

High frequency of autoantibody-secreting cells and long-lived plasma cells within inflamed kidneys of NZB/W F1 lupus mice

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Autoantibodies to double-stranded (ds) DNA represent a serological hallmark of systemic lupus erythematosus (SLE) and may critically contribute to the pathogenesis of lupus nephritis. Self-reactive antibodies might be partially produced by long-lived plasma cells (PCs), which mainly reside within the bone marrow and spleen. In contrast to short-lived PCs, long-lived PCs are extremely resistant to therapy and may sustain refractory disease courses. Recently, antibody-secreting cells were found within the inflamed kidneys of New Zealand black/white (NZB/W) F1 lupus mice as well as of patients with SLE. To analyze the longevity of the IgG-producing cells present in nephritic kidneys of NZB/W F1 mice we performed *in vivo* BrdU-labeling. We identified a higher frequency of long-lived than short-lived renal PCs, indicating that survival niches for long-lived PCs also exist within inflamed kidneys. Using ELISPOT assays, we found that on average 31% of renal IgG-producing cells reacted with dsDNA and 24% with nucleolin. Moreover, the frequencies of IgG-secreting cells specific for the autoantigens dsDNA and nucleolin were higher in the kidneys compared with those in the spleen and bone marrow.

Key words: Autoantibody-secreting cells · Autoimmunity · Long-lived plasma cells · Lupus nephritis



Supporting Information available online

Introduction

Autoantibodies critically contribute to the pathogenesis of various diseases including immune thrombocytopenia, autoimmune

hemolytic anemia, myasthenia gravis and systemic lupus erythematosus (SLE). The latter is a prototypic autoimmune disease, which can affect virtually all organs. Lupus nephritis is a frequent and serious complication. Anti-dsDNA antibody titers correlate with the clinical activity of the disease and there is accumulating evidence that anti-dsDNA antibodies are crucially involved in the pathogenesis of lupus nephritis [1, 2]. Anti-dsDNA and anti-nucleosome autoantibodies co-localize within the glomerular

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deposits in nephritic kidneys [2]. These immune complexes cause complement activation with the release of chemotactic factors, which is linked to recruitment of leukocytes [3]. Infiltrating inflammatory cells get further activated by Fc γ R-mediated mechanisms and essentially contribute to inflammatory organ destruction. These mechanisms lead to extensive inflammation and eventually renal lesions.

In lupus nephritis, cell infiltrates are predominantly composed of macrophages, dendritic cells, T and B lymphocytes including antibody-secreting plasma cells (PCs) [3]. The bone marrow (BM) and, to a lesser extent, the spleen represent the major homing sites of PCs, notably long-lived ones [4]. Additionally, a substantial number of PCs can be found in the mucosa, especially in the gut [5]. Antibody-secreting cells (ASCs) are also located in inflamed tissues, for instance within the nephritic kidneys of lupus mice and of SLE-patients [6–9] as well as in the synovial tissue of patients with rheumatoid arthritis [10]. Cassese et al. reported that after immunization of New Zealand black/white (NZB/W) F1 lupus mice with ovalbumin (OVA), OVA-specific antibody producing cells were initially found in the spleen [6]. Within weeks, they disappeared from the spleen and could then be detected in the BM and also within the inflamed kidneys. Hence, inflamed tissues may synthesize chemokines such as CXCL10, which recruit migratory plasmablasts to sites of inflammation. Apart from recent reports identifying cells secreting antibodies to histone H2B [8] and dsDNA [13], respectively, little is known about the antigen-specificity of ASCs within inflamed tissues. Also, it remained elusive whether inflammatory lesions can solely harbor short-lived PCs, or if they can also support the survival of long-lived PCs.

Non-dividing long-lived PCs play a critical role in maintaining protective antibody concentrations and may account for the majority of serum IgG [4]. These long-lived PCs may be located in niches providing survival factors such as APRIL or BAFF, stroma-derived factor-1 (SDF-1), IL-6, TNF- α , CD44 signaling, etc. to maintain continuous antibody production over time [11].

Here, we further characterize the renal ASCs in the course of experimental lupus. Remarkably, we not only identified short-lived, but also long-lived, PCs within the inflamed kidneys of NZB/W F1 mice, a mouse model resembling many features of SLE [12]. Moreover, we show that the frequencies of cells secreting IgG autoantibodies against dsDNA and nucleolin were significantly increased within nephritic kidneys when compared with those of the spleen and BM.

Results and discussion

Short- and long-lived PCs home to nephritic kidneys of NZB/W F1 mice

PCs can be detected within the inflamed kidneys of SLE patients and lupus mice; however, these ASCs have not yet been thoroughly characterized. Immunohistochemical staining on paraffin-sections of perfused kidneys from nephritic NZB/W F1

mice using anti-CD138 (Supporting Information Fig. 1A and B) showed PCs located within the renal tubulointerstitial tissue of medulla as well as cortex and often formed small clusters, similar to previous observations [6, 13].

Next we investigated if nephritic kidneys can harbor both short- as well as long-lived PCs. As shown in Fig. 1A, CD138⁺ intracellular κ and λ light chain⁺ PCs were detected at significantly increased numbers in aged lupus mice when compared with young, still healthy NZB/W F1 (8-wk-old) mice and >30-wk-old C57BL/6 mice. These results confirm the presence of significant numbers of PCs within the inflamed renal tissue in accordance to recently published data [8, 13]. To differentiate between short- and long-lived PCs we added bromodeoxyuridine (BrdU) to the drinking water, resulting in BrdU incorporation into the DNA of all proliferating cells including differentiating PCs, whereas non-replicating long-lived PCs did not incorporate BrdU. Long- and short-lived PCs as well as other proliferating and resting lymphocyte subpopulations in spleen and BM served as positive and negative controls for BrdU staining (Supporting Information Fig. 1C and data not shown). Remarkably, BrdU-positive short-lived as well as BrdU-negative long-lived PCs were both detected in the kidneys of lupus mice with established nephritis (Fig. 1B). The frequency of renal long-lived PCs was even higher than the frequency of short-lived PCs (Fig. 1C). In contrast to the recent data generated by Espeli et al., which only suggested the presence of long-lived PCs in kidneys based on the absence of the cell cycle marker Ki67 on the majority of renal PCs [13], we have directly demonstrated using BrdU incorporation that a large proportion of renal PCs had not been in S phase for the previous 2 wk. Taken together, these observations suggest that especially long-lived PCs contribute to the local antibody production in lupus nephritis. Our data are consistent with the concept that the inflammatory milieu supports the long-term survival of PCs, which either differentiate locally or migrate into the inflamed sites. It is yet unclear which factors within the inflamed kidneys are critical for the long-term survival of renal PCs; however, at least some well-known survival factors including IL-6 and TNF are locally expressed in lupus nephritis [14]. Furthermore, aberrant expression of APRIL and BAFF by B cells including PCs in SLE may contribute to PC survival also in nephritic kidneys [9]. In contrast to long-lived PCs within the BM and spleen, it is possible that long-lived PCs could be eliminated from inflamed organs by conventional immunosuppressive and anti-inflammatory drugs such as cyclophosphamide and glucocorticoids, because conditional “inflammatory survival niches” may disappear due to treatment. Also, spontaneous resolution of inflammation might deprive local PCs from their inflammation-mediated survival factors and thereby reduce the transiently increased total PC number to normal homeostatic levels.

Renal ASCs are predominantly directed against dsDNA and nucleolin

OVA-specific PCs were detected within nephritic kidneys of NZB/W F1 mice a few weeks post immunization, implying that

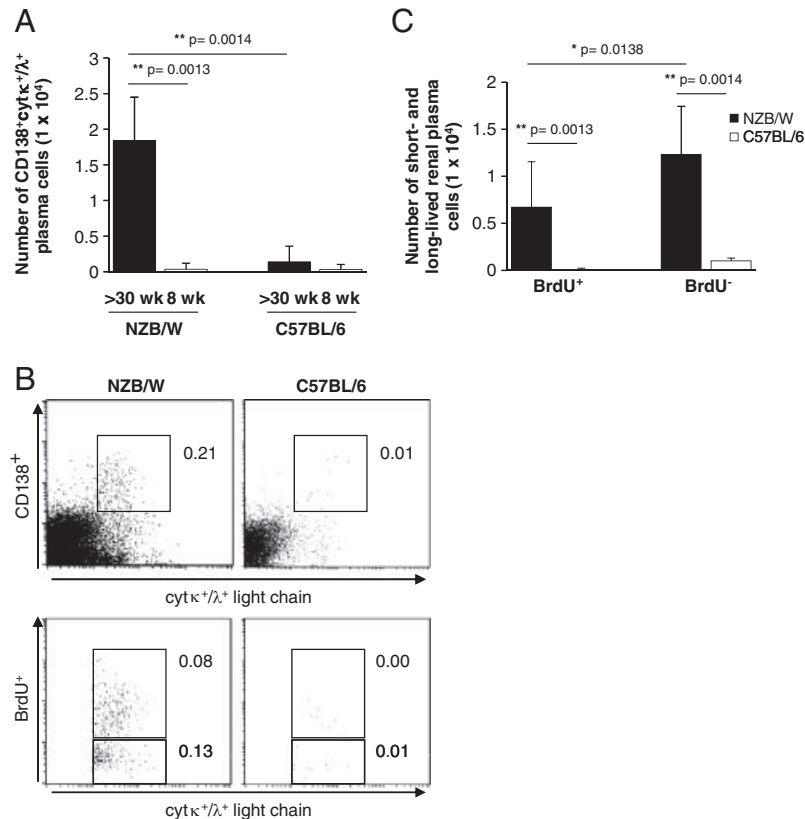


Figure 1. Short-lived and long-lived PCs in nephritic kidneys of NZB/W F1 mice. Single cell suspensions of renal tissue were analyzed for specific PC markers by flow cytometry and short-lived as well as long-lived PCs were differentiated by BrdU incorporation. (A) Total cell numbers of CD138⁺, intracellular κ/λ -light chain⁺ PCs per kidney in >30-wk-old ($n=12$) and 8-wk-old NZB/W F1 mice ($n=5$) compared with >30-wk-old ($n=5$) and 8-wk-old C57BL/6 mice ($n=5$) are shown. (B) Representative flow cytometric analysis of short-lived and long-lived PCs isolated from the kidney of a >30-wk-old NZB/W F1 mouse (left panels) versus a >30-wk-old C57BL/6 mouse (right panels). Gated PCs (upper panel) were discriminated in BrdU⁺ short-lived and BrdU⁻ long-lived PCs (lower panel). Numbers next to the region gates represent percentages of detected PCs with regard to the total cell numbers. (C) Bar graphs of flow cytometric analyses of the total cell numbers of CD138⁺, cytoplasmic κ/λ ⁺, BrdU⁺ short-lived as well as BrdU⁻ long-lived PCs per kidney of >30-wk-old NZB/W F1 and >30-wk-old C57BL/6 mice. (A and C) Horizontal bars represent mean+SD from three independent experiments. Statistical analyses were performed using Mann–Whitney *U* test.

migratory plasmablasts get recruited to the inflamed tissue, independently of their antigen specificity [6]. Using ELISPOT we analyzed the total numbers of IgG-secreting cells and, in parallel, the numbers of cells secreting antibodies to dsDNA and nucleolin. Nucleolin is a protein forming ribonucleoprotein-particles, as it is the case with several other autoantigens in SLE. IgG antibodies to nucleolin are found in approximately 40% of SLE sera and are relatively specific for SLE [[15], Wellmann et al., manuscript in preparation]. In spleen and BM of >30-wk-old NZB/W F1 mice the numbers of PCs secreting autoantibodies to dsDNA and nucleolin were significantly increased compared to age-matched C57BL/6 non-autoimmune mice (Supporting Information Fig 2A).

To determine whether PCs secreting IgG to dsDNA and nucleolin make up the majority of IgG-secreting cells in nephritic kidneys, we analyzed the total numbers of IgG-secreting cells and the numbers of cells secreting IgG antibodies to dsDNA and nucleolin. ELISPOT with single cell suspension from >30-wk-old female NZB/W F1 mice displaying high titers of anti-dsDNA autoantibodies and proteinuria resulted in significantly increased numbers of infiltrating IgG-secreting cells in their inflamed

kidneys when compared to young healthy NZB/W F1 and to non-autoimmune C57BL/6 mice (Fig. 2A). Most importantly, a large fraction of autoreactive cells produced antibodies reacting with dsDNA (31%) and/or nucleolin (24%) (Figs. 2B, C and 3B). Hence, autoantibodies, especially anti-dsDNA antibodies involved in the pathogenesis of lupus nephritis, are produced within the inflamed organ. Previous experiments revealed enriched anti-dsDNA antibodies after elution of immunoglobulins from glomeruli, we now demonstrate the existence and disease-dependent appearance of these presumably pathogenic ASCs in the renal tissue of lupus mice [16]. Similar to our results, Espeli et al. recently identified anti-dsDNA secreting cells in inflamed kidneys of NZB/W F1 mice. However, they neither analyzed additional autoantigens such as nucleolin nor compared frequencies of autoreactive PCs in kidneys with their frequencies in spleen and BM [13]. Our results suggest that, in addition to circulating anti-dsDNA IgG produced elsewhere, IgG antibodies produced by PCs that have infiltrated inflamed kidneys also contribute to lupus nephritis. Possibly, the absence of autoantibody production with high local antibody

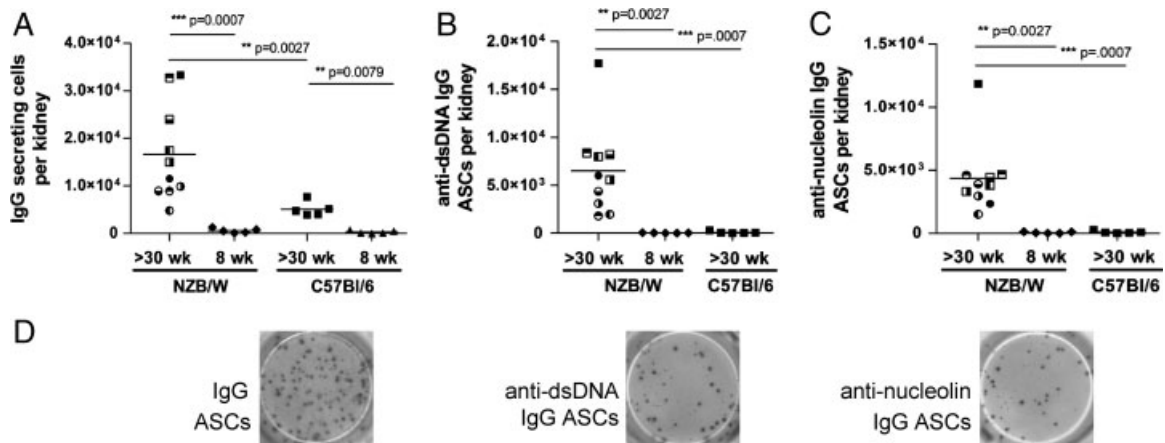


Figure 2. ASCs within inflamed kidneys of NZB/W F1 mice. ELISPOT results (A–C) indicate total numbers of IgG-ASCs as well as autoreactive anti-dsDNA IgG- and anti-nucleolin IgG-secreting cells detected per kidney in >30- and 8-wk-old NZB/W F1 mice compared with >30- and 8-wk-old C57BL/6 mice. The results of three independent experiments are shown. Corresponding data points for each individual aged NZB/W F1 mouse are identified by a filled square or circle with a unique pattern. Horizontal bars indicate the mean. Statistical analyses were performed using Mann–Whitney *U* test. (D) Representative wells of antigen-specific ELISPOT assays are shown.

concentrations within kidneys could account for the variable or mild nephritogenicity of certain transferred anti-dsDNA antibodies in mouse models [17]. However, the pathogenic relevance of in situ production of autoantibodies yet needs to be determined.

Autoreactive PCs are enriched in renal tissue

Next, we compared the total cell numbers and relative frequencies of cells secreting IgG, anti-dsDNA-IgG and anti-nucleolin-IgG in nephritic kidneys with their frequencies in the spleen and femoral BM (Fig. 3A and B). Interestingly, the percentage of autoreactive PCs within the population of all IgG-secreting cells was increased in the nephritic kidneys of lupus mice with advanced disease compared to spleen and BM (Fig. 3B). Furthermore, a comparison of antigen-specific PCs within each individual mouse seems to indicate that a low frequency of splenic auto-ASCs correlated with an increased frequency within the kidneys and vice versa. Although a preferential migration of autoreactive PCs from the spleen into the inflamed kidneys might explain these findings, this model lacks experimental evidence. It is yet unclear if at least a fraction of the autoreactive renal PCs has actually differentiated in situ within the kidneys, or whether the inflamed organ more efficiently attracts migratory plasmablasts and young PCs than BM and spleen [18].

Concluding remarks

ASCs critically contribute to antibody-mediated autoimmune diseases such as SLE. Especially long-lived PCs, which are resistant to conventional treatments, might be responsible for refractory disease courses. Autoantibodies to dsDNA are most likely involved in the pathogenesis of lupus nephritis. Here, we

demonstrated that short-lived as well as long-lived PCs populate nephritic kidneys of NZB/W F1 mice. Importantly, our data indicate that nephritic kidneys can provide survival niches for long-lived PCs. In addition, we detected a substantial amount of PCs secreting autoantibodies against dsDNA and nucleolin within inflamed kidneys of NZB/W F1 mice, implying that at least some of the autoantibodies deposited in nephritic kidneys are produced in situ. Moreover, the frequency of cells secreting antibodies to dsDNA and nucleolin is enriched in nephritic kidneys when compared to spleen and BM.

Materials and methods

Mice

Animal experiments were approved by the government of Mittelfranken (Regierung von Mittelfranken, AZ 54-2532.1-13/08). Female NZB/W F1 mice were bred under specific pathogen-free conditions at the animal facility of the University of Erlangen-Nuremberg. C57BL/6 mice were purchased from Janvier (Le Genest St. Isle, France). NZB/W F1 mice of >30 wk of age were screened for proteinuria using a dip stick assay (Albustix, Siemens Healthcare Diagnostics, USA). Mice with a semiquantitative proteinuria graded at least 300 mg/dL together with markedly increased anti-dsDNA serum titers ($OD_{495} > 0.8$) were considered to have advanced nephritis. Renal tissues from nephritic mice, 8-wk-old healthy NZB/W F1 mice and >30-wk-old as well as 8-wk-old C57BL/6 mice were digested in a solution containing 2 mg/mL collagenase D; 0.1 mg/mL deoxyribonuclease I (Roche, Mannheim, Germany) and 10 mM HEPES in RPMI medium supplemented with 5% FCS at 37°C for 60 min. Single-cell suspensions from spleen, BM (both femurs) and kidneys were analyzed by flow cytometry and ELISPOT assay.

BrdU labeling of proliferating cells

Mice were fed for 14 days with drinking water containing BrdU (0.8 mg/mL; Sigma-Aldrich, Taufkirchen, Germany) and 2% saccharose (Roth, Karlsruhe, Germany). Incorporated BrdU was detected in PC populations using the BrdU flow kit (BD Biosciences, Heidelberg, Germany).

Flow cytometric analyses

To define the PC population cells of the digested kidneys were stained with anti-CD138-APC (BD Pharmingen, USA). Then cells

were permeabilized using Fix & Perm Cell Permeabilization Kit (Caltag Laboratories, Hamburg, Germany) according to the manufacturer's instructions and stained with anti-Ig-kappa-PE as well as anti-Ig- λ -PE (Southern Biotech, USA). The labeled cells were analyzed using a BD FACS Calibur and the Cell Quest™ software.

Quantification of ASCs by ELISPOT assay

Kidneys were thoroughly rinsed, with 0.9% sodium chloride solution. Initial experiments demonstrated no significant differences between perfused and rinsed kidneys in terms of T, B and PC numbers that could be recovered from kidneys. ELISPOT

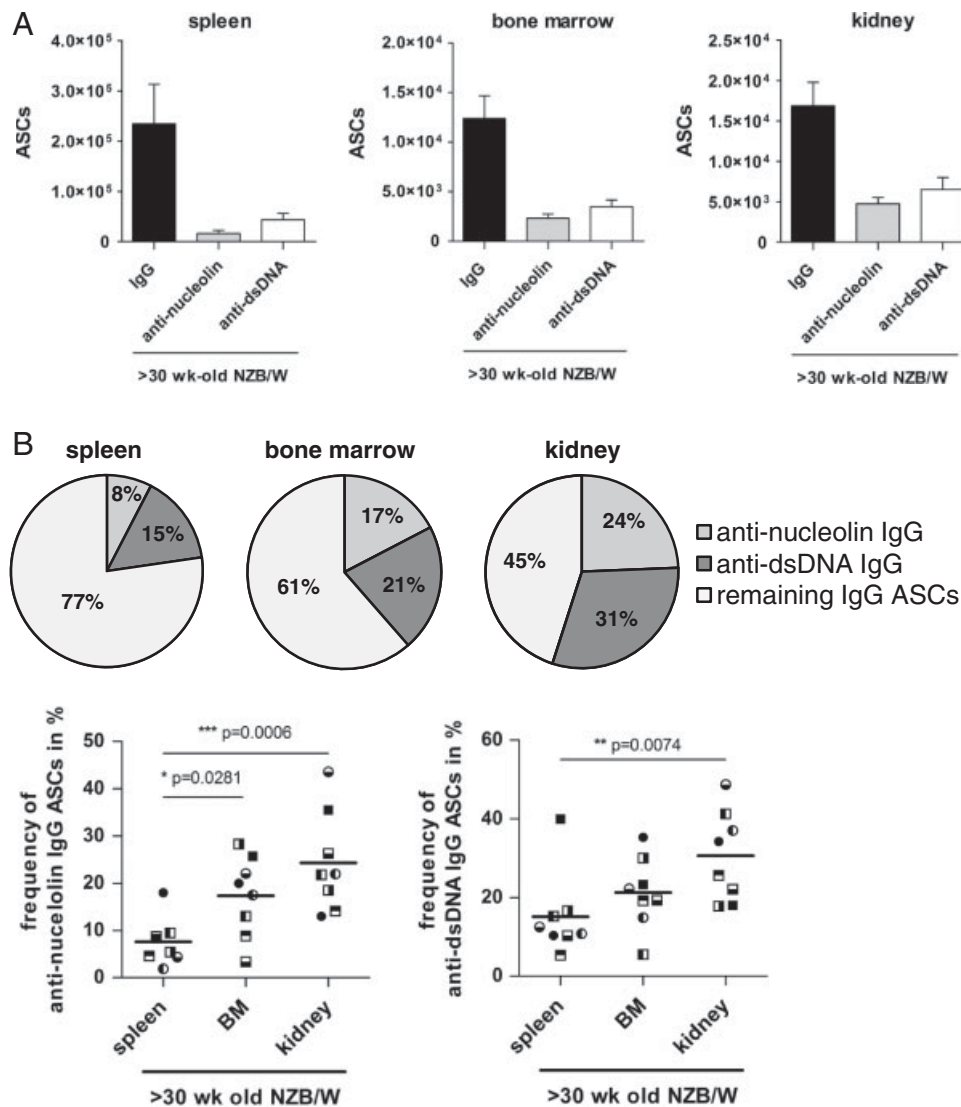


Figure 3. Enriched auto-ASCs in renal tissue of diseased NZB/W F1 mice. (A) Quantification of total numbers of IgG, anti-dsDNA and anti-nucleolin ASCs from the same donor mice as detected by ELISPOT in spleen, two femurs BM and per kidney of >30-wk-old lupus mice ($n=8$). Horizontal bars represent mean + SEM. (B) Percentages of autoreactive anti-dsDNA- and anti-nucleolin-secreting cells with regard to the total cell numbers of IgG-secreting cells are represented in a pie chart for each analyzed organ and summarized in dot plot graphs to evaluate the proportion of autoreactive cells. Relative frequencies of anti-dsDNA- and anti-nucleolin-specific IgG-ASCs detected by ELISPOT in the spleens, BMs and kidneys of >30-wk-old lupus mice with regard to the total cell numbers of IgG-secreting cells are shown. Data points of each individual aged NZB/W F1 mouse are represented by a filled square or circle with a unique pattern throughout the graphs. Horizontal bars represent mean values. Three independent experiments were pooled. Statistical analyses were performed using the two-tailed paired T-test.

multiscreen plates (Millipore, Billerica, MA, USA) were coated with anti-mouse IgG (1 µg/mL, Southern Biotech) or precoated with poly-L-lysine (Sigma) followed by calf thymus DNA (20 µg/mL, Sigma) or highly purified recombinant nucleolin (10 µg/mL, Diarect, Freiburg, Germany) kindly provided by U. Wellmann (University Erlangen-Nuremberg, Germany). Purity of recombinant nucleolin was assessed by silver staining (Supporting Information Fig. 2B). After blocking with 2% FCS in PBS, single cell suspensions from kidney, spleen and BM from two femurs were incubated for 2 h at 37°C. Plates were washed and incubated with HRP-goat antibody to mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h at 20°C. Spots were detected by tetramethylbenzidine substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) and analyzed using an automatic ELISPOT reader (AID Diagnostics, Strassberg, Germany) and AID ELISPOT reader software 4.0.

Statistical analysis

Data were analyzed using either a non-parametric Mann–Whitney *U* test or a two-tailed paired *T*-test using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Both, the Kolmogorov–Smirnov test and the Shapiro–Wilks test were applied to test for a normal distribution. Significant differences are indicated as * for $p < 0.05$, ** for $p < 0.01$ and *** for $p < 0.001$.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: ASCs: antibody secreting cells · NZB/W: New Zealand black/white · PCs: plasma cells · SLE: systemic lupus erythematosus

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