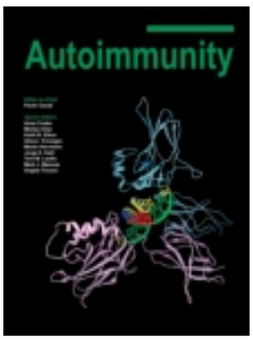

Autoimmunity

ISSN: 0891-6934 (Print) 1607-842X (Online) Journal homepage: http://www.tandfonline.com/loi/iaut20

Distinct phenotypes of plasma cells in spleen and bone marrow of autoimmune NOD.B10.H2b mice

Ewa A. Szyszko, Kathrine Skarstein, Roland Jonsson & Karl A. Brokstad

To cite this article: Ewa A. Szyszko, Kathrine Skarstein, Roland Jonsson & Karl A. Brokstad (2011) Distinct phenotypes of plasma cells in spleen and bone marrow of autoimmune NOD.B10.H2b mice, Autoimmunity, 44:5, 415-426, DOI: 10.3109/08916934.2010.545847

To link to this article: http://dx.doi.org/10.3109/08916934.2010.545847

Published online: 21 Feb 2011.

Autoimmunity, August 2011; 44(5): 415–426

ISSN 0891-6934 print/1607-842X online
DOI: 10.3109/08916934.2010.545847

informa
healthcare

Distinct phenotypes of plasma cells in spleen and bone marrow of autoimmune NOD.B10.H2b mice

EWA A. SZYSZKO[1,2], KATHRINE SKARSTEIN[2], ROLAND JONSSON[1,3], & KARL A. BROKSTAD[1]

[1]*Broegelmann Research Laboratory, The Gade Institute, University of Bergen, Bergen, Norway,* [2]*Section for Pathology, The Gade Institute, University of Bergen, Bergen, Norway, and* [3]*Department of Rheumatology, Haukeland University Hospital, Bergen, Norway*

(*Submitted 29 October 2010; revised 26 November 2010; accepted 3 December 2010*)

Abstract
Long-lived plasma cells (PCs) residing in the bone marrow (BM) are important producers of protective antibodies. However, when reacting against self-antigens, these PCs produce autoantibodies that contribute to progression of autoimmune diseases such as Sjögren's syndrome (SS). By using a murine model of primary SS, the NOD.B10.H2b mice, we characterized phenotype and generation of PCs at different stages of the pSS disease progression. In general, the PC population found in the NOD.B10.H2b mice expressed high amounts of MHCII, IgG, and BrdU. We further demonstrate the presence of both short- and long-lived PCs in autoimmune spleen and in autoimmune BM. A long-lived PC subset was also found in the spleen and BM of non-autoimmune BALB/c mice, which have not been treated with any immunological agent. Quantitative investigation of splenic and BM PCs revealed that in the NOD.B10.H2 mice, splenic PCs migrate not only to the BM but possibly also to the sites of inflammation. Finally, BM in the aged NOD.B10.H2b mice (40-week-old) presented significantly higher quantities of long-lived PCs than BM of BALB/c mice.

Keywords: *Plasma cells, long-lived plasma cells, autoimmunity, Sjögren's syndrome, NOD.B10.H2b mice*

Introduction

A range of autoimmune diseases such as Sjögren's syndrome (SS), rheumatoid arthritis, and systemic lupus erythematosus (SLE) are characterized by the production of systemic and organ-specific autoantibodies [1–3]. Circulating autoantibodies have, therefore, a central role in today's diagnosis of these diseases. The importance of B cells in the pathogenesis of autoimmunity and in the production of autoantibodies has resulted in the development of therapies based on targeting B cells and early plasma cells (PCs) [4,5]. Such therapies are, however, insufficient in altering the long-lived PC pool, and there is still a persistent autoantibody production in autoimmune patients after treatment [6–9]. The effect of these autoantibodies and the involvement of long-lived PCs in autoimmune diseases are still not fully understood. However, the existence and contribution of long-lived PCs to the progression of autoimmunity have been determined to some degree in murine models of SLE [10]. In light of these findings, long-lived PCs should be characterized in more detail and taken into consideration when designing new therapies for autoimmune diseases.

Long-lived PCs are primarily located in the bone marrow (BM) [11,12], although they have also been detected in spleen and at sites of inflammation [13,14]. The majority of long-lived PCs isolated from the BM are IgG secreting cells with somatically mutated V genes, and, therefore, it has been postulated that they are high-affinity cells descended from germinal centers. The lifespan of PCs varies from a few days to months and even years [12,14], depending on their intrinsic properties as well as capability of finding and migrating to the sites that

Correspondence: E. A. Szyszko, Broegelmann Research Laboratory, The Gade Institute, University of Bergen, Haukeland University Hospital, The Laboratory Building, N-5021 Bergen, Norway. Tel: +47 55 97 46 22. Fax: +47 55 97 58 17. E-mail: ewa.szyszko@gades.uib.no

contain the so-called survival niches. Migration is facilitated by many factors, i.e. chemokines. Interaction between CXCR4 chemokine receptor and CXCL12 factor expressed by stromal cells is one of the factors important in PCs migration to the BM [15,16].

In spite of active migration of PCs, only low numbers of PCs are found in the human circulation. This makes PCs difficult to access in humans. Since murine models have proved to be the valuable tools for exploring biological processes of human diseases, we have utilized NOD.B10.H2b (a model of pSS-like disease) and BALB/c mice in order to characterize long-lived PCs both in autoimmune and non-autoimmune spleen and in BM compartments. In this study, we demonstrated that long-lived PCs are present not only in the spleen and BM of autoimmune mice but also in the spleen and BM of non-autoimmune mice. The presence of long-lived PCs in non-autoimmune mice that have not been treated with any immunological agent has until now not been explored.

We have further detected distinct PCs' phenotypes within the two mice strains. In NOD.B10.H2b mice, PCs produced significantly higher levels of IgG and the autoimmune PCs were the only ones that produced antinuclear antibody (ANA)-specific antibodies. Furthermore, PCs from autoimmune mice were mostly IgG + cells with highly proliferative activity. Most importantly, BM in the aged NOD.B10.H2b mice (40-week-old) presented significantly higher quantities of long-lived PCs than BM of BALB/c mice, indicating supportive survival environment present in the BM of autoimmune mice.

Materials and methods

Mice

Female NOD.B10.H2b and BALB/c mice were purchased from Jackson Laboratories and Taconic (Denmark), respectively, and housed in an individually ventilated caging system at the animal facility at the University of Bergen. Mice were divided into different age groups with 10 NOD.B10.H2b and eight BALB/c mice included in each group and sacrificed at 11, 17, 24, 32, and 40 weeks of age. The study was approved by the National Animal Research Authority of Norway (# 2006014BB).

BrdU labeling

Since it has been shown that the level of BrdU positive PCs stabilize after 10 days of BrdU feeding [10], each group of mice was given BrdU in drinking water (protected from light and changed every second day) continuously for 11 days before sacrifice.

Stimulated saliva collection

Day before sacrifice, each mouse was weighed and treated with Ketalar (0.75 mg/100 g body weight) and Dormitor (0.01 mg/100 g body weight) dissolved in phosphate buffer saline (PBS). Salivary flow was stimulated by an intraperitoneal injection of pilocarpine (0.05 mg/100 g of body weight) and saliva was collected with micropipette for 10 min. The volume of each sample was calculated as saliva volume/body weight (μl/g). The saliva samples were stored at −70°C until further analysis.

Glucose level measurements and serum collection. At the day of sacrifice, mice were starved for 2 h and euthanized by CO_2 asphyxiation. Blood was collected by intracardiac puncture and glucose level was measured using the HemoCue Glucose 201 room temperature (RT) test kit (HemoCue Norge, Oslo, Norway). A glucose level > 300 mg/dl was considered as diabetic. The blood was allowed to clot overnight at 4°C. Serum was separated after centrifugation for 10 min at 1000*g* and stored at −70°C until further analysis.

Enzyme-linked immunosorbant assay

For quantitative detection of circulating anti-SSA/Ro and anti-SSB/La autoantibodies, a commercially available kit was used (Alpha Diagnostic, San Antonio, TX, USA). The ELISAs were carried out as recommended by the manufacturer. In brief, serum samples were diluted 1:100, added to the SSA/Ro or SSB/La antigen-coated plates, and incubated for 30 min at RT. Plates were washed 3 × and incubated for 30 min with goat anti-mouse IgG HRP conjugate. After additional washing, TMB substrate was added for 15 min. The reaction was stopped by a stop solution and the optical densities (ODs) were read at 450 nm. Concentrations of autoantibodies were calculated as micrograms per milliliter.

For detection of total IgG in serum, ELISA plates (Greiner) were coated overnight at 4°C with PBS containing 2 μg/ml of goat anti-mouse IgG antibody (Southern Biotech, Birmingham, AL, USA). The plates were blocked with PBS and 20% fetal bovine serum (FBS) for 1 h at RT. Sera were added in twofold dilutions, and standard IgG antibody (Sigma Aldrich, St. Louis, MO, USA) was tested in twofold dilution. After incubation for 2 h at RT, the plates were washed with PBS containing 0.05% Tween (PBS/T) and subsequently incubated with 0.5 μg/ml of biotinylated goat anti-mouse IgG antibody (Southern Biotech, Birmingham, AL, USA) for 1 h at RT. The plates were washed and ExtrAvidin peroxidase diluted 1:1000 in PBS/FBS was added to each well. After 1-h incubation, the reaction was developed by adding

OPD (0.4 mg/ml) and H_2O_2 (0.8 mg/ml) in dH_2O. The reaction was stopped after 5 min by the addition of 1 M H_2SO_4 and the ODs were read at 492 nm. The total IgG concentrations were calculated as micrograms per milliliter.

Measurement of ANAs

Hep-2 cells were seeded on the cover glasses and incubated at 37°C in humidified 5% CO_2 incubator until confluent. Cells were fixed with 4% paraformaldehyde, washed, and incubated at −20°C for 1 h with methanol. After washing with distilled H_2O (dH_2O), cells were blocked for 1 h with PBS containing 2% BSA and 0.05% Tween. The murine sera were diluted 1:50, added to the Hep-2 fixed glasses, and incubated for 1 h at RT. After washing, the cells were covered with FITC-conjugated goat anti-mouse IgG (Sigma Aldrich, St. Louis, MO, USA) diluted 1:100 and DAPI diluted 1:5000 for 1 h at RT.

Flow cytometry

Spleen and femur bones were collected from each mouse. Spleens were punctured with a syringe at each end, and cells were washed out with PBS. Cells for the BM analysis were harvested from the femur bones by flushing the marrow cavity with PBS. Cell suspensions were carefully layered on top of the Lymphoprep and centrifuged for 30 min at 800*g* at RT. The bands containing the mononuclear cells were removed and washed twice with PBS by centrifuging at 300*g* for 10 min. Cell counting and cell viability was determined by Casy cell counter system (Schärfe System, Reutlingen, Germany). Cells were resuspended in FACS buffer (PBS containing 1% FBS, 2 mM EDTA, and 0.01% azide), and cell concentration was adjusted to 1×10^6 per sample. Cells were incubated with affinity purified anti-mouse CD16/32 antibodies that blocks low-affinity Fc receptor expressed among other B cells.

The following fluorochrome-conjugated monoclonal antibodies were used: CD138-PE (clone 281-2, Miltenyi), CD38-PE-Cy5 (clone 90, eBioscience), BrdU-FITC (clone 3D4, BD Pharmingen), Bcl-2-FITC (clone 3F11, BD Pharmingen), IgG2a/2b-FITC (clone R2-40, BD Pharmingen), R&D Systems), IgM-APC (clone 1B4B1, Southern Biotec), MHCII-FITC (clone NIMR-4, Southern Biotec), and CXCR4-APC (clone 247506). Stained cells were analyzed using BD FACSCanto I (BD Bioscience, San Jose, USA). FlowJo software (Tree Star, Inc., Ashland, USA) was used in analyzing collected data. Mean fluorescence intensity (MFI) analysis was used to define density expression of each molecule on the surface of PCs.

Statistical analysis

Kolmogorov–Smirnov test was used to determine if the data was normally distributed. Statistical differences in the cytokine means for each time point were calculated by either the two-tailed Student's *t*-test or the Mann–Whitney *U*-test. A *p*-value of ≤ 0.05 was considered significant.

Results

Impaired salivary secretion

Dysfunction in the secretory activity of salivary glands is an important component of pSS in humans. In our murine study, we observed reduction in saliva secretion in NOD.B10.H2b mice at week 17 with further significant reduction detected by week 24 (Figure 1a). Intriguingly, significant increase in saliva production was observed in BALB/c mice at week 24 and 40 compared with younger BALB/c mice (Figure 1a).

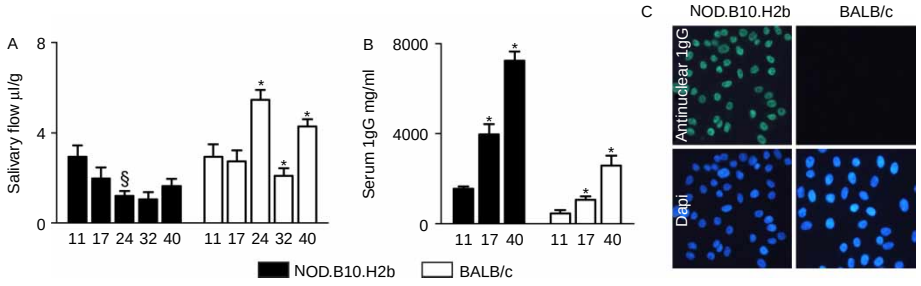

Figure 1. Salivary flow and antibody measurements in NOD.B10.H2b and BALB/c mice. (A) Saliva was collected from each mouse for 10 min after injection of pilocarpine. The volume of each sample was measured and data are presented as salivary flow rate [µl saliva/g body weight] for 10 or 8 NOD.B10.H2b and BALB/c mice, respectively, for each time point. The Kolmogorov–Smirnov test and Student's *t*-test were used in analyzing the data with ($p < 0.05$); § significantly lower than week 11; ⋆ significantly higher/lower than younger mice. (B) Serum IgG levels measured by ELISA method in 11-, 17- and 40-week-old NOD.B10.H2b and BALB/c mice. The Kolmogorov–Smirnov test and Student's *t*-test were used in analyzing the data with ($p < 0.05$); ⋆significantly higher/lower than younger mice. (C) Presence of ANAs was tested in 11-, 17- and 40-week-old NOD.B10.H2b and BALB/c mice by incubating sera from mice with Hep-2 cells. ANAs were detected only in NOD.B10.H2b mice at week 17 and 40. Representative results from one 40-week-old NOD.B10.H2b mouse and one 40-week-old BALB/c mouse are presented.

NOD.B10.H2b is a model for pSS-like diseases that in contrast to the NOD mice does not develop diabetes [17]. Thus, in order to make sure that our NOD.B10.H2b mice were diabetes free, we measured the blood glucose level. The glucose levels in the NOD.B10.H2b mice were stable (with variation between 200 and 250 mg/dl) throughout the whole study period and comparable with glucose level detected in age-matched BALB/c mice (data not shown).

Detection of circulating autoantibodies

Quantitative levels of circulating SSA/Ro and SSB/La autoantibodies and total IgG were tested in 11-, 17-, and 40-week-old mice. Both SSA/Ro and SSB/La autoantibodies were detected in NOD.B10.H2b and BALB/c mice; however, no significant differences were found between the two mice strains. Levels of SSA/Ro autoantibodies (with range of 38–76 μg/ml) were significantly higher than levels of SSB/La autoantibodies (with range of 0.83–2.13 μg/ml) in both mice strains. Interestingly, significant increase of both SSA/Ro and SSB/La autoantibodies was detected with increasing age (data not shown).

In contrast to SSA/Ro and SSB/La autoantibodies, concentrations of total IgG in serum of NOD.B10.H2b mice were threefold higher compared with BALB/c mice at all three time points tested (Figure 1b), thus mirroring the common elevated IgG levels seen in pSS patients. Equally to SSA/Ro and SSB/La autoantibodies, levels of circulating IgG increased with increasing age in both mice strains.

Qualitative tests for detection of circulating IgG antibodies against nuclear proteins showed presence of ANAs in NOD.B10.H2b mice already from week 17, with high reactivity at week 40 (Figure 1c), thus indicating an autoimmune type of inflammation in NOD.B10.H2b mice. Circulating antibodies from BALB/c mice showed no reaction with nuclear proteins at any time points tested (Figure 1c).

Identification of PCs in spleen and BM

Long-lived PCs are thought to develop mainly from germinal center B cells or memory B cells in secondary lymphoid organs such as spleen. Additionally, high amounts of long-lived PCs have been detected in the BM. Therefore, we isolated and characterized PCs from both spleen and BM of autoimmune NOD.B10.H2b mice and non-autoimmune BALB/c mice. PCs in both compartments were defined by CD138 expression and lack of CD38 expression ($CD138^+CD38^-$ cells) [18] (Figure 2a).

The number of PCs increased significantly from week 11 to 17, indicating a high proliferative activity in the spleen of NOD.B10.H2b mice during this time frame (Figure 2a,b). After 17 weeks, the number of PCs dropped to a level that was significantly lower than previously observed (Figure 2a,b). BALB/c mice showed a very different pattern, with PCs increasing from week 11 with a peak at week 24 (Figure 2b). Thereafter, the levels of PCs gradually decreased with low levels detected at week 40 (Figure 2b). At week 40, there were significantly more PCs in the spleen of NOD.B10.H2b compared with BALB/c mice.

In the BM of NOD.B10.H2b mice, high frequencies of PCs were detected at week 11 and 17, indicating increased migration of PCs to BM at these time points. Significant reduction of PCs was noted at week 24, followed by a slight increase at week 32 and 40 (Figure 2c). Different progression in the accumulation of PCs was observed in the BM of BALB/c mice. Significant increase of BM PCs was detected at week 24, followed by significant decrease by week 40 (Figure 2c). At week 40, there were significantly more PCs in the BM of NOD.B10.H2b compared with BALB/c mice.

In addition to being a PC marker, CD138 receptor is a novel marker for determining the final stage of PCs differentiation. Therefore, we examined the density of CD138 on PCs. We detected high CD138 density on splenic PCs at week 11 in both mice strains (Figure 2d), thus indicating presence of more mature phenotype of PCs in the spleen at week 11 than in the older mice. In the BM of NOD.B10.H2b mice, all PCs exhibited comparable densities of CD138 receptor (Figure 2e) pointing out to one particular type of PCs present in the BM.

PC phenotyping

Maturation level of splenic and BM PCs. MHCII is an important molecule in antigen presentation and, thus, is highly expressed by the B cells and less by the terminally differentiated PCs. Except for week 17 and 40, most of the splenic PCs in the NOD.B10.H2b mice expressed MHCII molecule (Figure 3a,b). At week 17 and 40, the distribution of $MHCII^+$ and $MHCII^-$ cells was equal. When investigating the density of MHCII molecules, we discovered high MFI on PCs from 11-week-old NOD.B10.H2b mice (Figure 3d). After week 11, the MFI dropped at week 17, rose again at week 24, and remained stable until week 40.

Between week 11 and 24, only 30–40% of PCs in the BALB/c mice were positive for MHCII molecule. Accordingly, low MHCII densities were detected at these time points. A shift in both percentages and density of MHCII molecule on PCs was observed by week 32 (Figure 3b,d), indicating generation of new PCs in the spleen of 32-week-old BALB/c mice.

The BM PCs were generally $MHCII^-$ (Figure 3a,c), indicating presence of higher numbers of terminally differentiated PCs in the BM compared with spleen. In NOD.B10.H2b mice, a significantly increased

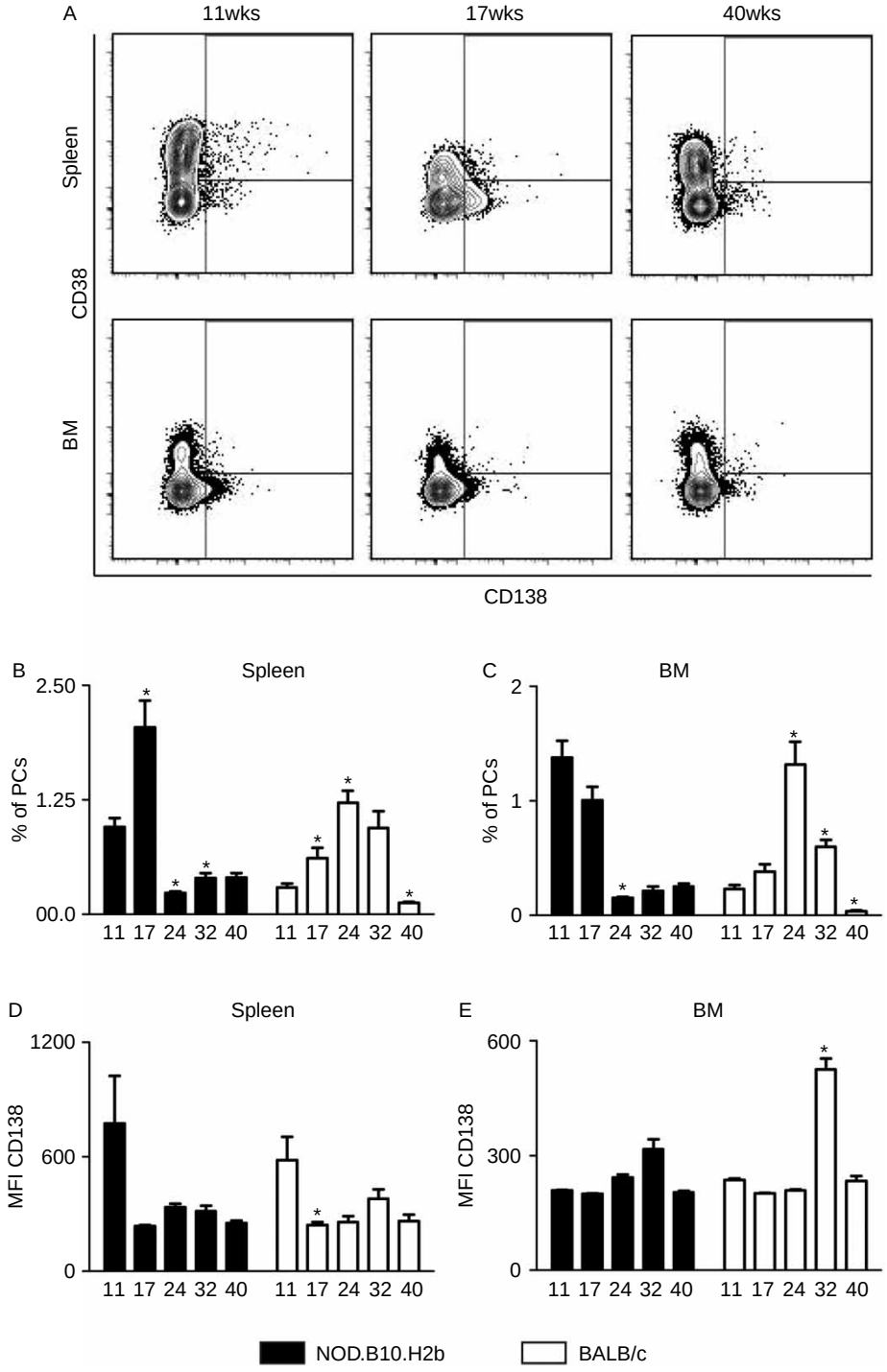

Figure 2. Splenic and BM PCs in NOD.B10.H2b and BALB/c mice. PCs from spleen and BM were identified as $CD138^{+}CD38^{-}$ cells. (A) Representative plots of 11-, 17- and 40-week-old NOD.B10.H2b mice. BALB/c mice presented comparable distribution pattern of $CD138^{+}CD38^{-}$ cells as NOD.B10.H2b mice (data not shown). Two gates are presented for each plot, $CD138^{+}CD38^{+}$ gate (possible early PCs) and $CD138^{+}CD38^{+}$ gate (mature PCs). Only mature $CD138^{+}CD38^{+}$ PCs have been further examined in this study. (B) PCs percentages of isolated splenocytes in 11-, 17-, 24-, 32- and 40-week-old NOD.B10.H2b and BALB/c mice. (C) PCs percentages of isolated BM cells in 11-, 17-, 24-, 32- and 40-week-old NOD.B10.H2b and BALB/c mice. (D,E) Density of CD138 receptor on the surface of PCs was tested in spleen and BM by MFI. (B–E) All data shown as mean ± SEM per sampling time for 10 or 8 NOD.B10.H2b and BALB/c mice, respectively. The Kolmogorov–Smirnov test and the Student's *t*-test were used in analyzing the data with ($p < 0.05$), *significantly higher/lower than younger mice.

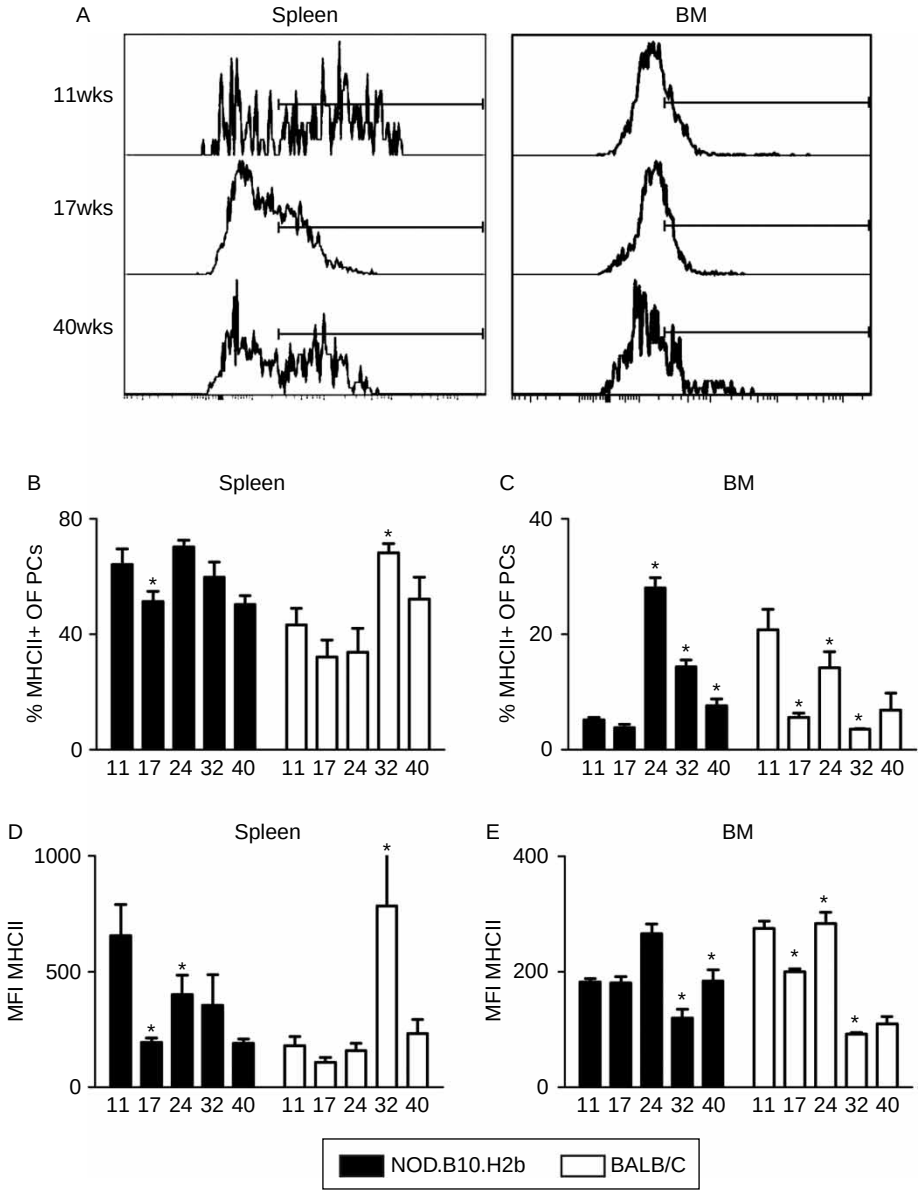

Figure 3. MHCII expression by splenic and BM PCs in NOD.B10.H2b and BALB/c mice. Expression of maturation marker and MHCII on PCs from spleen and BM were examined. (A) Representative plots of MHCII distribution within PC population in the spleen and BM of 11-, 17-, and 40-week-old NOD.B10.H2b mice. BALB/c mice presented comparable distribution pattern of MHCII as NOD.B10.H2b mice (data not shown). (B) Percentages of $MHCII^+$ cells within splenic PC subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (C) Percentages of $MHCII^+$ cells within BM PC subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (D,E) Density of MHCII molecule on the surface of PCs tested in spleen and BM by MFI. (B–E) All data shown as mean ± SEM per sampling time for 10 or 8 NOD.B10.H2b and BALB/c mice, respectively. The Kolmogorov–Smirnov test and Student's *t*-test were used in analyzing the data with ($p < 0.05$), ⋆significantly higher/lower than younger mice.

population of $MHCII^+$ PCs was observed at week 24. By week 32, the percentages of $MHCII^+$ PCs were significantly lower and continued to decline up to week 40. In the BALB/c mice, we observed a decrease in $MHCII^+$ PCs at week 17, but similar to NOD.B10.H2b, an increase of this phenotype was observed at week 24 (Figure 3c).

Immunoglobulin isotype of splenic and BM PCs. Since it has been established that long-lived PCs found in the BM are mostly high-affinity IgG expressing cells, whereas the majority of short-lived PCs are the IgM producing cells, we used immunoglobulin phenotyping to distinguish between the short- and long-lived PCs. Distribution of IgG and IgM cells in the spleen and BM of mice is shown in Figure 4a.

Interestingly, we detected comparable percentages of IgG expressing PCs in the spleen of NOD.B10.H2b mice between weeks 11 and 32.

A significant decrease in IgG^+ PCs was, however, observed in 40-week-old NOD.B10.H2b mice (Figure 4b). An opposite situation was observed in

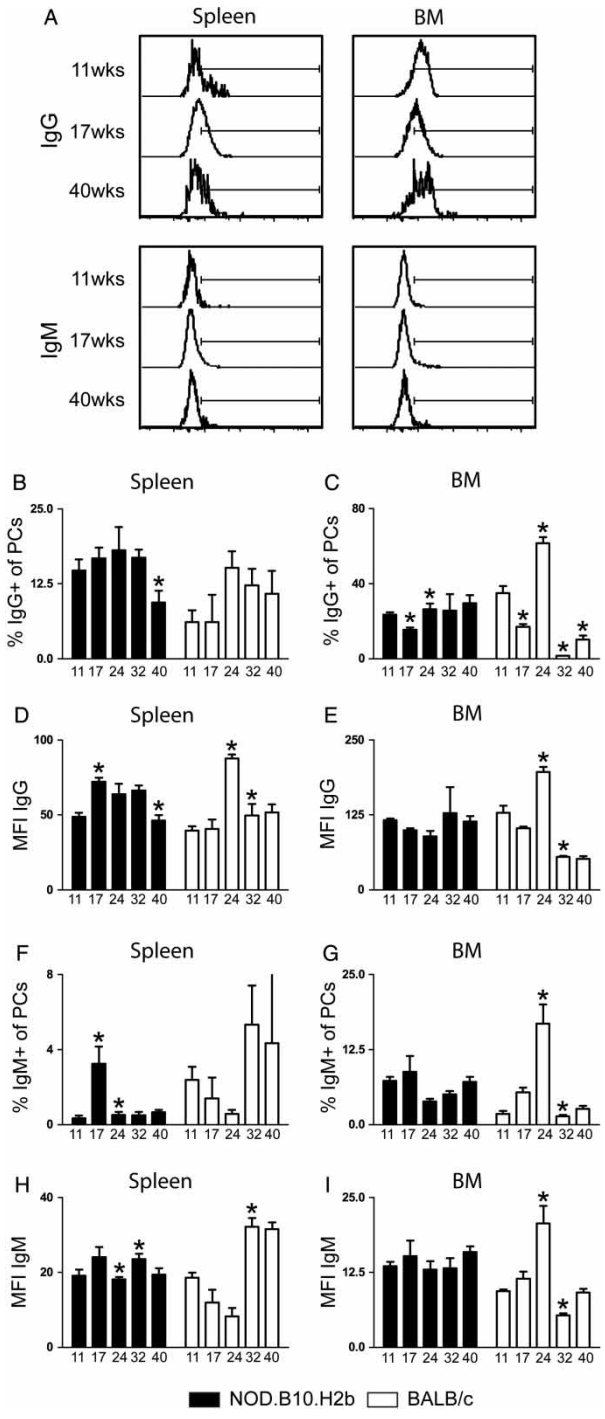

Figure 4. IgG and IgM expression by splenic and BM PCs in NOD.B10.H2b and BALB/c mice. Expression of IgG and IgM on PCs from spleen and BM were examined. (A) Representative plots of IgG and IgM distribution within PC population in the spleen and BM of 11-, 17-, and 40-week-old NOD.B10.H2b mice. BALB/c mice presented comparable distribution pattern of MHCII as NOD.B10.H2b mice (data not shown). (B) Percentages of IgG^+ cells within splenic PC subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (C) Percentages of IgG^+ cells within BM PC subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (D,E) Density of IgG molecule on the surface of PCs tested in spleen and BM by MFI. (F) Percentages of IgM^+ cells within splenic P^+C subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (G) Percentages of IgM^+ cells within BM PC subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (H,I) Density of IgM molecule on the surface of PCs tested in spleen and BM by MFI. (B–I) All data shown as mean ± SEM per sampling time for 10 or 8 NOD.B10.H2b and BALB/c mice, respectively. The Kolmogorov–Smirnov test and Student's *t*-test were used in analyzing the data with ($p < 0.05$), *significantly higher/lower than younger mice.

BALB/c mice, where an increase in IgG^+ PCs was observed in the spleen at week 24. The increase in IgG^+ PCs was already detected at 17 weeks; however, due to some outliners with very low percentages of IgG^+ PCs, the differences between 11- and 17-week-old mice and 17- and 24-week-old mice were not significant (Figure 4b).

In the BM up to 30% (in NOD.B10.H2b mice) and 61% (in BALB/c mice) of PCs were IgG^+ (Figure 4c). In BM of NOD.B10.H2b mice, we observed significant decrease in IgG^+ PCs at week 17 followed by an increase at week 24. Similarly, in BALB/c mice, a decrease in IgG^+ PCs at week 17 and increase at week 24 were observed. However, in contrast to the NOD.B10.H2b mice, a significant decrease in IgG^+ PCs was observed in BALB/c mice with low quantities of IgG^+ PCs detected at week 32 and 40 (Figure 4c). Density examination revealed some changes in the IgG densities on splenic PCs, but not on BM PCs in NOD.B10.H2b mice (Figure 4d,e). In BALB/c mice, high IgG densities were detected on PCs at week 24 both in spleen and BM (Figure 4d,e).

We detected high quantities of IgM^+ PCs in 17-week-old NOD.B10.H2b mice (Figure 4f). Interestingly, this is in accordance with the increase in the percentages of total PCs in the spleen of these mice (Figure 2b). The amounts of IgM^+ PCs decreased significantly at week 24. An increase of IgM^+ splenic PCs was observed in only some of the 32- and 40-week-old BALB/c mice and, thus, due to the great variations within these mice, the increase was not significant (Figure 4f). The BM subset of IgM^+ PCs was stable in NOD.B10.H2b mice, but shifted in BALB/c mice by significant increase at week 24 (Figure 4g). Examination of IgM densities reflected mainly changes seen in the quantities of IgM^+ PCs (Figure 4h,i).

Migration potential of PCs

Interactions between CXCR4 receptor on the surface of PCs and its ligand CXCL12 expressed among others by stroma in the BM have shown to be important in homing to the BM. Thus, we have examined expression of CXCR4 receptor on PCs in both spleen and BM of mice. Low quantifies of $CXCR4^+$ PCs were detected in spleen and BM of both NOD.B10.H2b and BALB/c mice (Figure 5a–c). We observed an increase in splenic $CXCR4^+$ PCs in 17-week-old NOD.B10.H2b mice; however, due to

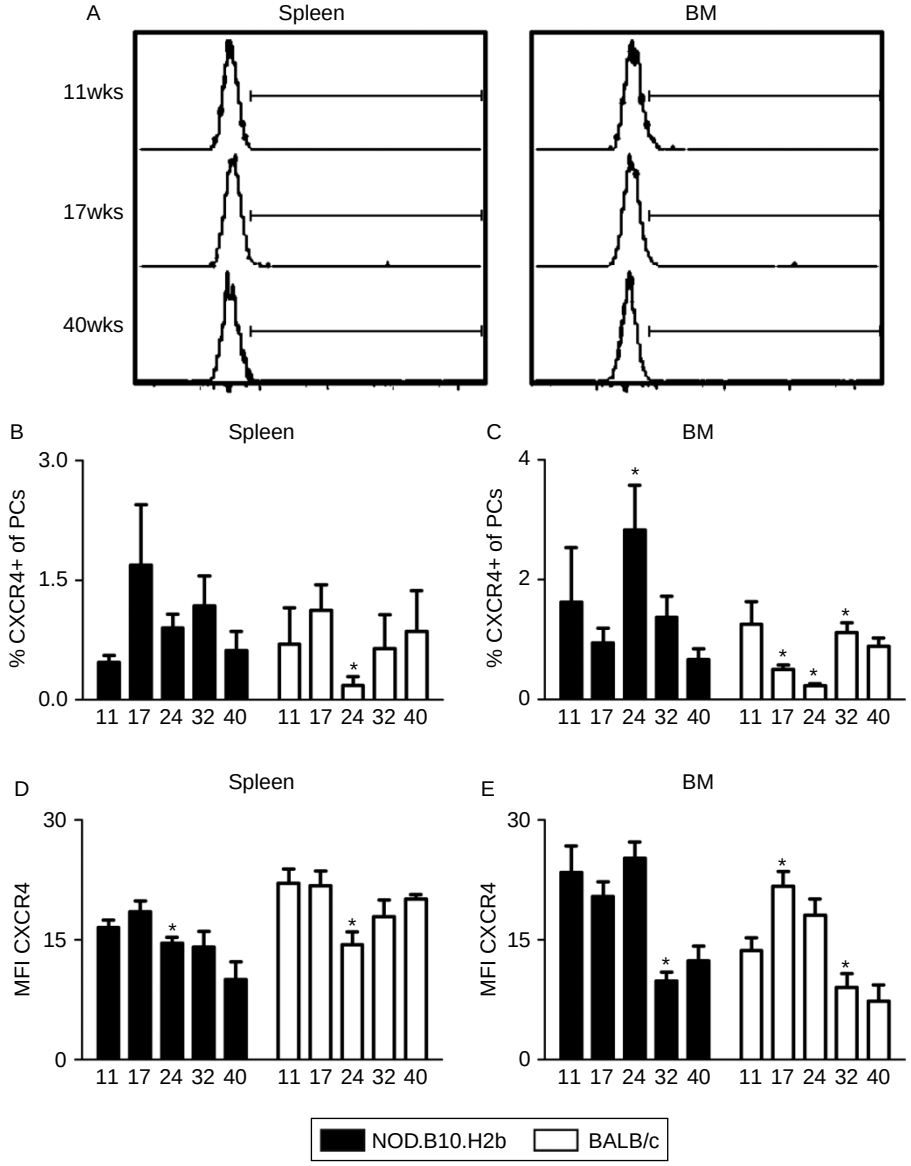

Figure 5. CXCR4 expression by splenic and BM PCs in NOD.B10.H2b and BALB/c mice. Expression of migration marker, CXCR4 on PCs from spleen and BM were examined. (A) Representative plots of CXCR4 distribution within PC population in the spleen and BM of 11-, 17-, and 40-week-old NOD.B10.H2b mice. BALB/c mice presented comparable distribution pattern of CXCR4 as NOD.B10.H2b mice (data not shown). (B) Percentages of $CXCR4^+$ cells within splenic PC subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (C) Percentages of $CXCR4^+$ cells within BM PC subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (D,E) Density of CXCR4 molecule on the surface of PCs tested in spleen and BM by MFI. (B–E) All data shown as mean ± SEM per sampling time for 10 or 8 NOD.B10.H2b and BALB/c mice, respectively. The Kolmogorov–Smirnov test and Student's *t*-test were used in analyzing the data with ($p < 0.05$), ⋆ significantly higher/lower than younger mice.

variations within the group, it was not significant (Figure 5b). In the BM, an increase in $CXCR4^+$ PCs was observed in the 24-week-old NOD.B10.H2b mice (Figure 5c), thus indicating immigration of these cells possibly from the spleen. In BALB/c mice, we observed significant reduction in $CXCR4^+$ PCs at week 24, thus pointing to emigration of these cells out of the spleen compartment (Figure 5b). This associates well with the increase of these cells in the BM of 32-week-old mice (Figure 5c).

Proliferation of splenic and BM PCs

To be able to distinguish between proliferating (short-lived) and non-proliferating (long-lived) PCs, incorporation of previously administrated BrdU was analyzed in the two mice strains (Figure 6). In NOD.B10.H2b mice, 50% of splenic PCs were $BrdU^+$ at week 11. Percentages of $BrdU^+$ PCs increased at week 17 and remained stable throughout weeks 24 and 32. A slight decrease in $BrdU^+$ PCs was detected at week 40; however, because of variation

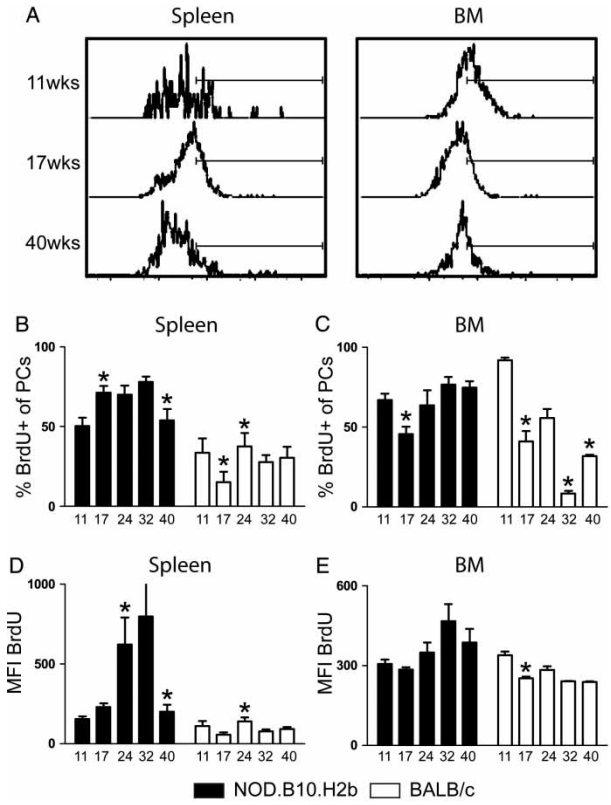

Figure 6. BrdU incorporation in splenic and BM PCs in NOD.B10.H2b and BALB/c mice. Mice were treated with BrdU in drinking water for 11 days before sacrifice. BrdU incorporation in proliferating PCs was tested by flow cytometry. (A) Representative plots of BrdU distribution within PC population in the spleen and BM of 11-, 17-, and 40-week-old NOD.B10.H2b mice. BALB/c mice presented comparable distribution pattern of MHCII as NOD.B10.H2b mice (data not shown). (B) Percentages of $BrdU^+$ cells within splenic PC subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (C) Percentages of $BrdU^+$ cells within BM PC subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (d,e) Density of BrdU molecule in PCs tested in spleen and BM by MFI. (B–E) All data shown as mean ± SEM per sampling time for 10 or 8 NOD.B10.H2b and BALB/c mice, respectively. The Kolmogorov–Smirnov test and Student's *t*-test were used in analyzing the data with ($p < 0.05$), *significantly higher/lower than in younger mice.

within the group, the decrease was not significant (Figure 6b).

In BALB/c mice, generally lower quantities of $BrdU^+$ PCs were detected compared with NOD.B10.H2b mice (Figure 6b), thus indicating less proliferation taking place in the spleen of these mice. Interestingly, the examination of BrdU density in the PCs from NOD.B10.H2b mice revealed high quantities of BrdU in the PCs at weeks 24 and 32, indicating increase in proliferative activity within the PC population at these time points (Figure 6d). The splenic PCs from BALB/c mice showed no differences in the density of BrdU in the five time points tested (Figure 6d).

In the BM of NOD.B10.H2b mice, the amounts of PCs with BrdU incorporation were stable throughout the whole study period (Figure 6c). The BrdU incorporation in the BM PCs from BALB/c mice was high at week 11. After week 11, the quantities of $BrdU^+$ PCs decreased and very low percentages were detected at week 32 (Figure 6c). The densities of BrdU in the BM PCs were stable throughout the whole study period in both mice stains (Figure 6e).

Survival of splenic and BM PCs

Survival of PCs is dependent on environmental factors and also on intrinsic properties of these cells. Bcl2 is an anti-apoptotic protein regulating process of cell survival, and therefore we examined expression of this factor in the splenic and BM PCs.

Low percentages of $Bcl2^+$ PCs were detected in the spleen of NOD.B10.H2b mice with a peak reached at week 17 (Figure 7a,b). Comparable quantities of $Bcl2^+$ PCs were observed in the spleen of BALB/c mice; however, a significant increase was observed at week 24 in these mice (Figure 7b).

Generally, higher percentages of $Bcl2^+$ PCs were detected in the BM compartment compared with spleen in both mice strains, indicating higher survival potential of BM rather than splenic PCs. In NOD.B10.H2b mice, the levels of $Bcl2^+$ PCs in the BM varied between 5 and 20%, with the most pronounced expression detected at week 24 (Figure 7c). In BALB/c mice, the percentages of $Bcl2^+$ PCs varied between 3 and 67, with highest levels of positive cells detected at week 11 (Figure 7c). These data indicate that even though there are high numbers of PCs with survival potential present in the BM, not all of these cells will be able to survive and retain in this compartment.

Investigation of Bcl2 expression showed increased Bcl2 densities in the BM PCs compared with splenic PCs, thus confirming possible higher survival potential of BM PCs. In the NOD.B10.H2b, the densities of Bcl2 in the PCs were stable in both spleen and BM. In contrast, BALB/c mice presented highest Bcl2 densities at week 24 in the spleen and week 11 in the BM (Figure 7d,e).

Discussion

During an immune response, two types of PCs may be formed: short-lived and long-lived PCs. The long-lived subset residing primarily in the BM is responsible for antibody titer persistence in the circulation. In autoimmune diseases, long-lived PCs can accumulate not only in the BM but also in secondary lymphoid organs and at sites of inflammation. Long-lived PCs secreting autoantibodies provide a therapeutic challenge because they are resistant to conventional treatments, in particular to immunosuppressive and anti-inflammatory drugs. In this study, we have investigated the generation of long-lived PCs and

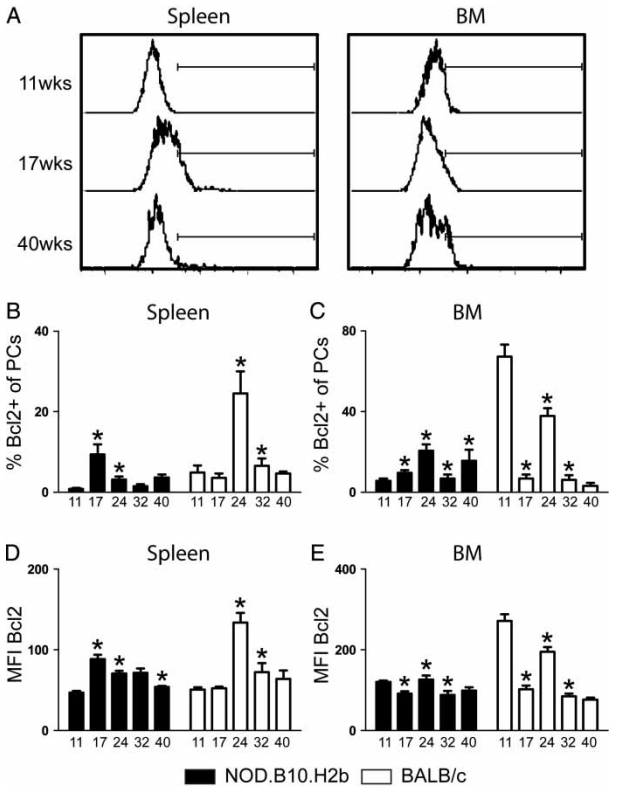

Figure 7. Bcl2 expression by splenic and BM PCs in NOD.B10.H2b and BALB/c mice. Expression of survival marker, Bcl2 inside PCs from spleen and BM were examined. (A) Representative plots of Bcl2 distribution within PC population in the spleen and BM of 11-, 17-, and 40-week-old NOD.B10.H2b mice. BALB/c mice presented comparable distribution pattern of Bcl2 as NOD.B10.H2b mice (data not shown). (B) Percentages of $Bcl2^+$ cells within splenic PC subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (C) Percentages of $Bcl2^+$ cells within BM PC subset in 11-, 17-, 24-, 32-, and 40-week-old NOD.B10.H2b and BALB/c mice. (D,E) Density of Bcl2 molecule inside (on the mitochondria and ER) PCs tested in spleen and BM by MFI. (B–E) All data shown as mean ± SEM per sampling time for 10 or 8 NOD.B10.H2b and BALB/c mice, respectively. The Kolmogorov–Smirnov test and Student's *t*-test were used in analyzing the data with ($p < 0.05$), ⋆significantly higher/lower than younger mice.

their possible role in development and progression of pSS-like disease in NOD.B10.H2b mice [17].

Consistent with other reports, the NOD.B10.H2b mice (model for pSS-like disease) presented decrease in salivary flow with increasing age [17,19–21]. In our study, we did not detect elevated serum levels of SSA/Ro and SSB/La autoantibodies in the autoimmune mice. This adds to the notion that there are probably different mechanisms regarding SSA/Ro and SSB/La autoantibody production in humans vs. mice [22,23]. However, NOD.B10.H2b mice had significantly higher ANAs detected in serum [20,21], indicating B cells involvement in autoimmune condition.

PC subset is a heterogenic subset comprising phenotypically different cells. In humans, BM PCs express high levels of CD138, CD38, CD27, and Bcl2 and reduced levels of CD19, CD45, and HLA-DR. However, a CD138- PC subset has also been detected [24]. To make it even more complicate, distinct phenotypes of PCs are presented in different compartments such as blood and tonsils [25]. In contrast to humans, terminally differentiated, mature PCs in mice do not express CD38 [18], and therefore, due to our primary interest in the mature, long-lived subset, we have chosen to define PCs as a $CD138^+CD38^-$ population.

Quantitative comparison of splenic PCs at different ages revealed a significant increase of PCs in 17-week-old NOD.B10.H2b mice. Simultaneously, a decrease in the density of CD138 on the surface of splenic PCs was detected in 17-week-old mice, pointing to the expansion of PC population by newly differentiated PCs. Furthermore, the increase in PCs was associated with loss of salivary secretion and a significant increase in the circulating IgG and ANAs. Detection of all these factors points to the fact that at week 17 the clinical phase of disease has already started in the NOD.B10.H2b mice.

PCs generated in the spleen will migrate to the BM [15,16]. In the BM of NOD.B10.H2b mice, the highest numbers of PCs were detected at weeks 11 and 17, indicating that the migration of PCs to the BM started before week 11, and thus the PC peak observed in the spleen at week 17 is most likely not the initial peak. Furthermore, the major decrease in BM PCs at week 24 implies that from the high amounts of splenic PCs produced at week 17, only a small amount will actually migrate to the BM. This could be due to the fact that many of the splenic PCs will die [10,26] before they reach BM [28] or that they migrate preferably to the sites of inflammation [27].

Intriguingly, a high number of PCs was detected in the BM of BALB/c mice at week 24. This finding suggests that most of PCs produced in the spleen of 17-week-old BALB/c mice possibly migrate to the BM of these mice. However, at week 40, there are four times less PCs in the BM of BALB/c mice than in the 40-week-old NOD.B10.H2b mice. This could be due to the still ongoing migration in the NOD.B10.H2b but not in the BALB/c mice or, alternatively, due to a more supportive survival environment present in the BM of autoimmune mice.

Analysis of MHCII expression revealed that BM PC population was of a more mature phenotype compared with splenic PC population in both mice strains. This is in agreement with human studies that showed a more mature PC in the BM compared with tonsils in humans [25]. Interestingly, we discovered that even though the levels of BM PCs in the 24-week-old autoimmune mice were very low compared with the other time points, the percentage of $MHCII^+$ PCs reached a peak at this time point. Based on this observation, we hypothesize that most of the

MHCII- PCs detected at week 17 probably lost the competition for the survival niches.

The high percentage of the less mature PCs ($MHCII^+$ cells) could further mean that these cells have newly migrated to the BM, indicating that BM of NOD.B10.H2b mice is a dynamic environment with continuous influx of PCs that compete for the survival niches and are often replaced by newly arrived PCs. This competition for the survival niches has been discovered in other studies [29,30]. Despite their importance, little is known about the distribution, components, and function of such niches.

Expression of CXCR4 on murine PCs is important in the homing of PCs to the BM [31]. In our study, only a small percentage of PCs expressed this chemokine receptor in both spleen and BM. This may be due to the fact that by identifying our PC population as $CD138^+CD38^-$, we are mostly looking at the more mature PCs and thus less migratory population [32]. However, we can still detect an increase of $CXCR4^+$ PCs in the BM in 24-week-old NOD.B10.H2b mice, thus indicating influx of new PCs to this site. At the same time, we observed a higher percentage of $CXCR4^+$ PCs in the spleen of 17-week-old NOD.B10.H2b mice, indicating presence of PCs with higher migration potential comparing with both younger and older NOD.B10.H2b mice. Additionally, there is a number of other chemokines that drives migration of PCs [32] that we did not test in this study.

Splenic PCs have often been considered as short-lived PCs. Nevertheless, a long-lived PC population has also been detected in the spleen of [13,14]. By using BrdU incorporation, we found stable percentages of possibly long-lived PCs in the spleen of both autoimmune NOD.B10.H2b and non-autoimmune BALB/c mice. Therefore, our results support the idea that splenic PCs are important contributors to the long-lived PC pool. In spite of stable levels of short-lived $BrdU^+$ PCs, examination of BrdU density revealed high PC proliferation activity in 24- and 32-week-old NOD.B10.H2b mice. This may reflect changes in the PCs population and generation of new PCs at these time points. In contrast, in the BM, approximately 60% of BM PCs were positive for BrdU at all time points with no differences in the BrdU densities. This indicates that PCs migrating to the BM are of a special maturation status.

In addition to the environment, the importance of intrinsic properties in the survival process of PCs has been presented in humans. For instance, long-lived BM PCs express higher amounts of Bcl2 than circulating PCs and the PCs found in the lymphoid organs [25]. Our finding confirms and extends this observation showing higher proportions of $Bcl2^+$ PCs as well as higher density of Bcl2 in the BM PCs compared with splenic PCs in both autoimmune NOD.B10.H2b and BALB/c mice. The low percentages of $Bcl2^+$ PCs that we found undermine, however, the theory about the important contribution of splenic PCs to the long-lived PC subset [10].

Our results suggest that PCs from spleen are more susceptible to apoptosis than BM PCs, although this should be examined in more detail. Intriguingly, in the context of autoimmunity, we found generally lower proportions of PCs expressing Bcl2 in the autoimmune NOD.B10.H2b compared with non-autoimmune BALB/c mice. Observing high accumulation of $Bcl2^+$ PCs in the salivary glands of pSS patients (own observations), one explanation of this phenomenon could be that the $Bcl2^+$ PCs in NOD.B10.H2b mice migrate to the site of inflammation instead of BM.

Conclusion

Our results demonstrate that non-proliferating, potentially long-lived PCs are present in the spleen and BM of both autoimmune NOD.B10.H2b and non-autoimmune BALB/c mice, indicating formation of long-lived PCs regardless of autoimmunity. Nevertheless, in the aged mice, there were significantly more PCs in the BM of NOD.B10.H2b strain, pointing to more supportive survival environment (with possibly more survival niches) present in the BM of autoimmune mice.

Acknowledgements

We gratefully thank Kjerstin Jakobsen and Marianne Eidsheim for technical assistance.

Declaration of interest: This work was supported by the Faculty of Medicine and Dentistry, University of Bergen, the L. Meltzer Foundation, the Strategic Research Program at Helse Bergen, the Western Regional Health Authority, and the Broegelmann Foundation. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

[1] Routsias JG, Vlachoyiannopoulos PG, Tzioufas AG. Autoantibodies to intracellular autoantigens and their B-cell epitopes: Molecular probes to study the autoimmune response. Crit Rev Clin Lab Sci 2006;43(3):203–248.

[2] Renaudineau Y, Jamin C, Saraux A, Youinou P. Rheumatoid factor on a daily basis. Autoimmunity 2005;38(1):11–16.

[3] Franceschini F, Cavazzana I. Anti-Ro/SSA and La/SSB antibodies. Autoimmunity 2005;38(1):55–63.

[4] Edwards JC, Cambridge G. B-cell targeting in rheumatoid arthritis and other autoimmune diseases. Nat Rev Immunol 2006;6(5):394–403.

[5] Eisenberg R, Albert D. B-cell targeted therapies in rheumatoid arthritis and systemic lupus erythematosus. Nat Clin Pract Rheumatol 2006;2(1):20–27.

[6] Cambridge G, Leandro MJ, Teodorescu M, Manson J, Rahman A, Isenberg DA, Edwards JC. B cell depletion therapy in systemic lupus erythematosus: Effect on autoanti-

body and antimicrobial antibody profiles. Arthritis Rheum 2006;54(11):3612–3622.

[7] Vallerskog T, Gunnarsson I, Widhe M, Risselada A, Klareskog L, van Vollenhoven R, Malmstrom V, Trollmo C. Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE. Clin Immunol 2007;122(1):62–74.

[8] DiLillo DJ, Hamaguchi Y, Ueda Y, Yang K, Uchidan J, Haas KM, Kelsoe G, Tedder TF. Maintenance of long-lived plasma cells and serological memory despite mature and memory B cell depletion during CD20 immunotherapy in mice. J Immunol 2008;180(1):361–371.

[9] Dorner T, Burmester GR. New approaches of B-cell-directed therapy: Beyond rituximab. Curr Opin Rheumatol 2008; 20(3):263–268.

[10] Hoyer BF, Moser K, Hauser AE, Peddinghaus A, Voigt C, Eilat D, Radbruch A, Hiepe F, Manz RA. Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice. J Exp Med 2004; 199(11):1577–1584.

[11] Slifka MK, Matloubian M, Ahmed R. Bone marrow is a major site of long-term antibody production after acute viral infection. J Virol 1995;69(3):1895–1902.

[12] Manz RA, Thiel A, Radbruch A. Lifetime of plasma cells in the bone marrow. Nature 1997;388(6638):133–134.

[13] Manz RA, Lohning M, Cassese G, Thiel A, Radbruch A. Survival of long-lived plasma cells is independent of antigen. Int Immunol 1998;10(11):1703–1711.

[14] Slifka MK, Antia R, Whitmire JK, Ahmed R. Humoral immunity due to long-lived plasma cells. Immunity 1998;8(3): 363–372.

[15] Kunkel EJ, Butcher EC. Plasma-cell homing. Nat Rev Immunol 2003;3(10):822–829.

[16] Cyster JG. Homing of antibody secreting cells. Immunol Rev 2003;194:48–60.

[17] Robinson CP, Yamachika S, Bounous DI, Brayer J, Jonsson R, Holmdahl R, Peck AB, Humphreys-Beher MG. A novel NOD-derived murine model of primary Sjogren's syndrome. Arthritis Rheum 1998;41(1):150–156.

[18] Oliver AM, Martin F, Kearney JF. Mouse CD38 is down-regulated on germinal center B cells and mature plasma cells. J Immunol 1997;158(3):1108–1115.

[19] Yamano S, Atkinson JC, Baum BJ, Fox PC. Salivary gland cytokine expression in NOD and normal BALB/c mice. Clin Immunol 1999;92(3):265–275.

[20] Gao J, Killedar S, Cornelius JG, Nguyen C, Cha S, Peck AB. Sjogren's syndrome in the NOD mouse model is an interleukin-4 time-dependent, antibody isotype-specific autoimmune disease. J Autoimmun 2006;26(2):90–103.

[21] Nguyen C, Cornelius J, Singson E, Killedar S, Cha S, Peck AB. Role of complement and B lymphocytes in Sjogren's syndrome-like autoimmune exocrinopathy of NOD.B10-H2b mice. Mol Immunol 2006;43(9):1332–1339.

[22] Nguyen KH, Brayer J, Cha S, Diggs S, Yasunari U, Hilal G, Peck AB, Humphreys-Beher MG. Evidence for antimuscarinic acetylcholine receptor antibody-mediated secretory dysfunction in nod mice. Arthritis Rheum 2000;43(10):2297–2306.

[23] Skarstein K, Wahren M, Zaura E, Hattori M, Jonsson R. Characterization of T cell receptor repertoire and anti-Ro/SSA autoantibodies in relation to sialadenitis of NOD mice. Autoimmunity 1995;22(1):9–16.

[24] Moser K, Tokoyoda K, Radbruch A, MacLennan I, Manz RA. Stromal niches, plasma cell differentiation and survival. Curr Opin Immunol 2006;18(3):265–270.

[25] Medina F, Segundo C, Campos-Caro A, Gonzalez-Garcia I, Brieva JA. The heterogeneity shown by human plasma cells from tonsil, blood, and bone marrow reveals graded stages of increasing maturity, but local profiles of adhesion molecule expression. Blood 2002;99(6):2154–2161.

[26] Smith KG, Hewitson TD, Nossal GJ, Tarlinton DM. The phenotype and fate of the antibody-forming cells of the splenic foci. Eur J Immunol 1996;26(2):444–448.

[27] Cassese G, Lindenau S, de Boer B, Arce S, Hauser A, Riemekasten G, Berek C, Hiepe F, Krenn V, Radbruch A, Manz RA. Inflamed kidneys of NZB/W mice are a major site for the homeostasis of plasma cells. Eur J Immunol 2001; 31(9):2726–2732.

[28] Sze DM, Toellner KM, Garcia de Vinuesa C, Taylor DR, MacLennan IC. Intrinsic constraint on plasmablast growth and extrinsic limits of plasma cell survival. J Exp Med 2000; 192(6):813–821.

[29] Manz RA, Hauser AE, Hiepe F, Radbruch A. Maintenance of serum antibody levels. Annu Rev Immunol 2005;23:367–386.

[30] Odendahl M, Mei H, Hoyer BF, Jacobi AM, Hansen A, Muehlinghaus G, Berek C, Hiepe F, Manz R, Radbruch A, Dorner T. Generation of migratory antigen-specific plasma blasts and mobilization of resident plasma cells in a secondary immune response. Blood 2005;105(4):1614–1621.

[31] Hargreaves DC, Hyman PL, Lu TT, Ngo VN, Bidgol A, Suzuki G, Zou YR, Littman DR, Cyster JG. A coordinated change in chemokine responsiveness guides plasma cell movements. J Exp Med 2001;194(1):45–56.

[32] Hauser AE, Debes GF, Arce S, Cassese G, Hamann A, Radbruch A, Manz RA. Chemotactic responsiveness toward ligands for CXCR3 and CXCR4 is regulated on plasma blasts during the time course of a memory immune response. J Immunol 2002;169(3):1277–1282.