27) (all purchased from BD PharMingen). At least 2 × 10^5 CD4^+ T cells were analyzed.

Serologic analysis

Antinuclear antibodies (ANAs) were assessed by indirect immunofluorescence on HEp-2 cells. Anti-dsDNA antibodies were detected by Crithidia luciliae immunofluorescence and commercial enzyme-linked immunosorbent assay (ELISA).

Statistical analysis

T- and B-lymphocyte subpopulation frequencies were calculated using CellQuest software (BD Biosciences). A paired t test was used to compare (per patient and immune parameter) pretransplantation and posttransplantation data using Graph Pad Prism 4 software (version 4.03; Graph Pad Software, San Diego, CA). Based on distributional assumptions, the Mann-Whitney U test was used to compare data from patients treated by ASCT with those from healthy controls and conventionally treated SLE patients. All P values were 2-sided; statistical significance was set at α = 0.05.

Results

Engraftment and leukocyte recovery

The median time for recovery to 0.5 × 10^9/L neutrophils and 10^9/L leukocytes in peripheral blood was 14 days. Platelets recovered to more than 20 × 10^9/L by a median of 12 days. No patient received unselected backup stem cell support. All patients had significantly reduced baseline lymphocyte counts (mean, 0.33 ± 0.14 × 10^3 cells/µL), reflecting lupus activity or side effects of immunosuppressive therapy. As in patients with hematologic diseases treated with a similar regimen,17 peripheral lymphocyte counts reconstituted slowly after ASCT and were still slightly reduced at the 6-month follow-up (0.94 ± 0.32 × 10^3 cells/µL). Mean absolute lymphocyte counts were back to normal at the 1-year follow-up (1.11 ± 0.29 × 10^3 cells/µL) and remained stable thereafter in responding patients.

Increased naive T cells after posttransplantation immune reconstitution

In SLE, pathogenic T-cell functions are thought to be mediated by autoreactive memory or memory effector CD4^+ T cells. Elimination of such cells in vivo by immunosuppression is therefore presumed to ameliorate autoimmune inflammation. Conversely, thymic reactivation is presumably required to reestablish central tolerance and to generate natural FoxP3^+ regulatory T cells. To assess the effect of immunosuppression and ASCT on the CD4^+ T-cell compartment, we first used the phenotypic markers CD45RA and CD45RO to...
discriminate between (CD45RA<sup>+</sup> CD45RO<sup>-</sup>) naive and (CD45RO<sup>+</sup> CD45RA<sup>-</sup>) memory CD4<sup>+</sup> T cells.

The longitudinal analysis of reconstituting naive and memory CD4<sup>+</sup> T cells is shown in Figure 1. At baseline, patients displayed a significant CD4<sup>+</sup> T-cell lymphopenia compared with age-matched healthy controls, which was attributable to both CD45RA<sup>+</sup> naive (median, 35/µL vs 288/µL, P < .001) and CD45RO<sup>+</sup> memory CD4<sup>+</sup> T cells (median, 51/µL vs 433/µL, P < .001), reflecting the disturbed T-cell homeostasis of active SLE (Figure 1A). In the regenerative phase, the memory phenotype (CD45RO<sup>+</sup>CD45RA<sup>-</sup>) was the predominant CD4<sup>+</sup> T-cell subset; there was a significant increase in this subpopulation at 6 months after treatment compared with baseline (median, 73.4% vs 53.3%, P = .116; Figure 1A) with a doubling of absolute counts (median, 121/µL vs 51/µL, P = .027; Figure 1B). Naive CD4<sup>+</sup> CD45RA<sup>+</sup> T-cell counts were low or undeterminable at that time but later increased continuously, reaching complete recovery 24 months after ASCT with significant higher values than before ASCT (median, 244/µL vs 35/µL, P = .014; Figure 1B). CD45RO<sup>+</sup> Th counts (Figure 1B) remained significantly diminished until the 4-year follow-up.

**Increased output of RTEs**

To determine whether CD45RA<sup>+</sup>CD45RO<sup>-</sup>CD4<sup>+</sup> T cells in the regenerated immune system were homeostatically expanded peripheral T cells or naive T cells newly generated in the thymus, we analyzed their expression of CD31, a surrogate marker of recent thymic emigrants (RTEs).<sup>18</sup> Six months after ASCT, 85.2% to 98.8% (median, 89.7%) of the CD45RA<sup>+</sup>CD4<sup>+</sup> T cells in responding patients coexpressed CD31; this was significantly more than CD4<sup>+</sup>/H9262P higher values than before ASCT (median, 244/H9262P/H11005 vs 51/mL, P < .031). The number of RTEs decreased only 98.8% (median, 89.7%) of the CD45RA<sup>+</sup>CD4<sup>+</sup> T cells (median, 51/H9262P/H11005 vs 35/µL, P = .001), reflecting the higher the patients' peripheral FoxP3 expression levels in CD4<sup>+</sup> T cells (median, 435/µL vs 164/µL, P = .016), and at 15 times the baseline levels (median, 435/µL vs 29/µL, P = .031). The number of RTEs decreased only in the patient who suffered a relapse 18 months after transplantation.

**The thymus contributes to the regeneration of FoxP3<sup>+</sup> regulatory T cells**

Regeneration of the CD4<sup>+</sup> T-cell (Treg) compartment was evaluated after ASCT by identifying peripheral blood CD4<sup>+</sup> T cells costaining brightly for CD25 and expressing intracellular FoxP3 (Figure 2A). Conventional treated patients with active SLE had significantly lower frequencies of peripheral FoxP3<sup>+</sup> Tregs than normal controls (median, 5.5% vs 8.0%, P = .001), as illustrated in Figure 2B. Those with inactive SLE had comparable, if not higher, frequencies of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells than the controls (median, 10.5% vs 8.0%, P = .048). FoxP3<sup>+</sup> CD4<sup>+</sup> T-cell frequencies in regenerated immunoablated ASCT patients at time points from 2 to 7 years after ASCT (as depicted in Figure 2A) were as high as those in normal controls (median, 9.4%, vs 8.0%, P = .229; Figure 2B). Overall, the absolute numbers of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells were similar in both groups (median, 62.8/µL vs 67.2/µL, P = .963; Figure 2C). However, the patients were heterogeneous with respect to numbers of FoxP3<sup>+</sup> CD4<sup>+</sup> T cells. The later the follow-up date, the higher the patients’ peripheral FoxP3<sup>+</sup> CD4<sup>+</sup> T-cell count (161.8/µL in patient 1 at 7 years) and the lower the count in patients examined at earlier time points after ASCT (31.1/µL in patient 7 at 2 years).

**Thymic output generates a new and diverse TCR repertoire**

CD4<sup>+</sup> T-cell diversity in the patients’ regenerating immune systems was analyzed with a panel of TCR VB-specific monoclonal antibodies by flow cytometry, as recently described.<sup>19</sup> At baseline, all patients analyzed (n = 4) showed significantly expanded TCR VB-expressing CD4<sup>+</sup> T cells at indicated times after ASCT in 5 patients and 1 control. (B) Median FoxP3 expression levels in CD4<sup>+</sup> T cells (as determined by flow cytometry in panel A) in 5 patients versus 14 healthy controls, 10 conventionally treated patients with active SLE (SLEDAI = 6), and 14 conventionally treated with inactive SLE (SLEDAI < 6). Group comparisons were performed using the Mann-Whitney U test. (C) FoxP<sup>3</sup> CD4<sup>+</sup> Tregs (median absolute counts, as determined by flow cytometry in panel A) in 5 patients versus 14 normal controls. A Mann-Whitney U test was used for group comparison.
expansion (patient 4, Vβ5.1 at 4 years and patient 6, Vβ12 at 2 years) after ASCT, the TCR Vβ profiles of CD4+ T cells remained stable and heterogeneous throughout follow-up. At the 3-year follow-up, the regenerated CD4+ T-cell TCR Vβ family usage was normal in all patients (Figure 3).

Early expansion of memory CD4+ T cells is not driven by autoantigens

The specificity of CD4+ memory T cells was analyzed by ex vivo short-term restimulation of whole blood with viral antigens and autoantigens, and subsequent enumeration of reactivated T cells expressing CD69 and IFN-γ (Th1 memory cells) or CD154 (all memory Th cells) during the early phase of immune reconstitution.15,16,21 Notably, these ex vivo restimulation assays were performed in patients with identified viral infections based on clinical symptoms and corresponding serologic findings (patient 1: varicella-zoster virus, patient 4: human herpes virus 6, patient 6: CMV reactivation, patient 7: herpes simplex virus 1). During viral infections, Th effector memory cells specific for VZV (patient 1) and CMV (patient 6) were readily detectable as CD69+ or CD154+ CD4+ T cells coexpressing IFN-γ. Inversely, T cells reacting to nucleosomes or SmD1 were not detectable early after ASCT (Figure 4).

### Table 3. Significantly expanded TCR Vβ-expressing CD4+ T cells at baseline and during follow-up after ASCT in patients 4 to 7

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>TCR</th>
<th>Before transplantation</th>
<th>3 months after transplantation</th>
<th>6 months after transplantation</th>
<th>1 year after transplantation</th>
<th>2 years after transplantation</th>
<th>3 years after transplantation</th>
<th>4 years after transplantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>BV5.1</td>
<td>5.5</td>
<td>8.1*</td>
<td>11.8*</td>
<td>8.9*</td>
<td>7.9</td>
<td>7.6</td>
<td>8.3*</td>
</tr>
<tr>
<td></td>
<td>BV5.2</td>
<td>1.7</td>
<td>2.4*</td>
<td>1.8</td>
<td>1.5</td>
<td>1.2</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>BV7.1</td>
<td>1.9</td>
<td>3.2*</td>
<td>1.9</td>
<td>2.7*</td>
<td>2.2</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>BV9</td>
<td>4.5</td>
<td>6.4*</td>
<td>4.3</td>
<td>3.5</td>
<td>3.0</td>
<td>3.3</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>BV13.6</td>
<td>1.5</td>
<td>6.7*</td>
<td>1.9</td>
<td>2.2</td>
<td>0.6</td>
<td>2.3</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>BV16</td>
<td>5.7*</td>
<td>0.5</td>
<td>0.3</td>
<td>0.6</td>
<td>0.2</td>
<td>1.1</td>
<td>0.7</td>
</tr>
<tr>
<td>5</td>
<td>BV14</td>
<td>8.4*</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>BV7.2</td>
<td>2.7*</td>
<td>1.7</td>
<td>1.4</td>
<td>1.9</td>
<td>1.6</td>
<td>1.4</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>BV12</td>
<td>2.1</td>
<td>2.3</td>
<td>2.5</td>
<td>2.2</td>
<td>2.7*</td>
<td>2.5</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>BV13.2</td>
<td>5.0*</td>
<td>3.5</td>
<td>2.9</td>
<td>3.4</td>
<td>3.4</td>
<td>3.1</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>BV20</td>
<td>3.7*</td>
<td>4.7*</td>
<td>1.1</td>
<td>1.5</td>
<td>2.1</td>
<td>1.4</td>
<td>2.1</td>
</tr>
<tr>
<td>7</td>
<td>BV4</td>
<td>2.6*</td>
<td>1.9</td>
<td>2.6*</td>
<td>2.2</td>
<td>2.3</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>BV12</td>
<td>2.1</td>
<td>3.4*</td>
<td>3.2*</td>
<td>2.5</td>
<td>1.7</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>BV13.1</td>
<td>5.2</td>
<td>12.9*</td>
<td>4.7</td>
<td>4.8</td>
<td>5.1</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

— indicates not applicable.

*Significant expansions according to criteria described in “Methods.”
Normalization of disturbed B-cell homeostasis after ASCT

Active SLE is characterized by marked B lymphocytopenia, which reportedly affects CD27^- naive B cells more than CD27^+ memory B cells. To evaluate the effect of immunoablation and ASCT on these B-cell disturbances, we analyzed peripheral blood B lymphocytes from regenerating immune systems for IgD, CD27, and CD20 expression. Before treatment, patients had significantly lower numbers of IgD^- naive B cells than normal controls (median, 4/µL vs 202/µL, P < .001; Figure 5) as well as a predominance of IgD^- memory B lymphocytes (median, 67.2% vs 23.5%, P = .003) and a prominent population of CD27^high CD20^- plasma blasts (median, 10.3% vs 0.9%, P = .006) in peripheral blood. After ASCT, B lymphocytes predominantly displayed a naive IgD^- phenotype. Complete numeric recovery of this subset was observed by 12 months after ASCT, with counts 50 times higher than at baseline (median,
IgD memory-phenotype B-cell frequencies drastically declined from a median of 67.2% at baseline to 7.0% within 6 months after ASCT (P = .002). During immune regeneration, IgD B-cell frequencies remained lower than in healthy controls over the entire follow-up period of up to 8 years (Figure 5A). IgD memory B-cell counts did not recover until 3 years after ASCT (Figure 5B). IgD memory B lymphocytes did not detectably expand before that time, except in the patient with the lupus flare (Figure 5A). CD20 CD27<sup>high</sup> plasma blast frequencies among CD19<sup>+</sup> B cells normalized within 6 months after ASCT in all patients analyzed, and normal levels persisted during the entire follow-up period (Figure 5C).

**Autoreactive and protective antibodies in serum are largely extinguished after ASCT**

All patients had ANAs and persistently high anti–double-stranded (anti-ds) DNA serum antibody titers before enrollment. After immunoablation and ASCT, anti-dsDNA antibodies disappeared in all patients within 1 month (Table 4) and recurred only in the patient with reactivated disease (patient 3). Four of 6 patients with a follow-up of at least 6 months after transplantation showed a decrease in ANA titers to negative or 1:80, which is regarded as clinically not significant (Table 4). From these 4 patients, only 1 (patient 4) showed relevant ANA recurrence, which persisted from the 3-year follow-up onward without clinical symptoms of SLE. In 2 patients, ANA persisted, albeit in significantly reduced titers.

Not only autoantibodies but also protective serum antibodies specific for measles, mumps, tetanus, and diphtheria disappeared in the immunoablated patients. All patients had received the World Health Organization-recommended vaccination before enrollment. Even though not all patients had reached protective levels of vaccine-specific antibodies before immunoablation, significant decreases in serum antibody levels for measles (P = .043, Figure 6A), mumps (P = .028, Figure 6B), tetanus toxoid (P = .048, Figure 6C), and diphtheria (P = .049, Figure 6D) were observed when tested 1 or 2 years after ASCT (Figure 6). The serologic data point to an effective depletion of long-lived plasma cells from the bone marrow. A bone marrow aspiration sample from 1 patient (patient 7), obtained early after ASCT (1 month), exhibited almost complete depletion of CD38<sup>+</sup>CD138<sup>+</sup> plasma cells with

![Table 4. Titer of ANAs and anti–dsDNA antibodies before and after ASCT](image)

<table>
<thead>
<tr>
<th>Patient no./antibodies</th>
<th>Before transplantation</th>
<th>1 month</th>
<th>6 months</th>
<th>12 months</th>
<th>24 months</th>
<th>36 months</th>
<th>48 months</th>
<th>60 months</th>
<th>72 months</th>
<th>84 months</th>
<th>96 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ANA 5120</td>
<td>320</td>
<td>Negative</td>
<td>Negative</td>
<td>320</td>
<td>80</td>
<td>160</td>
<td>320</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>dsDNA 8</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>ANA 5120</td>
<td>320</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>160</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>dsDNA 64</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>ANA 2560</td>
<td>160</td>
<td>80</td>
<td>80</td>
<td>5120</td>
<td>2560</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dsDNA 64</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>ANA 20 480</td>
<td>640</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>320</td>
<td>1280</td>
<td>1280</td>
<td>1280</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dsDNA 64</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>ANA 2560</td>
<td>320</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dsDNA 64</td>
<td>Negative</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>ANA 10 240</td>
<td>2560</td>
<td>2560</td>
<td>2560</td>
<td>5120</td>
<td>5120</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dsDNA 80</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>ANA 10 240</td>
<td>1280</td>
<td>1280</td>
<td>640</td>
<td>640</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>dsDNA 80</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

ANA indicates antinuclear antibodies, inverse titer; dsDNA, anti–double-stranded DNA antibodies (C. luciliae assay), inverse titer; and —, not applicable.

![Figure 6. Changes in protective antibody titers over time after ASCT](image)

A paired t test was used to compare pretransplantation and posttransplantation data.
only 0.03% among BM-MNC compared with a normal control bone marrow with 1.24% of such cells (Figure 5D).

Discussion

Immunoblation followed by ASCT is an emerging treatment option for patients with severe autoimmune diseases refractory to conventional therapies, including SLE. In accordance with previous reports, this regimen achieved long-term (up to 8 years) clinical and serologic treatment-free remissions in our lupus patients. Notably, these patients originally had high disease activity and a poor prognosis, reflected by high SLEDAI scores and persistent anti-dsDNA antibody titers. Although the clinical efficacy of this experimental therapy is rapidly becoming evident, it remains obscure how it works. Our detailed analysis of the long-term reconstitution of the patients’ immune systems with respect to the recurrence of T- and B-lymphocyte subsets and the course of serologic changes over time demonstrated successful depletion of autoreactive immunologic memory and the regeneration of a tolerant immune system from hematopoietic stem cells. Regeneration involved reactivation of the thymus and extensive renewal of antigen-receptors, in other words, a “resetting of the immunologic clock.”

Depletion of autoreactive immunologic memory by immunoblation was most drastically reflected in the complete disappearance of autoantibodies, particularly dsDNA-specific antibodies. Depletion of immunologic memory was not restricted to autoreactive memory. In addition, pathogen-specific serum antibodies for mumps, measles, tetanus, and diphtheria were largely extinguished. This drastic ablation of humoral memory suggests that the ATG used for immunoblation directly targets the plasma cells secreting these serum antibodies. It was recently shown that the plasma cells providing humoral memory are long-lived cells that reside mostly in the bone marrow, where they dwell in specialized niches providing essential survival signals. Recent in vitro experiments indicated that polyclonal rabbit ATG directly targets plasma cells and B cells via complement-mediated lysis and apoptosis. In line with this hypothesis, we were able to stain plasma cells ex vivo with the polyclonal rabbit ATG used for immunoblation (data not shown); moreover, plasma cells disappeared from bone marrow 1 month after immunoblation in 1 case. The depletion of long-lived plasma cells might be of particular relevance for the success of immunoablative therapy. It has been demonstrated that these plasma cells are resistant to immunosuppression by cyclophosphamide, irradiation, and CD20-mediated B-cell depletion. Hence, autoreactive long-lived plasma cells represent a key component of autoreactive immunologic memory. Persistent autoantibodies secreted by long-lived plasma cells could maintain chronic inflammation and accelerate autoimmunity. A retrospective survey by the European Blood and Marrow Transplant and European League Against Rheumatism Registry revealed that patients without complete loss of autoantibody responses after immunoblation and ASCT had higher rates of relapse. In our cohort, the only patient who relapsed became anti-dsDNA-negative after immunoblation, but anti-Ro/SSA and anti-La/SSB antibodies persisted until the relapse. From the other patients, only 1 had anti-Ro/SSA antibodies before enrollment (patient 6). Similar to the patient with the relapse, anti-Ro/SSA antibodies persisted in this patient after ASCT, albeit without evidence for SLE reactivation. So far, it is not clear why plasma cells secreting these autoantibodies seem to be more resistant to the immunoablative regimen and if their persistence characterizes patients with a higher risk for relapse.

T-cell reconstitution after immunoblation was characterized by continued generation of new naive CD4+ T cells for up to 8 years after ASCT. In particular, naive CD45RA-CD31+ T cells expressing CD31 with high overall clonal diversity of the CD4+ TCR repertoire were generated. These cells have been shown to be recent thymic emigrants. In the regenerated patients, absolute CD45RA-CD31+ naive CD4+ T-cell counts continuously increased to levels twice as high as those in age-matched controls, resembling those in young children. This observation supports the notion that, after immunoblation and ASCT, the naive CD4+ T-cell compartment is regenerated by thymic reactivation rather than by lymphopenic expansion of surviving naive T cells, emphasized earlier for patients undergoing immunoblation and ASCT for treatment of hematologic malignancies and multiple sclerosis. In our SLE patients, the finding is even more relevant in light of the disease- and treatment-related impairment of the naive T-cell compartment, which has been attributed to intrinsic impairment of thymic export. Immunoablation and ASCT obviously can correct this deficiency, rejuvenate the CD4+ T-cell compartment, and normalize naive T-cell homeostasis.

After monitoring the TCR Vβ family repertoire of the recurring CD4+ T-cell compartment, we observed a drastic change in clonal diversity of the TCR repertoire. The originally observed clonal expansions and deletions disappeared, suggesting that treatment had led to the ablation of expanded clones and to the generation of a complete repertoire of recent thymic emigrants.

Among CD4+ T cells, FoxP3+ regulatory T cells regenerated to frequencies and absolute numbers comparable with those in normal controls. The fact that regeneration of the Treg compartment was accompanied by the reappearance of naive T cells and recent thymic emigrants suggests that these regulatory T cells were generated in the thymus. Similar observations have been made in patients undergoing immunoblation and ASCT for juvenile idiopathic arthritis, suggesting a common mechanism of action of stem cell transplantation in different autoimmune diseases.

Whereas regeneration of thymic naive Th cells was delayed for up to 1 year after ASCT, mature CD45RO+ memory CD4+ T cells reappeared faster with on average a doubling of absolute counts at 6 months after transplantation compared with baseline values. However, their TCR Vβ repertoires were highly restricted, reflecting responses to a limited array of available antigens during lymphopenia. If peripheral T-cell expansion had involved lymphopenia-driven proliferation of memory T cells in response to low-affinity self-antigens, expansion of autoreactive T-cell clones should have been observed. However, we found no evidence of clonal expansion of autoreactive T cells specific for SLE-associated autoantigens, such as nucleosomes or SmD1. This implies that the early expansion of memory CD4+ T cells is not driven by autoantigens and, in particular, not by those involved in SLE. Rather, we showed that clonally expanded memory T cells reacted to virus-specific antigens in patients infected with specific viruses. This implicates protective pathogen-specific immune responses as a cause of clonal expansion of memory-phenotype T cells. The expansion of protective pathogen-specific T cells in response to treatment may contribute to the control of autoimmunity by
restricting the space available in the effector-memory compartment for autoreactive T cells, the expansion of which is driven by (weak) reactions to autoantigens.

Regeneration of the B lymphocyte compartment in the treated SLE patients resembled that of patients receiving ASCT for treatment of hematologic malignancies. The majority of repopulating B cells initially showed a naive (IgD⁺) phenotype. Memory (IgD⁻) B cells did not reappear until later. In the present study, this regeneration of the B-cell compartment was remarkable in view of the significant disturbances observed in our active SLE cohort before ASCT. These patients had shown naïve B-cell lymphopenia, relative predominance of phenotypically memory B cells, and expansion of CD27high, CD20+ plasma cell precursors. The complete normalization of these preexisting disturbances indicates that immunoblation had removed all autoreactive B cells. Apparently, the B-cell compartment also regenerates from stem cells after immunoblation and ASCT, and it is tolerant to self-antigens, including those that had been relevant in the patients before treatment.

In conclusion, this study provides direct evidence for a profound regeneration of the adaptive immune system in SLE patients after immunoblation and ASCT. All patients except 1 achieved long-lasting clinical and serologic remissions and are no longer reliant on immunosuppressive therapy. The 1 exception relapsed after having been in clinical remission for more than a year. The relapse might be the result of insufficient ablation of autoreactive immunologic memory, presentation of tolerance-breaking autoantigen forms to the regenerated immune system, or genetic predispositions that restart the disease in the regenerated immune system. Our findings would propose that chronic autoimmunity is not an endpoint depending on continuous treatment with specific anti-inflammatory agents but may be cured by combining specific targeting of autoreactive memory and effector cells with a reactivation of thymic activity.

Acknowledgments
This work was supported by grants from the Bundesministerium für Bildung und Forschung (01GI9944/DRFZ C4.1) and the Sonderforschungsbereiche (SFB) 650 TP12.

Authorship


Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Tobias Alexander, Department of Rheumatology and Clinical Immunology, Charité Universitätsmedizin, Charitéplatz 1, 10117 Berlin, Germany; e-mail: tobias.alexander@charite.de.

References


Depletion of autoreactive immunologic memory followed by autologous hematopoietic stem cell transplantation in patients with refractory SLE induces long-term remission through de novo generation of a juvenile and tolerant immune system

Tobias Alexander, Andreas Thiel, Oliver Rosen, Gero Massenkeil, Arne Sattler, Siegfried Kohler, Henrik Mei, Hartmut Radtke, Erika Gromnica-Ihle, Gerd-Rüdiger Burmester, Renate Arnold, Andreas Radbruch and Falk Hiepe