Bone marrow of NZB/W mice is the major site for plasma cells resistant to dexamethasone and cyclophosphamide: Implications for the treatment of autoimmunity

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Abstract

Antibodies contribute to the pathogenesis of many chronic inflammatory diseases, including autoimmune disorders and allergies. They are secreted by proliferating plasmablasts, short-lived plasma cells and non-proliferating, long-lived memory plasma cells. Memory plasma cells refractory to immunosuppression are critical for the maintenance of both protective and pathogenic antibody titers. Here, we studied the response of plasma cells in spleen, bone marrow and inflamed kidneys of lupus-prone NZB/W mice to high-dose dexamethasone and/or cyclophosphamide. BrdU+, dividing plasmablasts and short-lived plasma cells in the spleen were depleted while BrdU− memory plasma cells survived. In contrast, all bone marrow plasma cells including anti-DNA secreting cells were refractory to both drugs. Unlike bone marrow and spleen, which showed a predominance of IgM-secreting plasma cells, inflamed kidneys mainly accommodated IgG-secreting plasma cells, including anti-DNA secreting cells, some of which survived the treatments. These results indicate that the bone marrow is the major site of memory plasma cells resistant to treatment with glucocorticoids and anti-proliferative drugs, and that in inflamed tissues and secondary lymphoid organs can contribute to the autoreactive plasma cell memory. Therefore, new strategies targeting autoreactive plasma cell memory should be considered. This could be the key to finding a curative approach to the treatment of chronic inflammatory autoantibody-mediated diseases.

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1. Introduction

Antibodies secreted by plasma cells and their precursors, plasmablasts, are part of the humoral immune system. Pathogenic antibodies can contribute to chronic inflammatory diseases [1,2]. We have shown that long-lived plasma cells, also known as memory plasma cells, are refractory to cyclophosphamide and significantly contribute to autoimmunity [3]. The impact of these cells has been affirmed by the findings that depletion of plasma cell memory by immunoaablation with antithymocyte globulin followed by autologous stem cell transplantation in SLE patients [4] and by the proteasome inhibitor bortezomib in lupus-prone NZB/W mice [5] may result in deletion of autoimmunity. However, it was observed that in a few patients, autoantibodies do not disappear despite immunoaabative therapy. Therefore, the need arises to obtain more information about the location and stability of memory plasma cells, which have been thought to mainly reside in bone marrow under physiological conditions. Additionally, plasma cells have been detected in inflamed tissues like the kidneys of lupus nephritis mice [6,7], synovial tissue of patients with rheumatoid arthritis [8], salivary glands in Sjögren’s syndrome [9] and skin of scleroderma patients [10].

After antigenic challenge, activated and memory B cells from the spleen and other secondary lymphoid organs differentiate via proliferating plasmablasts into non-proliferating long-lived memory plasma cells. The numbers of splenic plasmablasts/short-lived plasma cells diminish significantly within 10–14 days of the immune challenge [11]. These cells thus deal only with acute
immune challenges. Plasmablasts are capable of homing to bone marrow via CXC4/CXCL12-mediated chemokine signaling, where they mature into sessile, non-proliferating long-lived plasma cells [12,13]. However, only a small fraction of plasmablasts become long-lived in the bone marrow, indicating the limited availability of survival niches for memory plasma cells in this tissue [11,14]. The bone marrow was already identified in 1980s as the major site of serum antibody production [15]. Bone marrow plasma cells maintain protective antibody memory for years after secondary immune challenges [16,17]. During inflammation, IFNγ induces upregulation of chemokine receptor CXCR3 on plasmablasts and its chemokine ligands CXCL9, 10 and 11 in inflamed tissues [18]. Consequently, plasmablasts can migrate into inflamed tissues. Maturation of plasma cells can be expected to take place in inflamed tissues as they express several plasma cell survival factors [1].

In order to determine the location and refractoriness of (auto-reactive) memory plasma cells to immunosuppression, we analyzed the spleen, bone marrow and inflamed kidneys of NZB/W mice after treatment with high-dose dexamethasone and/or cyclophosphamide. In this detailed study, we show that the susceptibility of plasma cells to these drugs could depend on the site where they reside. A fraction of plasma cells, including anti-dsDNA antibody-secreting cells, survived this treatment in inflamed kidneys. Moreover, while the spleen and inflamed kidneys contained both sensitive and resistant plasma cells, all bone marrow plasma cells including anti-DNA antibody-secreting cells were refractory to these drugs. Pathogenic antibodies secreted by plasma cells in the bone marrow and in inflamed tissues can therefore contribute to refractoriness of antibody-mediated diseases to immunosuppression with glucocorticoids and cytotoxic agents. Unlike the spleen and bone marrow, the kidneys contained almost exclusively class-switched IgG antibody-secreting cells.

2. Materials and methods

2.1. BrdU feeding and drug administration

Female NZB/W F1 mice were bred and maintained in specific pathogen-free conditions at the mouse facility of German Rheumatism Research Centre in Berlin. Twenty to 22-week-old mice with lupus nephritis and proteinuria were used for the experiment. In order to discriminate between proliferating plasmablasts/short-lived plasma cells and non-proliferating long-lived memory plasma cells, mice were fed bromodeoxyuridine (BrdU) dissolved in drinking water (1 mg/ml; Sigma–Aldrich/1% glucose) for a period of three weeks. BrdU is a thymidine analog that is incorporated by proliferating cells; therefore, it is taken up by plasmablasts/short-lived plasma cells but not by non-proliferating memory plasma cells. The BrdU water bottles were carefully protected from light and changed every three days. During the third week, the mice were divided into four groups treated with intraperitoneal doses of either vehicle (PBS 100 μl/day on all 7 days), dexamethasone (1 mg/kg/day on all 7 days), cyclophosphamide (30 mg/kg/day on 4 days) or dexamethasone plus cyclophosphamide. All the drugs were diluted in PBS to obtain the desired dose and concentration. At the end of treatment (and BrdU feeding), the mice were sacrificed by cervical dislocation, and their spleens, bone marrows and kidneys were taken for analysis. Plasma cells were analyzed in the organs of the different treatment groups by FACS and ELISPOT.

2.2. FACS analysis of splenic and bone marrow lymphocytes

Spleen and bone marrow (femur and tibia) samples were filtered through 70 μm cell strainers (BD Falcon) and washed twice with PBS/0.5% BSA to yield single-cell suspensions. The total number of leukocytes per spleen was determined using a CASY cell counter (Schräfe Systems). Femoral and tibial bone marrow leukocytes together constitute approximately 20% of the total bone marrow leukocytes in mice [15,19]. The total number of leukocytes per mouse bone marrow was therefore estimated by multiplying the number of femoral and tibial leukocytes by five. In order to block unspecific antibody binding during staining, the cells were first incubated with anti-CD16/CD32 (clone 2.4G2; BD Biosciences) and rat Ig (Biotrend) for 15 min on ice. The cells were then stained with anti-CD138–PE (clone 281–2; BD Biosciences) and anti-MHC II-biotin (clone M5/114) for 15 min and washed. Streptavidin–PerCP was used as a secondary reagent for developing biotin. This was followed by cell fixation/membrane permeabilization and staining for intranuclear BrdU using the BrdU Flow Kit (BD Biosciences) according to the manufacturer’s protocol. Anti-BrdU–FITC (clone 3D4; BD Biosciences) and anti-kappa light chain-Cy5 (clone 187.1; BD Clone) were used for intracellular staining. In the bone marrow, additional staining for intracellular kappa light chain was required for clear differentiation from other cells. Anti-B220-Cy5 (clone RA3.6B2), anti-CD4–FITC (clone GK 1.5), anti-CD8–PE (clone 53–6.7) were used for staining of B and T lymphocytes. Flow cytometer acquisition was performed on a FACS LSRII cytometer (BD Biosciences), and the data were analyzed using CellQuest software (BD Biosciences). Debris and red blood cells were excluded by electronic gating. The frequencies of BrdU– (proliferating short-lived plasmablasts and plasma cells) and BrdU– plasma cells (non-proliferating long-lived memory plasma cells) among all leukocytes in the spleen and bone marrow were determined by FACS. These frequencies were used to calculate the total number of plasma cells per organ.

2.3. Quantification of antibody-secreting cells (ASC) by ELISPOT

Functional activity of plasma cells in the spleen, bone marrow and kidneys of the treated mice and the classes of the antibodies they produced were determined by IgM and IgG ELISPOT assays. The organs were passed through 70 μm cell strainers twice to obtain single-cell suspensions. The suspensions were washed twice and re-suspended in RPMI 1640 medium supplemented with 10% FCS (Invitrogen), Goat anti-mouse IgM or goat anti-mouse IgG antibodies (Southern Biotech) diluted in PBS was used to coat the ELISPOT plates (Corning/Costar 96-well microplate, high binding capacity, flat bottom). Each well was coated with 50 μl of diluted coating antibodies (5 μg/ml in PBS) and incubated overnight at 4°C. The plates were then washed and blocked with PBS/3% BSA for 2 h at 37°C. Single-cell suspensions in medium were harvested into the coated wells of the ELISPOT plates and incubated for 2 h at 37°C/5% CO2. The plates were then washed and developed as described previously [6].

Anti-DNA antibody-secreting cells in the target organs were analyzed by DNA ELISPOT assay using a slightly modified protocol. In brief, ELISPOT plates were precoated with methylated BSA (10 μg/ml; Sigma–Aldrich, 2 h at 37°C), washed with PBS and subsequently coated with calf thymus DNA (10 μg/ml; Sigma–Aldrich) and incubated overnight at 4°C. The cell suspensions were then pipetted onto the coated plates, and ELISPOT was developed as described above and analyzed for DNA-specific antibody-secreting cells.

2.4. Migration assay

Migration assays were performed with plasma cells using the previously described protocols [12].
2.5. Statistics

Statistical analyses were performed using GraphPad Prism software. The effects of the different treatments were compared to those of the control group (vehicle) using a non-parametric test (Mann–Whitney) or unpaired t test.

3. Results

3.1. Splenic long-lived plasma cells are refractory to dexamethasone and/or cyclophosphamide

First, we analyzed the effects of the different treatments on plasma cells by flow cytometry. Splenic plasma cell counts in NZB/W mice are much higher than those in non-autoimmune mouse strains [6]. Although both cell types express the same surface marker-CD138, it was possible to distinguish them by FACS based on BrdU incorporation (Fig. 1a). Continuous BrdU feeding for 3 weeks allowed us to distinguish BrdU+ plasmablasts/short-lived plasma cells that had proliferated during these 3 weeks from BrdU− plasma cells that had formed earlier. Our previous kinetics studies showed that plasma cells that do not incorporate BrdU during the 3 weeks of feeding survive for several months without proliferating [3]. Hence, they can be classified as long-lived plasma cells.

During the last week of BrdU feeding, the mice received either dexamethasone or cyclophosphamide or both drugs. Frequencies of BrdU+ and BrdU− plasma cells in the spleen and bone marrow were determined by FACS and transformed into absolute numbers per organ for better comparability. Splenic BrdU+ plasmablasts/short-lived plasma cells were significantly depleted by dexamethasone and/or cyclophosphamide (3–4-fold reduction), whereas BrdU− long-lived plasma cells remained largely unaffected (Fig. 1b). Dexamethasone and cyclophosphamide alone were equally effective in depleting plasmablasts, and the combination of two drugs was not more effective than the single drugs. On the other hand, long-lived plasma cells in the spleen remained refractory to all treatment protocols. These results are in accordance with our original finding that splenic long-lived plasma cells are refractory to cyclophosphamide [3]. The current results additionally show that resistance of these cells is not limited to the anti-proliferative drug cyclophosphamide, but also applies to glucocorticoids.

3.2. Dexamethasone and/or cyclophosphamide deplete splenic IgG ASC more effectively than IgM ASC

ELISPOT assays were performed to determine the total number of IgM and IgG ASCs per spleen. Dexamethasone and cyclophosphamide, alone or in combination, significantly reduced the number of IgM and IgG ASC in the spleen (Table 1). IgG ASC were more susceptible to the drugs than IgM ASC. There was an only 2-fold reduction of IgM ASC compared to a 5-fold reduction of IgG ASC. Despite strong activation of the T-helper cell population in autoimmune lupus and the expected class switch to downstream antibody classes, the spleens of treated NZB/W mice contained more IgM ASC than IgG ASC. Also, most of the refractory splenic plasma cells produced IgM antibodies. This finding is in accordance with our earlier observation that most of the long-lived plasma cells in NZB/W mice secrete IgM [3].

3.3. Neither BrdU+ nor BrdU− bone marrow plasma cells are affected by immunosuppressive drugs

Nearly 80% of the bone marrow plasma cells were BrdU-negative i.e. did not proliferate during the 3 weeks of BrdU feeding (Fig. 2a). This result suggests that, despite chronic autoimmunity, the great majority of bone marrow plasma cells in NZB/W mice are non-proliferating cells with a relatively slow turn-over. Although this result does not allow for definitive half-life estimation, it suggests that plasma cells in NZB/W bone marrow can survive for several weeks to months, similar to the bone marrow plasma cells in non-autoimmune mouse strains [20,21]. Non-proliferating BrdU− bone marrow plasma cells were refractory to cyclophosphamide (as expected) as well as to dexamethasone. Unlike the BrdU+ plasma cells/plasmablasts in the spleen, which were significantly depleted in all treatment groups, those in the bone marrow were not significantly reduced by any of the treatment protocols. In this bone marrow population, cyclophosphamide alone did, however, induce a small reduction tendency, which was not statistically significant (P = 0.08) (Fig. 2b).

Since the BrdU+ plasma cells in bone marrow remained widely refractory to both drugs, we further analyzed MHC class II expression on these cells. MHC class II expression is normally high in plasmablasts but is lost as the cells differentiate into more mature long-lived memory plasma cells [3,11]. Here, we found that the majority of BrdU− plasma cells in bone marrow have down-regulated MHC class II expression, resembling more closely the phenotype of BrdU− plasma cells in the same tissue. Splenic BrdU−...
plasma cells, on the other hand, express high levels of MHC class II (Fig. 3a). The migration assays additionally showed that only splenic MHC class II-expressing plasma cells migrate towards signals from chemokines CXCL12 (SDF) and CXCL10 (IP10), while very few bone marrow cells with the phenotype of plasmablasts (BrdU+/ and upregulation of MHC class II) are attracted by CXCL12 (Fig. 3b and c). Together, these data suggest that the majority of BrdU+/ plasma cells in the bone marrow are probably recently formed long-lived plasma cells or plasma cells undergoing maturation to become long-lived.

The resistance of bone marrow plasma cells to immunosuppressive drugs demonstrated by FACS analysis was also apparent in bone marrow plasma cells analyzed by ELISPOT. Bone marrow IgM and IgG ASC remained resistant to cyclophosphamide and dexamethasone. As in the spleen, there were more IgM ASC than IgG ASC in the bone marrow and, unlike the spleen, both IgM and IgG ASC were resistant (Table 1).

### 3.4. Dexamethasone and cyclophosphamide are potent in spleen and bone marrow

As dexamethasone and cyclophosphamide did not reduce the number of bone marrow plasma cells, there was a need to determine (as a control) whether the drugs were effective in the bone marrow. For this purpose we analyzed the B220+/ B cells and CD4+/CD8+ T cells in the bone marrow and spleen after the treatment. As expected, splenic B- and T-cell cell counts decreased significantly after treatment with both dexamethasone and cyclophosphamide. There was an approximately 3-fold reduction of B220+ B cells (Fig. 4a) and 2-fold reduction of CD4+ T cells in the spleen (Fig. 4b), while the CD8+ T cells remained relatively unresponsive (Fig. 4c).

In the bone marrow, cyclophosphamide had a significant effect on lymphocytes, causing an approximately 6–7-fold reduction of B220+ cells (Fig. 4a) and 2-fold reduction of CD4+ T cells (Fig. 4b). However, bone marrow CD8+ T cells were resistant to cyclophosphamide (Fig. 4c). Dexamethasone alone produced an insignificant reduction of B220+ cells in the bone marrow (Fig. 4a) but a significant increase in the numbers and frequencies of CD4+ and CD8+ bone marrow T cells (Fig. 4b and c). This glucocorticoid effect of increasing bone marrow T cell count has already been demonstrated [22–24]. The effect of the combination of the two drugs on bone marrow B and T lymphocytes was similar to that of cyclophosphamide alone. These results demonstrate that cyclophosphamide and dexamethasone were active in the bone marrow.

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spleen (×10^5 ASC)</th>
<th>Bone marrow (×10^5 ASC)</th>
<th>Kidney (×10^5 ASC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>Vehicle</td>
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<td>4.78 ± 1.58</td>
<td>9.39 ± 0.78</td>
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<td>3.30 ± 0.60</td>
<td></td>
<td>0.48 ± 0.06</td>
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<tr>
<td></td>
<td>4.21 ± 1.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyclophosphamide</td>
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<td>0.69 ± 0.16***</td>
<td>8.60 ± 0.68</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>0.17 ± 0.03***</td>
</tr>
<tr>
<td></td>
<td>1.42 ± 0.25***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dexamethasone</td>
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<td>0.95 ± 0.16**</td>
<td>7.85 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>2.91 ± 0.46</td>
<td></td>
<td>0.23 ± 0.04**</td>
</tr>
<tr>
<td></td>
<td>3.45 ± 1.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combination*</td>
<td>2.74 ± 0.39**</td>
<td>0.46 ± 0.11***</td>
<td>9.55 ± 1.14</td>
</tr>
<tr>
<td></td>
<td>3.15 ± 0.73</td>
<td></td>
<td>0.21 ± 0.03***</td>
</tr>
<tr>
<td></td>
<td>1.97 ± 0.50</td>
<td></td>
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</tbody>
</table>

The asterisks denote significant depletion of ASC compared to the corresponding control group: *P < 0.05, **P < 0.01, ***P < 0.001 (n = 10, Mann–Whitney test).

* Cyclophosphamide plus dexamethasone.

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**Fig. 2.** Analysis of different treatments on bone marrow plasma cells. (A) Representative FACS dot plots of bone marrow plasma cells from each treatment group. Bone marrow plasma cells were identified by CD138 surface and intracellular kappa light chain staining. The plasma cells were distinguished as BrdU+ or BrdU– cells. (B) Effects of the different treatment protocols on total numbers of BrdU+ and BrdU– bone marrow plasma cells are shown. The bars represent mean numbers ± SEM of plasma cells (n = 10 mice per treatment group). Statistics was performed to test for differences between the treatment groups and the vehicle control group (Mann–Whitney test). Abbreviations: DEX dexamethasone, CYC cyclophosphamide and DEX + CYC dexamethasone plus cyclophosphamide.

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3.5. Inflamed kidneys contain mainly IgG ASC that are partly susceptible to cyclophosphamide and less susceptible to dexamethasone

With increasing age and inflammation, large numbers of ASC accumulate within the kidneys of NZB/W mice [6,7,25]. Interestingly, in contrast to the spleen and bone marrow, the kidneys of six-month-old NZB/W mice suffering from full-blown disease contained much higher frequencies of IgG ASC than IgM ASC. There was an approximately two-fold reduction of IgM ASC (P < 0.05) in the kidneys of mice treated with cyclophosphamide and/or dexamethasone (Table 1). However, IgG ASC were affected only in kidneys treated with cyclophosphamide alone. Dexamethasone had no significant effect on IgG ASC in this inflamed organ. Notably, nearly half of the kidney plasma cells survived all immunosuppressive treatment regimes.

3.6. Bone marrow is the major site of autoreactive plasma cell memory

Antibodies binding to double-stranded (ds) DNA are a hallmark of human and murine SLE. ELISPOT analysis was performed to determine the numbers of anti-DNA ASC per organ of mice in the control and treatment groups. Equal numbers of anti-DNA ASC reside in the spleen and bone marrow of 5-month-old NZB/W F1 mice (Table 2), while their inflamed kidneys contained approximately 10 times fewer anti-DNA ASC. Nearly all renal anti-DNA ASC were IgG-producing cells, but huge inter-individual variations in total counts were observed. IgM anti-DNA ASC were nearly absent from the kidneys.

Within the spleen and bone marrow, the frequency of IgM anti-DNA ASC was similar to that of IgG anti-DNA ASC (Table 2). All the treatment protocols used in this study significantly depleted splenic autoreactive ASC of both classes to the same extent (5–6-fold). However, bone marrow IgM and IgG anti-DNA ASC remained refractory to cyclophosphamide and/or dexamethasone. These results are in accordance with the observation that some BrdU+ splenic plasma cells are affected by the treatments while bone marrow plasma cells are completely resistant.

In contrast to the bone marrow and spleen, the numbers of IgG anti-DNA ASC in the kidneys were about 9 times higher than the IgM anti-DNA ASC counts (Table 2). Cyclophosphamide and dexamethasone led to a considerable decrease in both Igg and IgM anti-DNA ASC populations in the kidney (Table 2). However, the renal anti-DNA ASC showed huge variations between individual mice within each treatment group, making an interpretation of these results difficult. In any case, it is worth noting that a fraction of anti-DNA ASC in this inflamed organ was refractory to the treatment protocols. These results demonstrate that inflamed tissues can maintain autoreactive anti-DNA ASC, which survive glucocorticoid and cyclophosphamide therapy.

4. Discussion

Our detailed analysis shows that memory plasma cells are refractory to treatment with dexamethasone and/or cyclophosphamide. This was clearly demonstrated in the spleen, where memory plasma cells not incorporating BrdU were not affected by these treatments, whereas BrdU-positive plasmablasts characterized as proliferating cells were significantly reduced. Furthermore, the combination of the two drugs did not result in a synergistic effect; that was also true for plasma cells in bone marrow and inflamed kidneys. These results seem to be surprising as the mode of action differs between cyclophosphamide and glucocorticoids.
induction of apoptosis of lymphocytes [27], irrespective of their proliferation status. Dexamethasone is used in the treatment of multiple myeloma, in which it induces apoptosis of malignant plasma cells [28,29]. Moreover, glucocorticoids inhibit the transcription factor NF-κB, which is involved in the production of pro-inflammatory cytokines and plasma cell survival factors like TNFα and IL-6 [30].

Our data reveal the bone marrow as the major source of drug-resistant antibody production. Neither cyclophosphamide nor dexamethasone depleted BrdU+ and BrdU− plasma cells (including anti-DNA antibody-secreting cells) in bone marrow. Resistant autoreactive memory plasma cells in these tissues could therefore contribute to refractory disease and disease relapses and could prevent disease cure [2].

The question is why splenic BrdU+ plasmablasts/plasma cells were strongly depleted while bone marrow BrdU+ cells were not affected by dexamethasone and/or cyclophosphamide. A possible explanation could be that splenic BrdU+ cells represent a different stage of plasma cell development than BrdU+ plasma cells in the bone marrow. While the majority of splenic BrdU+ cells are dividing plasmablasts, bone marrow BrdU+ plasma cells could be cells that recently entered the bone marrow as plasmablasts and differentiated into mature plasma cells in this microenvironment. They might have remained BrdU+ after incorporating BrdU during the last cell division just prior to migration to the bone marrow. The phenotypic characteristics of these bone marrow BrdU+ plasma cells, like low expression of MHC class II and the absence of migration towards CXCL10 and 12, more closely resemble those of BrdU− plasma cells in the same organ. This supports the assumption that at least some BrdU+ plasma cells in bone marrow are rather mature memory plasma cells.

However, the results do not exclude that the bone marrow provides niches for dividing BrdU+ plasmablasts and that the environment protects them from drug-induced cell death. Environment-mediated drug resistance is a well-known phenomenon from tumor biology and multiple myeloma [31]. The bone marrow provides survival signals for mature plasma cells, e.g., from CXCL12 and IL-6. Both cytokines are produced by bone marrow stromal cells and have been implicated in mediating drug resistance for malignant plasma cells. Since plasmablasts are attracted by CXCL12 [12] and plasma cells are co-located with CXCL12-expressing stromal cells in the bone marrow [32,33], their survival there could be supported even in the presence of dexamethasone and/or cyclophosphamide. In this context, it should be noted that BAFF and APRIL enhance plasma cell survival by signaling through the BCMA receptor; BCMA is expressed by bone marrow plasma cells and causes upregulation of anti-apoptotic proteins on these cells [34]. BAFF and APRIL are strong activators of NF-κB binding activity and have been shown to induce upregulation of anti-apoptotic proteins Bcl-2 and Mcl-1 in malignant plasma cells. More interestingly, BAFF and APRIL can rescue primary myeloma cells and other myeloma cell lines from dexamethasone-induced apoptosis [35]. Therefore, elevated levels of BAFF in NZB/W mice [36] may partially contribute to upregulation of anti-apoptotic proteins on bone marrow plasma cells, which in turn are capable of mediating dexamethasone resistance. Microarray data on long-lived plasma cells (from healthy donors) generated in our laboratory confirms upregulation of Bcl-2 and other anti-apoptotic proteins on these cells [37].

In the experiments described here, treatment of NZB/W mice with dexamethasone and/or cyclophosphamide for one week did not affect bone marrow and splenic memory plasma cell counts. However, the same treatments effectively depleted splenic BrdU+ plasmablasts/plasma cells, showing a direct cytolytic effect of dexamethasone during this period. The study does not exclude the

Cyclophosphamide is an alkylating agent, which is metabolized by liver enzymes to yield its active metabolite, phosphoramide mustard [26]. The metabolite induces DNA interstrand and DNA-protein crosslink formation, which is fatal to proliferating cells. This explains the susceptibility of proliferating plasmablasts and the resistance of non-proliferating memory plasma cells to cyclophosphamide-mediated depletion [26]. The immunosuppressive effect of glucocorticoids, on the other hand, is due to the
possibility of a reduction of memory plasma cells occurring during long-term treatment. Such a treatment could be expected to diminish the production of inflammatory cytokines that strongly support the survival of plasma cells. It should, however, be noted that 1 mg/kg of dexamethasone is an extremely high dose for daily glucocorticoid treatment of autoimmune patients. Moreover, preliminary experiments revealed that one-month treatment of NZB/W mice with up to 10 mg/kg prednisolone does not affect bone marrow or splenic memory plasma cells. Our results are in accordance with the work of Benner et al., who demonstrated the resistance of antigen-specific and total antibody responses in the bone marrow of non-autoimmune mice treated with extremely high doses of dexamethasone. One week of treatment with doses as high as 16 mg/kg body weight and a single extremely high-dose injection of 144 mg/kg body weight did not affect total and antigen-specific antibody responses in the bone marrow of the mice [38,39].

Our results show that approximately equal numbers of anti-DNA antibody-secreting cells (ASC) reside in the spleen and bone marrow of NZB/W mice. In general, the response of anti-DNA ASC to immunosuppressive treatment was similar to that of total ASC populations in the respective organs. Despite the strong depletion of splenic anti-DNA ASC by immunosuppressive drugs, bone marrow anti-DNA ASC remained refractory. Remarkably, the bone marrow contains higher numbers of IgM anti-DNA ASC than IgG anti-DNA ASC. This could be explained by earlier findings that younger NZB/W mice produce predominantly IgM anti-DNA antibodies, and that the class switch to IgG anti-DNA antibodies occurs with increasing age and progressing disease [40,41]. In accordance with our concept of plasma cell niches [1], the majority of bone marrow niches for memory plasma cells are occupied by IgM anti-DNA ASC. This interpretation is in line with the finding that IgG ASC are abundantly present in inflamed kidneys, forming 90% of all plasma cells and plasmablasts. In contrast to bone marrow, the niches for plasma cells in kidneys arise late in relation with manifestation of inflammatory kidney disease in mice older than 5 months [6,7,25,42]. At this stage of development, there are more IgG plasmablasts capable of finding a niche in inflamed kidneys for further maturation and survival. Interestingly, the proportion of renal autoreactive plasma cells substantially increases in very old NZB/W mice with severe nephritis [7,25]. The question of whether the predominance of IgM-secreting cells in bone marrow or of IgG-secreting cells in inflamed kidneys is also influenced by tissue-specific factors remains unclear. Nearly half of the ASC in kidneys survive immunosuppressive treatment, suggesting that remaining cells are mature, long-lived plasma cells protected in this environment by inflammatory pro-survival signals. It can also not be excluded that the drugs have restricted access to some kidney plasma cells. Unlike B cells in kidneys, which are localized in distinct foci, plasma cells are scattered across the whole organ on kidney cross-sections [Panne D. et al., unpublished]. Therefore, plasma cells located in poorly perfused areas may not be eliminated by the treatment. This might explain why some autoantibodies such as scleroderma-specific autoantibodies in systemic sclerosis [43] or anti-Ro/SSA in SLE [44] can be detectable after drastic immunoablative regimens followed by autologous stem cell transplantation.

The microenvironment contributing to the survival niches for plasma cells in inflamed tissues is not completely understood. CXCL12, the ligand of CXCR4, is upregulated in inflamed kidneys of NZB/W mice [45]. In salivary glands of patients with Sjögren’s syndrome, plasma cells are localized in close proximity to CXCL12 and IL-6 producing cells such as epithelial cells, infiltrating mononuclear cells and adipocytes. Plasma cells themselves can highly express BAFF and APRIL, as can be observed in active SLE patients. This was also demonstrated for renal plasma cells in lupus nephritis [46]. BAFF and APRIL are ligands for BCMA, which is highly expressed on mature plasma cells and is essential for plasma cell survival [34].

The results of this study bring us one step closer towards understanding why immunosuppressive therapy is not able to cure systemic autoimmune diseases and why severe active disease can be refractory to treatment. They support our assumption that an autoreactive plasma cell memory located mainly in the bone marrow but also in secondary lymphoid tissues and inflamed organs contributes to the maintenance of autoimmunity. This plasma cell memory is responsible for autoantibody secretion independent of autoantigen stimuli and T cell help. B-cell depletion with monoclonal anti-CD20 antibody also does not affect this long-lived plasma cell compartment since plasma cells do not express CD20. Many reports show that autoantibodies can be resistant to B-cell-depletion therapy. Autoantibodies disappearing in response to B-cell-depletion therapy must be secreted by plasmablasts or short-lived plasma cells since their formation depends on continuous B cell activation and differentiation to autoantibody-secreting cells [2].

Because of its high stability, the elimination of the plasma cell memory in the bone marrow and inflamed tissues is a big challenge for new therapeutic approaches in autoimmunity. It was recently reported that the proteasome inhibitor bortezomib efficiently depletes short- and long-lived plasma cells from the bone marrow and spleen of NZB/W mice. As a consequence, serum anti-DNA antibody titers are reduced and renal disease ameliorated, resulting in prolonged survival of these mice [5]. First experiences with bortezomib in refractory SLE patients are promising [47,48]. Recent publications have also shown that antithymocyte globulin is able to deplete plasma cells [49]. Immunoablative therapies including antithymocyte globulin followed by transplantation of autologous hematopoietic stem cells can almost completely deplete memory plasma cells including autoantibody-secreting cells [44]. Future therapeutic strategies should therefore consider targeting auto-reactive plasma cell memory in bone marrow and inflamed tissues. This may include strategies for specific depletion of pathogenic

### Table 2

Number of anti-DNA ASC per organ in the different treatment groups compared to the control group (vehicle). Mean and SEM of anti-DNA IgM and IgG ASC per spleen, bone marrow and kidney are shown.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Spleen</th>
<th>Bone marrow</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>Vehicle</td>
<td>5282 ± 1318</td>
<td>7815 ± 3640</td>
<td>9381 ± 1166</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>1345 ± 287**</td>
<td>1000 ± 185***</td>
<td>8240 ± 1158</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1255 ± 163***</td>
<td>1275 ± 325**</td>
<td>9378 ± 1329</td>
</tr>
<tr>
<td>Combination*</td>
<td>850 ± 135***</td>
<td>563 ± 107***</td>
<td>6757 ± 1051</td>
</tr>
</tbody>
</table>

The asterisks denote significant depletion of ASC compared to the corresponding control group: *P < 0.05, **P < 0.01, ***P < 0.001 (n = 10, Mann–Whitney test).

* Cyclophosphamide plus dexamethasone.
plasma cells, manipulation of plasma cell survival and early prevention of the development of pathogenic plasma cell memory [2]. It is particularly pleasing to contribute this paper to the dedicated issue of Dr. Pierre Youinou, as Dr. Youinou has contributed so much to the diagnosis and treatment of autoimmune disease. This paper, as with the other dedicated manuscripts for distinguished autoimmunologists, is an attempt to provide an in depth overview as well as experimental data on an issue that is critical in immunology and rheumatology, much like the issues dedicated to Drs. Ian Mackay, Noel Rose, Harry Moutsopoulos and Chella David [50–58].

5. Conclusion

Aberrant production of autoantibodies is a hallmark of autoimmune diseases. Both short-lived plasmablasts/plasma cells and long-lived memory plasma cells can secrete pathogenic autoantibodies. The bone marrow is the major site of long-lived memory plasma cells including autoantibody-secreting cells, which are resistant to glucocorticoids and anti-proliferative drugs. Besides bone marrow, inflamed tissues and secondary lymphoid organs can accommodate autoreactive long-lived memory plasma cells and may thus contribute to the generation of pathogenic autoantibodies in spite of immunosuppression. These data provide an explanation why conventional immunosuppressive therapies 1) cannot cure chronic autoimmune disease and 2) are not effective in severe refractory autoimmune disease. Future therapeutic strategies should therefore consider targeting autoreactive long-lived memory plasma cells in bone marrow and inflamed organs. This could be the key to finding a curative approach to the treatment of chronic autoimmune autoantibody-mediated diseases.

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References


