Title page

Subset size, activation threshold and distribution of autoreactive MZ and FO B cells do not differ in a sex specific manner in the NZB/W F1 murine lupus model.

An experimental mouse study.

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Subset size, activation threshold and distribution of autoreactive MZ and FO B cells do not differ in a sex specific manner in the NZB/W F1 murine lupus model.

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Abstract

Objectives: Systemic lupus erythematosus shows a strong sex bias preferentially affecting females and B cells are thought to play a pivotal role in its pathogenesis. Here, we compared the splenic B cell compartments, their autoreactivity and activation threshold of female and male NZB/W F1, a murine lupus model reflecting sex bias observed in SLE patients.

Methods: Autoantibody levels and the amount of autoantibody secreting cells were determined using ELISA and ELISPOT. Flowcytometry and immunofluorescence were applied to analyse the composition of the splenic B cell pool. Purified Follicular (FO) and marginal zone (MZ) B cells were stimulated and the frequency of autoreactive cells was determined. Finally, the proliferative response of FO and MZ B cells upon stimulation was assessed using CFSE dilution and [³H]-Thymidin incorporation.

Results: Higher autoantibody titers were detected in female NZB/W F1, which were mainly produced in the spleen. Analyzing the composition of the splenic B cell subsets, no differences were found prior to disease development. Autoreactive dsDNA-specific B cells were mostly found in the MZ compartment, while SmD1₍₈₃₋₁₁₉₎ reactive cells were more evenly distributed. Equal frequencies of autoreactive B cells

were found in female and male mice and no difference in the response to polyclonal stimuli of the cells of both sexes was detected.

Conclusions: No differences in the composition or functionality of splenic B cells were observed that account for the different disease course in both sexes.

Introduction

Autoimmune diseases such as Systemic Lupus Erythematosus (SLE) show a strong gender bias, with 9 times more women than men being affected. Females are especially prone to develop lupus in the so called child bearing years, the period in life that is characterized by enhanced production of sex hormones (1). The risk of developing a lupus flare is further increased during pregnancy, when the endogenous levels of estrogens further increase (2-3).

Similar to humans, most mouse models for SLE mirror the “preference” of lupus for females (4). Female New Zealand Black x New Zealand White F1 mice (NZB/W F1) mice develop a lupus like syndrome earlier and more severely than their male siblings. In the NZB/W F1 model application of estrogens accelerates the disease (5-6), while androgens (7), antiestrogens (8) and ovariectomy (9) have a protective effect.

Besides sex hormones, non-hormonal factors may add to the preponderance of SLE in females. Many genes playing key roles in the regulation of the immune system such as Foxp3, CD40Ligand (CD40L) and Toll like receptor 7 (TLR7) are expressed on the X-chromosome (10). It has been proposed that sex differences are attributable to a gene dosage effect, like it has been shown for TLR7 and lupus (11).

One of the hallmarks of SLE is the production of autoantibodies against nuclear components such as double stranded deoxyribonucleic acid (dsDNA) and the SmD1 peptide (amino acid 83-119) (SmD1₍₈₃₋₁₁₉₎) by autoreactive B cells (12-13). Sex

specific factors influencing the autoantibody production have been hypothesized, however the cellular mechanisms remain elusive. Mature conventional B cells in the secondary lymphoid organs can be mainly divided into marginal zone (MZ) B cells and follicular (FO) B cells. Studies using transgenic mice suggest that autoreactive B cells are preferentially selected into the MZ B cell compartment, which helps maintaining immune tolerance (14). Further experiments with gene modified mice provided evidence that sex hormones influence the signaling strength of the B cell receptor and thereby modify the selection and survival of autoreactive B cells (15). Especially estrogen has been shown to expand the MZ compartment, activate transgenic MZ B cells and support the production of autoantibodies (16). Other studies demonstrated that female cells are more easily activated by certain TLR ligands than male cells (17).

In the present study, we probed the hypothesis that the sex differences in lupus are attributable to changes in the composition and functional properties of B cells. The NZB/W F1 mouse model for SLE was used to determine autoantibody levels, size and architecture of the splenic B cells compartments, frequency of autoreactive cells and stimulatory response in female and male B cells. We conclude that the sex discrepancy in lupus is not due to altered B cell properties.

Materials and Methods

Mice

NZB/W F1 (NZB x NZW F1) mice were obtained from the Bundesinstitut für Risikobewertung in Marienfelde, Berlin, Germany. Mice were kept in a specific pathogen free (SPF) environment at the DRFZ (German Rheumatology Research Centre), Berlin. In this mouse strain, young mice showed no urinary abnormalities. In contrast, all female mice at an age > 30 weeks demonstrated a marked proteinuria (300-2000 mg/dl) as detected by Multistix (Bayer Diagnostics, Germany) and were considered to have active nephritis and advanced lupus disease. Male mice up to 6 months of age showed no urinary abnormalities. All experiments were conducted with the approval of the local authorities Landesamtes für Gesundheit und Soziales Berlin (T0016/08).

Enzyme-linked immunosorbent assay (ELISA)

Serum samples of female and male mice were acquired monthly upon an age of 34 weeks. An anti-dsDNA and anti-SmD1₍₈₃₋₁₁₉₎ ELISA was used to analyse serum concentrations of autoantibodies. Sera were analysed in triplicates as described previously (13, 18).

Isolation of cells

Spleen cells were isolated from NZB/W F1 at different ages (between 6 and 36 weeks) as specified. Mice were sacrificed by cervical dislocation. Spleens were mashed through a 70µmØ cell strainer (BD Biosciences, Germany) and the cell suspension was washed once with PBS/BSA. Renal infiltrating cells, thymus cells, peritoneal cavity cells and bone marrow cells were isolated from aged mice. Kidneys were mashed through a 70µmØ cell strainer (BD Biosciences, Germany) and the cell

suspension was washed once with PBS/BSA. Renal lymphocytes were separated from other cells and cell detritus by density centrifugation using Percoll 1.124 (Biochrom AG, Germany) in 40% and 70% v/v dilution with RPMI media (PAA Laboratories, Germany). After centrifugation, lymphocytes were isolated and immediately washed twice with PBS/BSA. To isolate peritoneal cavity cells the peritoneum was opened and the cavity was gently washed with 10ml cold PBS containing 0.5% bovine serum albumin (PBS/BSA). Thymus cells were mashed through a 70µmØ cell strainer, for isolation of bone marrow cells both femurs were washed out using a syringe. Erythrocytes were eliminated by lysis using a solution containing 0.83%NH₄Cl and 0.01M TRIS-HCl at pH 7.5 ([Red blood cell lysing solution](#)) followed by washing with PBS/BSA. Renal and thymus cells were pooled from 5 mice to have sufficient cell numbers for analysis.

Quantification of antibody secreting B cells by Enzyme Linked Immuno Spot Technique (ELISPOT)

For quantification of antigen specific cells, purified FO and MZ cells were stimulated for 3 days with 10µg/ml LPS (Sigma-Aldrich, Germany) or with a combination of 10µg/ml anti-mouse CD40 IgG clone FGK-45 and 20ng/ml IL-4 (R&D, Germany) in complete RPMI medium containing 2mM L-Glutamine, 100 units/ml penicillin G, 100µg/ml streptomycin, 50µg/ml Gentamycin (PAA Laboratories, Germany), 50 µM 2-mercaptoethanol (Invitrogen, Karlsruhe, Germany), 10% FCS (Sigma-Aldrich, Germany). To determine dsDNA specific antibody secreting cells (ASC), high binding 96-well plates (Corning, USA) were first coated with 10µg/ml methylated-BSA (Sigma-Aldrich, Germany) for 24h and then coated with 10µg/ml calf thymus DNA (Sigma-Aldrich, Germany) in PBS for 24h. For determination of anti SmD1₍₈₃₋₁₁₉₎ specific ASC, high binding 96-well plates were coated with SmD1 peptide (aa 83-

119) (Department of Biochemistry, Charité, Germany) in a concentration of 10µg/ml in PBS overnight. To detect total ASC, plates were coated with goat anti-mouse Ig (Southern Biotech, USA). Plates were blocked with complete RPMI medium, activated cells incubated on the plates and washed of after 16-24h. After extensive washing bound antibodies were detected with goat anti-mouse Ig-biotin (Southern Biotech, USA). After washing Streptavidin-Alkaline phosphatase (AP) (Roche, Germany) was added, and AP activity was visualized with developing buffer containing 5-Bromo-4-chloro-3-indol-phosphat (BCIP) and 0.6% Agarose (Sigma-Aldrich, Germany). ASC were enumerated as blue spots using an inverted microscope (Leitz, Germany). Percentages of antigen specific B cells were calculated by dividing the number of specific ASC by the number or total ASC (%Ag specific ASC = Ag specific ASC/total ASC x100).

To analyze the number of IgM and IgG specific ASC directly *ex vivo*, cells were transferred in complete RPMI medium immediately after isolation without stimulation onto ELISPOT plates. Cells were washed of after 24h of incubation and the amount of total IgM and IgG and the amount of dsDNA and SmD1₍₈₃₋₁₁₉₎ specific IgM and IgG producers were determined. IgM antibodies were detected with µ-chain specific goat anti-mouse IgM-biotin (Southern Biotech, USA), IgG was detected using γ-chain specific goat anti-mouse IgG-biotin (Southern Biotech, USA).

Flow cytometric (FACS) analysis of the cells

Surface markers were stained by incubating single-cell-suspensions with staining antibodies for 20min, non-bound antibodies were removed by washing with PBS/BSA. To minimize unspecific binding, Fc Receptors were blocked using a surplus of unconjugated anti-Fc-gamma antibody clone 2.4G2 (DRFZ, Berlin). The following antibodies were used: anti-mouse CD21-Cy5 clone 7G6 (DRFZ, Berlin),

anti-mouse CD23-PE clone B3/B4 (DRFZ, Berlin), anti-mouse CD19-FITC clone 1D3 (DRFZ, Berlin). Dead cells were stained with Propidium Iodide (PI) (Sigma, Germany). Cells were analysed using a Calibur flow cytometer (BD Pharmingen, Germany) and data were analysed using Flojo Software (Tree Star, USA).

Immunofluorescence

Spleens were frozen in Tissue-Tek OCT (Sakura, Staufen) compound and stored at -70°C . Frozen tissue sections of $7\mu\text{m}$ were prepared, fixed in acetone for 10 min, and air dried. Splenic sections were pre-treated with PBS containing 3% BSA to block unspecific binding. The sections were stained with anti-mouse IgD-FITC clone 11-26c.2a (BD Pharmingen, Germany) and μ -chain specific goat anti-mouse IgM-bio (Southern biotech, USA), Streptavidin-Rhodamin (Southern biotech, USA), was used to visualize biotin. Finally, slides were counterstained using DAPI (2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride)(Sigma, Germany).

Cell sorting

MZ and FO B cells were purified using a FACSDiva or FACS Aria (BD Pharmingen, Heidelberg, Germany) cell sorter. To purify splenic MZ and FO B cells, splenocytes were stained for CD19, CD21, CD23 and PI. Purity of the isolated MZ and FO B cells was routinely $\geq 95\%$.

Cell proliferation

FO and MZ B cells purified by FACSsorter were labeled with carboxy-fluorescein-diacetate-succinimidyl ester (CFSE) (Molecular Probes, USA) at a final concentration of $0.5\mu\text{M}$ for 3 min at room temperature. After labelling, the cells were stimulated with $1\mu\text{g/ml}$ LPS, $1\mu\text{g/ml}$ Imiquimid (Invivogen, USA) or $1\mu\text{g/ml}$ CPG (5'- tcc atg

acg ttc ctg acg tt -3', Invivogen, USA) or a combination of 10µg/ml anti-CD40 IgG clone FGK-45 and 20ng/ml IL-4 in complete RPMI for 3 days. Cell proliferation was analyzed by CFSE dilution using flowcytometry. Alternatively, after 2d 1µCi [³H]Thymidine was added to the cultures and 16h later cells were harvested onto filter-bottom UniFilter-96 GF/B plates using a FilterMate cell harvester. Subsequently 40µl scintillation fluid was added to the cells harvested on the filter plate. The amount of incorporated [³H]Thymidine was measured by a Top-count NXT liquid scintillation counter as counts per minute (cpm).

Statistics

Means, standard deviations, Mann-Whitney test, Wilcoxon test and students T Test were calculated using Graphpad Prism 3.0 (GraphPad Software, San Diego, USA). To calculate p values, the Mann-Whitney test was applied except when otherwise indicated. p-values of ≤ 0.05 were considered significant. In order to compare ELISPOT experiments and proliferative response data a ratio was calculated. Each value was divided by the mean value for female and male cells (ratio = value/(mean female + male), with 1 indicating identical response rate for female and male cells.

Results

Female NZB/W F1 develop higher titers of autoantibodies which are mainly produced in the spleen.

Consecutive autoantibody levels were determined in a group of female and male NZB/W F1 until an age of 34 weeks (n=5 in each group). As expected, female mice presented autoantibodies against the nuclear autoantigens dsDNA and SmD1₍₈₃₋₁₁₉₎ earlier and at higher titers than their male siblings (Figure 1 A and B). IgM autoantibodies showed a continuous increase in female mice over the period monitored, while IgG autoantibodies showed a sharp increase at an age between 22 and 26 weeks. The increase in IgG autoantibodies was followed by the development of nephritis, at an age of 26 weeks the female mice had developed relevant proteinuria which was not observed in male mice until the end of the observation period (p 0.0003 at week 26, t test).

At an age of 34 weeks, mice were sacrificed and the sites of antibody production determined (n=5 in each group). In the spleen an average of $21.16 \pm 13.97 \times 10^6$ IgM producers per spleen were detected in female mice compared to $11.81 \pm 3.64 \times 10^6$ in male mice, although this difference did not reach significance (p=0.22). By comparing IgG producers, female mice had significantly more IgG ASC per spleen than male mice ($3.31 \pm 1.95 \times 10^6$ IgG producers versus $0.12 \pm 0.05 \times 10^6$, respectively, p=0.0079). In line with the higher amount of total antibody producing cells, more than 10-fold higher amounts of IgM and IgG anti-dsDNA and anti-SmD1₍₈₃₋₁₁₉₎ producers were found in the spleen of female mice (p<0.01 for all), (Figure 1 C-F). Furthermore we observed a higher frequency of IgM autoantibodies in the spleen of female mice (dsDNA IgM AFCs females $0.51 \pm 0.26\%$, males $0.06 \pm 0.02\%$, p<0.01) (IgM SmD1₍₈₃₋₁₁₉₎ AFCs females $0.5 \pm 0.14\%$, males $0.01 \pm 0.01\%$, p<0.01). There was no significant difference in the frequency of splenic anti-dsDNA IgG autoantibody producers (anti-

dsDNA IgG females $1.3 \pm 0.34\%$, males $1.3 \pm 0.76\%$, $p=1.0$), although there was a trend towards a higher frequency of anti-SmD1₍₈₃₋₁₁₉₎ IgG secretors in female mice (females $1.2 \pm 0.26\%$, males $0.51 \pm 0.45\%$, $p=0.0952$).

The second major site of antibody production was the bone marrow of the mice. Interestingly we found no significant difference neither in the amount of IgM producers (females $164.1 \pm 36.1 \times 10^3$, males $163.7 \pm 112.6 \times 10^3$, $p=0.4206$), IgG producers (females $13.0 \pm 9.4 \times 10^3$, males $14.7 \pm 6.6 \times 10^3$, $p=0.8413$), nor IgG or IgM autoantibody producers (Figure 1 C-F). Furthermore, the bone marrow of female mice contained significantly lower frequencies of autoantibody producers than the splenic compartment (dsDNA IgM $0.1 \pm 0.05\%$, dsDNA IgG $0.46 \pm 0.06\%$, SmD1₍₈₃₋₁₁₉₎ IgM $0.03 \pm 0.04\%$, SmD1₍₈₃₋₁₁₉₎ IgG $0.18 \pm 0.12\%$, $p<0.01$ for IgM dsDNA, IgM SmD1₍₈₃₋₁₁₉₎ and IgM dsDNA, $p<0.05$ for IgG SmD1₍₈₃₋₁₁₉₎).

In the peritoneal cavity of female mice significantly more cells spontaneously producing dsDNA IgM autoantibodies were found than in male mice ($p<0.05$) (Figure 1 C-F). Furthermore more antibody producing cells in the inflamed kidneys of female mice (42.2×10^3 IgM AFCs and 300 IgG AFCs) were detected compared to male mice (7.8×10^3 IgM AFCs and 2 IgG AFCs, pooled cells from 5 mice). Higher ASC numbers in females were also found in the thymus (187×10^3 IgM AFCs and 297×10^3 IgG AFCs compared to 110×10^3 IgM AFCs and 115×10^3 IgG AFCs in males, pooled cells from 5 mice).

Changes in the composition of the female and male splenic B cell compartment with progressive age.

The size of the splenic B cell compartment and the proportion of FO and MZ B cells were determined in female and male mice at different ages. In 6 week old mice we observed no difference in the percentage or the total amount of splenic B cells, FO B

cells (CD19⁺CD21^{low}CD23^{high}), MZ B cells (CD19⁺CD21^{high}CD23^{low}) or other B cells (CD19⁺CD21⁻CD23⁻) (Figure 2 B and D) (n=10 in each group, no proteinuria). With progressive age, a decrease in the percentage of splenic FO B cells in female NZB/W F1 was detected. At an age of 12-14 weeks, FO B cells accounted for 63.9 ±2.8% of all splenic B cells compared to 63.4 ±6.0% in males (p=0.8534)(n= 6 female and 10 male mice), and in 6 month old mice this figure had decreased to 53.5 ±2.0% in female and 60.4 ±1.1% in male NZB/W F1 (p=0.0012)(Figure 2 C and E)(n= 6 female and 7 male mice).

In line with this quantitative observation, no qualitative differences in the splenic architecture were detected using immunofluorescence (n=3 in each group). In splenic section of young mice, FO cells (IgD^{high}IgM^{low}) and MZ cells (IgD^{low}IgM^{high}) showed identical distribution patterns. Immunofluorescence of splenic sections of 6-8 month old female and male mice revealed that the majority of MZ B cells had left the marginal zone around the follicles and migrated to the interfollicular zone and into the germinal center. In contrast, the FO B cells still mainly remained in the follicular structures around the germinal center (Figure 3).

Female and male NZB/W F1 mice do not differ in the frequency of autoreactive FO or MZ cells.

Splenic FO and MZ cells of healthy mice at an age of 8-14 weeks were purified, stimulated with LPS and the frequency of autoreactive cells determined using ELISPOT (n=6 female and male mice analysed in 4 separate experiments). B cells reactive with dsDNA were mainly found in the MZ compartment (mean 0.95% of all ASC), while FO B cells contained less cells with specificity for dsDNA (mean 0.56%, p<0.05 Wilcoxon matched pairs test) (Figure 4 B). SmD1₍₈₃₋₁₁₉₎ reactive cells were found in similar frequencies in the MZ and FO B cell compartment and these cells

were found in lower frequencies than dsDNA B cells (mean 0.05% of all MZ ASC, 0.06% of all FO ASC, $p=0.8$)(Figure 4 C). Similar results were obtained when the cells were stimulated with a combination of anti-CD40L and IL-4.

Comparison of the frequency of autoreactive B cells between female and male mice revealed no difference for dsDNA or SmD1₍₈₃₋₁₁₉₎ reactive MZ and FO B cells (Figure 4 D-F). In order to compare different experiments, a stimulation ratio was calculated, with a value of 1 indicating identical frequencies of autoreactive cells. For LPS activated female MZ cells the ratio for dsDNA reactive cell was 1.015 ± 0.181 compared to 0.985 ± 0.160 for males ($p=0.75$), and for FO cells 1.014 ± 0.204 opposed to 0.986 ± 0.128 ($p=0.59$), (Figure 4 D). Similar result were obtained for SmD1₍₈₃₋₁₁₉₎ reactive cells (Figure 4 E). Neither variation of the stimulation regime (LPS or anti-CD40L + IL-4) nor of the age of the mice (female mice aged 22 weeks and older with nephritis as indicated by proteinuria and age matched male mice, $n=4$) resulted in a significant difference in the frequency of autoreactive splenic B MZ or FO B cells between the two sexes of NZB/W F1.

B cells of female and male origin do not differ in their activation threshold or in their proliferative response upon stimulation

In order to compare the activation threshold of female and male B cells purified FO and MZ cells from pre-diseased mice (age 12 weeks) were stimulated with LPS, CPG, Imiquomid and anti-CD40L + IL-4 (pooled cells from 8 mice, 4 separate experiments). Using CFSE dilution and [³H]-Thymidin incorporation there were no differences in the activation threshold or proliferative response neither upon stimulation with various TLR ligands (LPS, CPG, Imiquomid) nor upon stimulation with anti-CD40L and IL-4 (Figure 5 A-D). Comparing the different experiments by calculating the proliferative ratio of female and male cells (with 1 indicating identical

proliferation), no significant difference was detected neither for MZ (Figure 5 E) nor FO B cell (not shown). In line with these observations, no difference in the number of antibody producing cells upon stimulation of purified female and male FO and MZ B cells with LPS or anti-CD40L + IL-4 was observable (Figure 5 F and G).

Discussion

In the present study we analysed the composition of the splenic B cell compartments, the frequency of autoreactive B cells and the activation threshold of B cells from female and male NZB/W F1 mice. No sex specific differences prior to overt lupus disease were evident.

One hallmark of lupus is the occurrence of autoantibodies against nuclear components such as dsDNA or SmD1₍₈₃₋₁₁₉₎ (12-13, 19), however the exact events leading to the development of SLE are ill defined. Notably, the disease shows a strong sex bias as SLE affects nine times more women than men and murine models almost unequivocally reflect this sex bias (1, 4). In the NZB/W F1 model, female mice succumb to a lupus like disorder earlier than their male siblings, show a more accelerated course and decess at a younger age (4). Given the small genetic difference between female and male NZB/W F1 and the overt different disease course, we found it appealing to determine the cellular basis for the latter.

As expected, female NZB/W F1 developed autoantibodies earlier and at higher levels than male mice. In line with the autoantibody levels, female mice presented signs of nephritis at a time point, when their male siblings showed no urinary abnormalities at all. In female mice, the spleen turned out to be the major site of (auto)antibody producing cells, containing more than 100 times more total antibody producers and autoantibody producers than the bone marrow of two femurs. Given that 12-16% of the total bone marrow is found in the femurs of mice (20), even extrapolating the amount of ASC in the femurs to the total bone marrow mass falls short of the amount of splenic antibody producers of female NZB/W F1.

The splenic B cells were further analyzed to determine whether a sex specific difference in the B cell composition or functionality was responsible for the observed difference in autoantibody levels and disease course. In the NZB/W F1 and other

mouse models of lupus, an expansion of the MZ B cell compartment has been described and associated with SLE (21-22). Consistent with these reports we found an expanded MZ B cell compartment, with 15-22% of the splenic B cells presenting an MZ B cell phenotype. However, neither a quantitative nor qualitative difference in the splenic B cell compartments comparing female and male NZB/W F1 mice was observed. Subtle differences arose in the course of the disease, but those differences are likely rather a consequence of the accelerated disease in females and thus not the primary reason for the clinical sex difference. Similarly we recently reported that in pre-diseased young NZB/W F1 mice there is no difference in the frequency of autoreactive B1a B cells in the peritoneal cavity, which however arises in the course of the generalized autoimmunity in these mice (23).

In previous reports MZ B cells have been shown to be easier activated than FO B cells and to harbour B cells with autoreactive specificity (14, 21, 24). Our present study is in line with these previous findings, as we found more dsDNA reactive cells in the MZ than the FO compartment and MZ B cells proved to be easier activated and to proliferate more upon stimulation.

Sex specific factors such as hormones have been reported to be involved in the selection and survival of autoreactive B cells (15-16). We determined whether NZB/W F1 mice of both sexes would differ in the frequency or subset distribution of autoreactive B cells. In the present study, no differences in the percentage or subset distribution of B cells autoreactive with the two canonical SLE autoantigens dsDNA and SmD1₍₈₃₋₁₁₉₎ were detected. Previous reports described that estrogen leads to an expansion of the MZ B cell compartment and selection of autoreactive B cells into the MZ B cell pool (16). It was shown that the expansion of the MZ compartment was mediated via the estrogen- α and $-\beta$ receptor, while the increased survival of high affinity anti-dsDNA B cells was mediated solely via the estrogen- α receptor (25). The

discrepancy of our results and these reports may be owed to the used model, as these previous studies have used genetically modified mice and the B cell selection of transgenic B cells might be skewed artificially. On the other hand a limitation of our study is that we had no means of determining the fine specificity and affinity of the autoreactive cells analysed. Therefore, our approach using the ELISPOT technique to determine the frequency of autoreactive cells could have missed a hypothetical difference in the affinity of the autoantibodies produced.

B cells in SLE are known to be hyperreactive and differences in the activation threshold of female and male cells have been claimed (17, 26). Activation of B cells via intracellular TLR's sensing RNA and DNA (TLR 7 and 9) is likely to play a pivotal role in the pathogenesis of SLE. In addition B cells producing autoantibodies in SLE display the features of B cells that have received T cell help (27). In our experiments no difference in the proliferation of female and male B cells was observed, neither upon stimulation via the intracellular TLR's 7 and 9, the extracellular LPS sensing TLR 4 nor upon activation with anti-CD40 plus IL-4. These results indicate that female and male B cells do not differ in their activation threshold and proliferative response upon activation.

In summary, we show that female and male NZB/W F1 mice do not differ in their splenic B cells composition, activation threshold or frequency of dsDNA- or SmD1₍₈₃₋₁₁₉₎ autoreactive B cells prior to disease development. Therefore the question remains, why female mice show earlier and higher autoantibody production and accelerated disease course. Based on our results we speculate that sex specific differences in the activation of the immune cascade downstream of the analyzed B cells properties may account for the overt clinical differences. Estrogens have been shown to act on T cells, which may in turn lead to a stronger B cell activation (28). Additionally sex hormones may influence cell survival and the amount of antibodies

produced (29). Others have reported a direct effect of estrogen on the expression of transcription factors, which may further promote progression of autoimmunity (30). Considering the marked clinical difference between females and males, analysing sex specific differences will warrant further future insights into the pathogenesis of lupus.

List of abbreviations

ASC - Autoantibody secreting cells

CFSE - Carboxy-fluorescein-diacetate-succinimidyl ester

Cpm -Counts per minute

DAPI -(2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride)

dsDNA – double stranded deoxyribonucleic acid

ELISA- Enzyme-linked immunosorbent assay

ELISPOT - Enzyme Linked Immuno Spot Technique

FO - Follicular

MZ - Marginal zone

NZB/W F1 - (NZB x NZW F1) mice

PI - Propidium Iodide

SLE- Systemic lupus erythematosus

SmD1₍₈₃₋₁₁₉₎ - SmD1 peptide, amino acid 83-119

TLR - Toll like receptor

Competing interests

The authors declare not to have any competing interests.

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Figure 1. Female mice showed increased titers of autoantibodies, which were mainly produced in the spleen. **A.** IgM dsDNA and SmD1₍₈₃₋₁₁₉₎ autoantibody titers in male and female NZB/W F1 mice. A significant difference was observed as early as 14 weeks of age. **B.** IgG dsDNA and SmD1₍₈₃₋₁₁₉₎ autoantibody titers in NZB/W F1 mice of both sexes. Female mice exhibited a sharp increase in IgG autoantibody titers while male mice hardly developed any IgG autoantibodies in the observation period. **C and D.** Anti-dsDNA IgM and IgG producing B cells per organ in female and male mice. The spleen was the major site of dsDNA autoantibody production. **E and F.** IgM and IgG anti-SmD1₍₈₃₋₁₁₉₎ autoantibody production in different organs. In females more than 100fold more autoantibody producing cells were detected compared to the bone marrow of one pair of femurs.

Figure 2. No a priori difference in the quantitative composition of splenic B cells subsets between female and male NZB/W F1. **A.** Example plot showing CD21^{low}CD23^{high} FO B cells, CD21^{high}CD23^{low} MZ B cells and CD21⁻CD23⁻ B cells (gated on PI⁻CD19⁺lymphocytes). **B.** Frequency of total B cells in the spleen and percentage of FO, MZ and CD21⁻CD23⁻ B cells in the spleen of 6 week young mice showed now difference in the subset distribution between female and male NZB/W F1. **C.** In aged mice there was a significant difference in the frequency of FO B between both sexes, all other subsets were equally distributed. **D.** Absolute number of B cells and the different B cells subsets in 6 week young female and male NZB/W F1, no differences were observed. **E.** Total amount of splenic B cells and B cell subsets in 6 month old NZB/W F1. No significant difference between female and male mice was detected.

Figure 3. Splenic sections of female and male NZB/W F1 at 6-7 weeks and 6-8 month of age stained with anti-IgD (green), anti-IgM (red) and DAPI (blue)(100x magnification). No difference in the splenic architecture of the B cell compartment in young female and male NZB/W F1 prior to disease development was observable. Immunofluorescence staining of splenic sections showed FO B cells (IgD^{high}, green) and MZ B cells (IgM^{high}, red) in the follicular structures. In old mice the FO B cells still formed follicular structures, while the MZ B cells were mainly found outside the marginal zone. (n=3 mice in each group).

Figure 4. Distribution of autoreactive B cells in the different B cell subsets in female and male NZB/W F1 mice. **A.** Example of splenic MZ and FO cells purified by FACS sorter. **B.** dsDNA reactive B cells were enriched in the MZ compartment ($p < 0.05$). **C.**

SmD1₍₈₃₋₁₁₉₎ reactive B cells were detected at similar frequencies in the MZ and FO B cell compartment. **D.** Female and male mice showed no difference in the frequency of dsDNA reactive B cells in the MZ or FO B cell compartment. To pool data from different experiments a stimulation ratio was calculated, with 1 indicating identical frequencies of autoreactive cells. **E.** No difference in the frequency of SmD1₍₈₃₋₁₁₉₎ reactive B cells in the splenic B cell subsets as indicated the stimulation ratio. **F.** Example ELISPOT showing anti-dsDNA ASC produced by MZ and FO B cells from female and male donors upon stimulation of equal cell numbers. No difference in the amount of spots was observable. (Displayed are the results of 6 and 12 week old, pre-diseased mice).

Figure 5. B cells from female and male donors did not differ in their stimulation threshold or proliferative response upon stimulation. **A.** Example experiment of purified MZ B cells stimulated with increasing concentrations of LPS. Proliferation was assessed as [³H] incorporation, no difference between female and male cells was detectable. **B.** Stimulation of B cells with TLR9 agonist CPG, no difference in [³H] incorporation between the B cells of both sexes. **C.** Stimulation of MZ B cells with the TLR7 agonist Imiquimid. **D.** Stimulation of female and male FO B cells with a combination of anti-CD40L and IL-4 mimicking T cell help. No difference in proliferative response. **E.** Pooled results from experiments using [³H] incorporation or

CFSE dilution to assess proliferation. Proliferative response is displayed as a proliferative ratio, with 1 indicating identical proliferation of female and male mice. For all assessed TLR agonists the proliferative ratio did not differ significantly between both sexes. **F and G.** Number of ASC produced by 0.5×10^6 MZ or FO cells upon 3 days of stimulation with LPS showed no difference in the amount of antibody producers.