CD22 regulates early B cell development in BOB.1/OBF.1-deficient mice

Tatjana Samardzic[1], Judith Gerlach[2], Kerstin Muller[1], Dragan Marinkovic[1], Jochen Hess[1], Lars Nitschke[2] and Thomas Wirth[1]

[1] Department of Physiological Chemistry, Ulm University, Ulm, Germany
[2] Institute for Virology and Immunobiology, Würzburg University, Würzburg, Germany

BOB.1/OBF.1 (also called OCA-B), a B lymphocyte-specific transcriptional coactivator, is recruited to octamer-containing promoters by interacting with the Oct-1 or Oct-2 proteins. BOB.1/OBF.1-deficient mice show impaired secondary immunoglobulin isotype secretion and complete absence of germinal centers. Furthermore, numbers of splenic B cells are reduced due to a developmental block at the transitional B cell stage in the bone marrow. We found that surface expression of CD22 is selectively increased on B lineage cells in the bone marrow of BOB.1/OBF.1-deficient mice. CD22 is known as a negative regulator of B cell receptor signaling. We therefore investigated whether defects in B cell development in the BOB.1/OBF.1-deficient mice might be due to CD22 up-regulation. Mice were generated lacking both genes. In BOB.1/OBF.1×CD22 double-deficient mice, numbers of transitional B cells in the bone marrow were normal. Consequently, double-deficient mice also had normal B to T cell ratios in the spleen. We show that BOB.1/OBF.1$^{-/-}$ B cells were incapable to induce BCR-triggered Ca^{2+} mobilization. This Ca^{2+}-signalling defect was restored in BOB.1/OBF.1×CD22 double-deficient B cells. Nevertheless, double-deficient animals were unable to mount humoral immune responses and to form germinal centers. Finally, we demonstrate that CD22$^{-/-}$ splenic B cells proliferate independently of BOB.1/OBF.1 upon stimulation with LPS. These studies suggest that the B cell differentiation defect observed in BOB.1/OBF.1$^{-/-}$ mice is BCR-signal dependent. However, the impairment in germinal center formation is caused by a different mechanism.

Key words: B lymphocyte / Cell surface molecule / Transcription factor / Cellular differentiation / Signal transduction

Received	29/5/02
Accepted	18/6/02

1 Introduction

Octamer-dependent transcription is critical at various stages of B cell development [1]. Octamer motifs are found in virtually all immunoglobulin heavy chain and light chain promoters and in addition in various enhancer elements present in immunoglobulin genes. The activity of promoter octamer motifs is conferred by either of two DNA binding proteins, namely Oct1 or Oct2, and a B cell restricted co-activator protein BOB.1/OBF.1 (also known as OCA-B) [2–8]. Inactivation of BOB.1/OBF.1 in the mouse germ line revealed several distinct functions of this transcriptional co-activator in the process of the B-lymphoid development, differentiation and immune response. In BOB.1/OBF.1-deficient mice early B cell development in the bone marrow occurs comparable to wild-type mice. However, the number of transitional B cell is significantly reduced in the bone marrow [1]. Accordingly, the number of transitional B cells arriving in the spleen is diminished in BOB.1/OBF.1$^{-/-}$ mice compared to wild-type controls [1, 9]. Furthermore, BOB.1/OBF.1$^{-/-}$ mice are greatly impaired in the production of secondary immunoglobulins [7, 10]. As could be shown, this defect is not due to a failure of the isotype switching process, but rather to reduced levels of transcription from normally switched immunoglobulin heavy-chain loci [10]. Additionally, no germinal centers can be found in BOB.1/OBF.1-deficient mice [7, 9, 10]. This is consistent with several observations indicating that the level of BOB.1/OBF.1 protein is up-regulated in germinal center B lymphocytes [11, 12]. Up-regulation of BOB.1/OBF.1 in the germinal centers is not only due to transcriptional induction [11, 12] but also by a post-translational mecha-

[I 23198]

The first two authors contributed equally to this work.

0014-2980/02/0909-2481$17.50+.50/0

nisms [13, 14]. Recent investigations demonstrate that the transcriptional co-activator regulates the expression of the chemokine receptor BLR1, which is a major regulator of the microenvironmental homing of B cells in lymphoid organs [15]. So far it is still unclear, how BOB.1/OBF.1 influences the early stages of B-lymphoid differentiation. When we compared the cell surface phenotype of wild-type and BOB.1/OBF.1-deficient bone marrow B cells, we realized that CD22 is up-regulated in cells lacking BOB.1/OBF.1, suggesting that a higher expression of this inhibitory molecule may cause the observed developmental block.

The expression of CD22, a B lineage-restricted adhesion receptor, begins at the pre-B cell stage [16, 17] and steadily increases during development in the bone marrow. The role of CD22 during early B cell development is largely unknown, while mature, long-lived B cells require CD22 for recirculating back to the bone marrow [18]. Furthermore, CD22 acts as an inhibitory accessory molecule modulating the BCR-initiated signal. The negative role of CD22 was proven by the generation of CD22 knockout mice [16, 19–21]. Upon BCR cross-linking in the CD22-deficient B cells, an increased and prolonged Ca^{2+} signal can be measured [16, 19–21]. In $CD22^{-/-}$ mice, mature B cells circulate in a pre-activated stage and show a pronounced susceptibility to apoptosis, as well as an increased turnover rate [16]. Independently of the BCR-initiated signal, CD22-deficient B cells respond with increased proliferation upon LPS treatment. So far, the mechanisms resulting in this augmented proliferation are unclear. Since BOB.1/OBF.1 expression is induced in B cells upon LPS stimulation, we checked the expression level of the co-activator in $CD22^{-/-}$ B cells upon LPS treatment and could detect a much higher BOB.1/OBF.1 protein level compared to control B cells. The overexpression of CD22 on BOB.1/OBF.$1^{-/-}$ pre-B cells and the increased protein level of BOB.1/OBF.1 in $CD22^{-/-}$ B cells upon LPS stimulation encouraged us to cross BOB.1/OBF.$1^{-/-}$×$CD22^{-/-}$ mice, to analyze whether their signaling pathways are functionally connected.

2 Results

2.1 Increased surface expression of CD22 on B cell precursors in BOB.1/OBF.$1^{-/-}$ mice

To further investigate the impairment in B cell differentiation in BOB.1/OBF.$1^{-/-}$ mice, expression of various cell surface molecules of wild-type and BOB.1/OBF.$1^{-/-}$ bone marrow and spleen B cells were compared. We performed three-color stainings of bone marrow cells isolated from wild-type and BOB.1/OBF.$1^{-/-}$ mice. By these stainings an increased expression level of the B-lineage specific cell surface protein CD22 was observed (Fig. 1A). Concordant with previous data [16, 17], staining of wild-type bone marrow cells with CD22 specific antibody separated the B220/$CD45^+$ B cell into three different CD22-expressing populations. High expression level of CD22 characterized mature, recirculating B cells ($B220^{high}IgM^{low}$), whereas immature ($B220^{low}IgM^{med}$) and transitional B ($B220^{low/high}IgM^{high}$) cells expressed an intermediate and pre-B cells ($B220^{low}IgM^{neg}$) a low level of CD22. Bone marrow B cells isolated from BOB.1/OBF.1-deficient mice showed increased expression of CD22 (Fig. 1A). Interestingly, this increase of CD22 expression in BOB.1/OBF.1-deficient B cells was most pronounced on the pre-B cells and immature B cells in the bone marrow, but also found on $CD22^{high}$-expressing mature B cells in the bone marrow and the spleen (Fig. 1A, B). In contrast, the pre-B cell markers BP-1 and CD25 were expressed at normal levels in BOB.1/OBF.$1^{-/-}$ mice (Fig. 1C). Furthermore, the $B220^+HSA^{high}$ population (pre-B and immature B cells) expressed a similar HSA level in both types of mice (Fig. 1C).

2.2 CD22 deficiency rescued development of transitional B cells in BOB.1/OBF.$1^{-/-}$ mice

CD22 has been shown to act as a negative regulator of the BCR-initiated signal in mature B cells [22]. Since small changes of the CD22 expression level can already show a phenotype [23], we considered the possibility that the up-regulation of CD22 expression on B cell precursors may be responsible for the developmental block observed in the bone marrow of BOB.1/OBF.1-deficient mice. To further investigate this hypothesis, BOB.1/OBF.$1^{-/-}$ mice were crossed with mice deficient in CD22 and the bone marrow of BOB.1/OBF.1×CD22 double-deficient mice was analyzed. As has been previously described [1], development of pro-, pre- and immature B cells in BOB.1/OBF.$1^{-/-}$ mice occurred normal, while the number of transitional and recirculating B cells was reduced. CD22 deficiency did not interfere with B cell development in the bone marrow, resulting in normal numbers of transitional B cells [16]. However, recirculating B cells were almost absent due to a homing defect of CD22-deficient B cells [18]. Using anti-B220 and anti-IgM antibodies allowed us to distinguish pro- and pre- ($B220^{low}IgM^{neg}$), immature ($B220^{low}IgM^{med}$), transitional ($B220^{low/high}IgM^{high}$) and recirculating ($B220^{high}IgM^{low}$) B cells. Bone marrow B cells isolated from BOB.1/OBF.1×CD22 double-deficient mice revealed a significantly augmented number of transitional B cells compared to BOB.1/OBF.$1^{-/-}$ mice, almost achieving wild-type level (Fig. 2). These data were consistent with the hypothesis that the defect of early B cell development observed in BOB.1/OBF.$1^{-/-}$ animals might be due to the

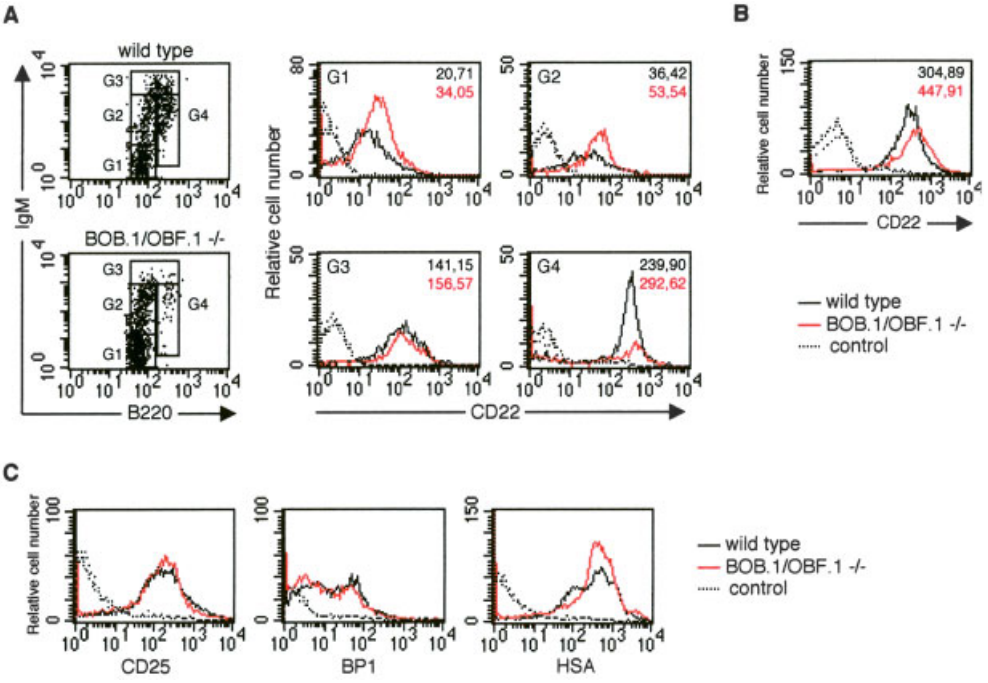

Fig. 1. Altered CD22 expression pattern in the bone marrow of BOB.1/OBF.1$^{-/-}$ mice. (A) Bone marrow cells from wild-type and BOB.1/OBF.1$^{-/-}$ mice were stained with anti-B220-FITC, anti-IgM-biotin and anti-CD22-PE antibodies. Lymphocytes were gated for pro- and pre B cells (B220^{low}IgM^{neg}, G1), immature B cells (B220^{low}IgM^{low}, G2), transitional (B220$^{low/high}$IgM^{high}, G3) and mature, recirculating B cells (B220^{high}IgM^{low}, G4). Histograms present the CD22 expression levels of BOB.1/OBF.1$^{-/-}$ and wild-type mice of the indicated gates. Mean fluorescence intensities (MFI) for wild-type (black) and BOB.1/OBF.1-deficient (red) samples are given. (B) Splenocytes from wild-type and BOB.1/OBF.1 $^{-/-}$ mice were gated for B220^{+} cells and a histogram showing CD22 staining with MFI is given. (C) Expression of CD25, BP1 and HSA on B220^{+} bone marrow cells from wild-type and BOB.1/OBF.1$^{-/-}$ mice. Data are representative out of four experiments.

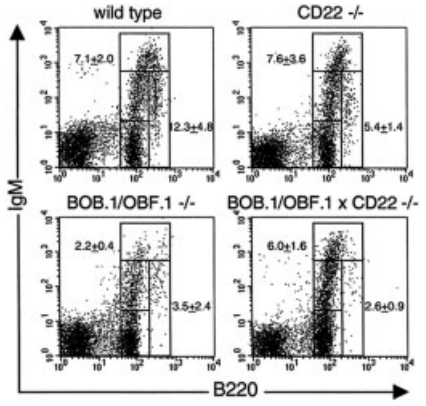

Fig. 2. Development of B cells in the bone marrow was rescued in BOB.1/OBF.1×CD22 double-deficient mice. Bone marrow cells were isolated from wild-type, CD22$^{-/-}$, BOB.1/OBF.1$^{-/-}$, and BOB.1/OBF.1×CD22$^{-/-}$ mice, stained with the combination of anti-B220-FITC and anti-IgM-PE antibodies and analyzed by flow cytometry. One representative out of 6 staining is shown. Mean percentages with SD of transitional (B220$^{low\text{-}med}$IgM^{high}) and recirculating (B220^{high}IgM^{low}) B cells are given.

increased inhibitory influence on the BCR-signal, since CD22 was higher expressed on precursor B cells. Nevertheless, the defect in recirculating B cells in the bone marrow of BOB.1/OBF.1$^{-/-}$ mice could not be rescued in double knockout mice.

2.3 Rescued B to T cell ratio in the spleen of BOB.1/OBF.1×CD22 double–deficient mice

BOB.1/OBF.1-deficient mice revealed a two- to fourfold reduced number of splenic B cells [1, 7, 9]. Since crossing of BOB.1/OBF.1×CD22 deficiencies led to virtually normal numbers of transitional B cells in the bone marrow, further investigations were done to clarify, whether this rescue might result in the appearance of normal B cell numbers in the periphery. Indeed, comparing T and B cell populations showed that CD22 deficiency increased the B to T cell ratio in the spleen of BOB.1/OBF.1$^{-/-}$ mice (Fig. 3A). To analyze the different B cell subpopulations in the spleen of BOB.1/OBF.1×CD22 double-deficient mice, anti-IgM versus anti-IgD stainings

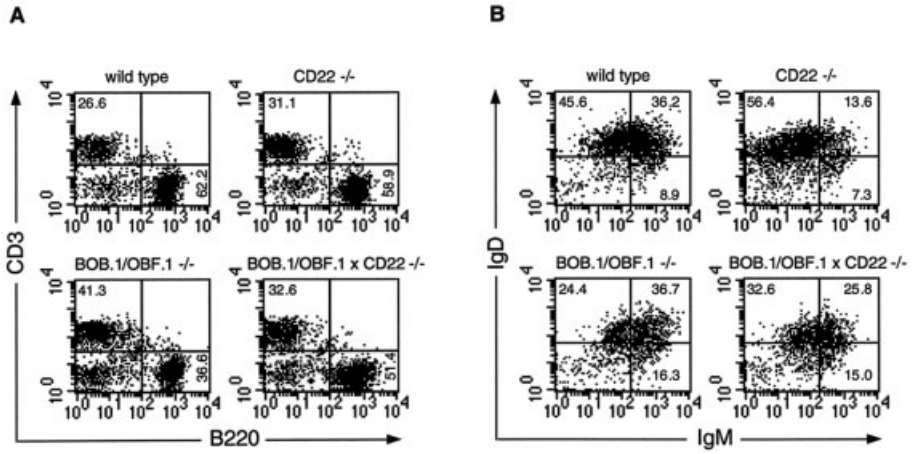

Fig. 3. B to T cell ratio in the spleen of BOB.1/OBF.1×CD22 double-deficient mice comparable to wild-type mice. (**A**) Splenic B cells from mice with indicated genotype were stained with anti-B220-FITC and anti-CD3-PE. Mean percentages of T and B cells are given. (**B**) Splenic cells are stained with anti-B220-biotin, anti-IgM-PE and anti-IgD-FITC antibodies. $B220^+$ cells were gated and mean percentages of total B cells, transitional T1 ($IgM^{high}IgD^{low}$), transitional T2 ($IgM^{high}IgD^{high}$) [26] and mature ($IgM^{low}IgD^{high}$) B cell populations from one representative experiment are denoted. Mean percentages of mature ($IgM^{low}IgD^{high}$) B cells obtained in four independent experiments were 47.67±3.99 (wild-type), 48.81±5.46 ($CD22^{-/-}$), 21.88±4.16 ($BOB.1/OBF.1^{-/-}$) and 34.81±4.43 (BOB.1/OBF. 1×$CD22^{-/-}$).

were done. BOB.1/OBF.1 deficiency resulted in the reduction of $IgM^{low}IgD^{high}$ mature splenic B cells. In contrast, CD22 deficiency enhanced maturation of B cells in the periphery, since in $CD22^{-/-}$ mice the relative number of $IgM^{low}IgD^{high}$ cells was slightly increased [16]. In the spleen of BOB.1/OBF.1×CD22 double-deficient mice the population of $IgM^{low}IgD^{high}$ mature B cells increased, but did not reach wild-type level (Fig. 3B).

2.4 Ca^{2+} mobilization was restored in BOB.1/OBF.1×CD22 double-deficient splenic B cells

Having shown the developmental rescue of transitional B cells in the bone marrow and an increase of mature B cells in the spleen of BOB.1/OBF.1×CD22 double-deficient mice, we looked for possible mechanisms. Therefore, the signaling capacity of the BCR of $BOB.1/OBF.1^{-/-}$ and $BOB.1/OBF.1\times CD22^{-/-}$ B cells was compared. Splenocytes of $BOB.1/OBF.1^{-/-}$, $CD22^{-/-}$, double-deficient and control mice were stimulated by cross-linking the BCR and the initiated Ca^{2+} mobilization was measured (Fig. 4). As expected, the Ca^{2+} mobilization of $CD22^{-/-}$ splenic B cells was augmented, whereas in $BOB.1/OBF.1^{-/-}$ B cells Ca^{2+} mobilization stayed just above background level. Thereby we demonstrated for the first time, that defective BCR signaling in BOB.1/OBF.1-deficient B cells was caused by a lack of Ca^{2+} mobilization. When analyzing BOB.1/OBF.1×CD22 double-deficient splenic B cells, the Ca^{2+} level was even

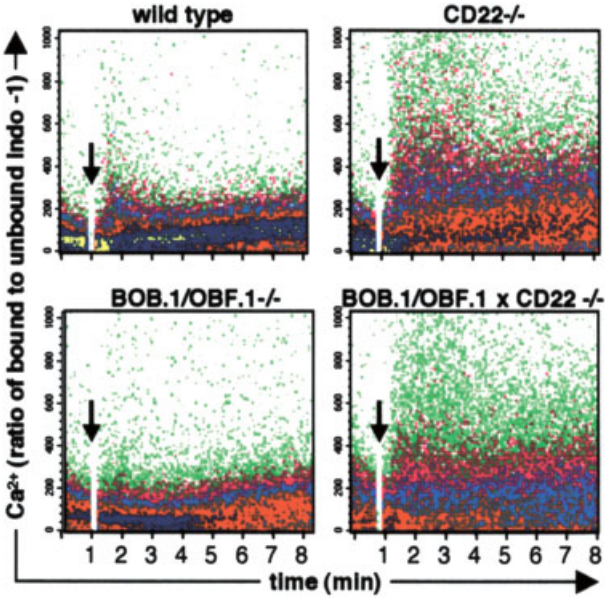

Fig. 4. CD22 deficiency rescued Ca^{2+} mobilization in $BOB.1/OBF.1^{-/-}$ splenic B cells. Splenic B cells of wild-type, $CD22^{-/-}$, $BOB.1/OBF.1^{-/-}$, and BOB.1/OBF.1×CD22 double-deficient mice were stimulated with 10 μg/ml B7–6 (anti-IgM antibody) at the time point indicated by the arrow within the figure. Ca^{2+} mobilization can be determined by the ratio of bound to unbound Indo-1. One typical experiment out of five with identical results is shown.

higher than in control B cells, but not reaching the level of CD22-deficient B cells. We could show that the almost undetectable Ca^{2+} signal of BOB.1/OBF.1$^{-/-}$ B cells was enhanced to at least wild-type level by deleting CD22.

2.5 Antigen-dependent immune responses in BOB.1/OBF.1×CD22$^{-/-}$ mice

BOB.1/OBF.1 is not only involved in B cells development, but also has an important role in antigen-dependent B cell responses. In the absence of BOB.1/OBF.1, thymus-dependent immune responses and germinal center formation do not take place [7, 9, 10]. To analyze, whether antibody responses were rescued in BOB.1/OBF.1×CD22 double-deficient mice, we tested T cell-dependent immune response. Therefore, mice of all genotypes were immunized twice with TNP-ovalbumin (TNP-OVA) and serum titers of anti-TNP specific IgM and IgG_1 antibodies were measured. While immunization of wild-type and CD22$^{-/-}$ mice with TNP-OVA led to a strong anti-TNP IgG_1 production, BOB.1/OBF.1$^{-/-}$ mice showed no primary IgG1 response and only a minor secondary response in this experiment. Similarly, double-deficient mice generated no TNP-specific IgG1antibodies (Fig. 5). However, both BOB.1/OBF.1$^{-/-}$ and double knockout mice mounted a significant IgM primary response to TNP-OVA (not shown). Also, total Ig levels of all isotypes were similarly decreased in BOB.1/OBF.1 and double knockout mice (data not shown). Finally, the spleens of TNP-OVA immunized mice were analyzed for the presence of germinal centers. Staining with PNA revealed germinal centers in the spleens of wild-type and CD22$^{-/-}$ mice. As expected, germinal centers were not detectable in the splenic follicles of immunized BOB.1/OBF.1$^{-/-}$ mice (Fig. 6). Likewise, no germinal centers were observed in PNA-stained spleen sections from immunized double-deficient mice.

2.6 LPS stimulation induced an earlier onset and increased expression of BOB.1/OBF.1 in CD22$^{-/-}$ splenic B cells

CD22-deficient B cells showed an enhanced proliferative response upon treatment with the mitogen LPS [16, 20]. Trying to elucidate the mechanism for this, we looked at downstream LPS signaling pathways including the transcriptional co-activator BOB.1/OBF.1. Purified splenic B cells from wild-type and CD22$^{-/-}$ mice were stimulated for 12, 24, 36 or 48 h with LPS. The proliferative response as well as the expression level of BOB.1/OBF.1 were examined. In Fig. 7 the expression of BOB.1/OBF.1 at the protein level is shown. While unstimulated CD22$^{-/-}$

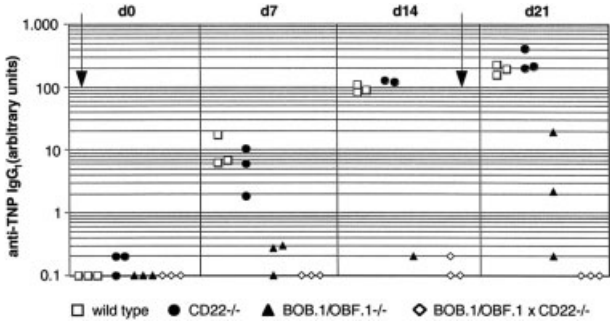

Fig. 5. Analysis of T cell dependent immune response. Wild-type, CD22$^{-/-}$, BOB.1/OBF.1$^{-/-}$, and BOB.1/OBF.1×CD22 double-deficient mice were immunized i.p. with 10 μg/ml TNP-OVA at day 0, and boosted at day 14. Blood of the immunized mice was taken at days 0, 7, 14, and 21 and immune response was determined by measuring TNP-specific IgG1 levels by ELISA. Each symbol represents one individual. The arrows indicate the day of TNP-OVA injection. For day 14 only the indicated mouse sera were available. One typical out of two experiments is shown.

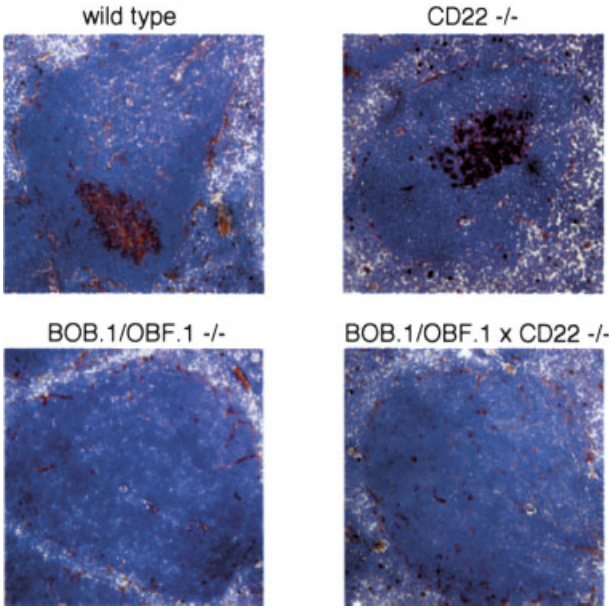

Fig. 6. Analysis of germinal centers formation. Wild-type, CD22$^{-/-}$, BOB.1/OBF.1$^{-/-}$, and BOB.1/OBF.1×CD22 double-deficient mice were immunized i.p. with 10 μg/ml TNP-OVA at day 0, and boosted at day 14. At day 21 mice were killed and spleens were subjected to histological stainings. Spleen sections, 4 μm thick, were stained with biotin-labeled PNA. Binding of PNA to germinal centers is visualized using streptavidin-HRP and AEC compound as a substrate. Sections were counterstained with hematoxylin and analyzed using Leica microscope and OpenLab software.

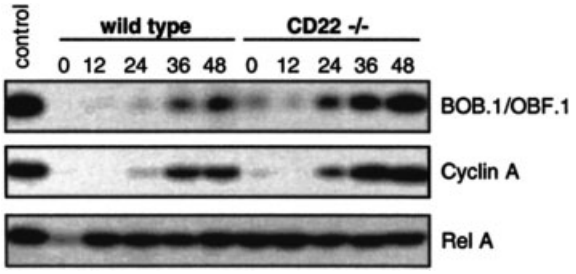

Fig. 7. Increased BOB.1/OBF.1 protein level in CD22$^{-/-}$ B cells upon LPS stimulation. T cell depleted splenic B cells out of CD22$^{-/-}$ and wild-type mice were stimulated with 15 μg/ml LPS. Proliferation was stopped at the indicated time points by lysing the cells. Comparable protein concentrations were analyzed by staining the Western blot with anti-BOB.1/OBF.1 and anti-Cyclin A antibodies. Rel A staining was performed as loading control. Unstimulated B cells are indicated as time point zero and untreated PD31 cells were used as positive control.

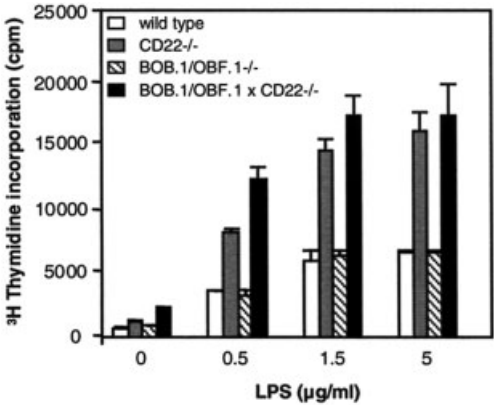

Fig. 8. BOB.1/OBF.1×CD22 double-deficient splenic B cells proliferate comparably to CD22$^{-/-}$ B cells upon stimulation with LPS. Proliferative response of wild-type, CD22$^{-/-}$, BOB.1/OBF.1$^{-/-}$, or BOB.1/OBF.1 × CD22 double-deficient splenic B cells was measured as [^{3}H]thymidine incorporation upon 24-h stimulation with the indicated concentration of LPS. The graph shows the mean of triplicates and the corresponding standard deviations.

or control B cells expressed BOB.1/OBF.1 at comparable low levels, the amount of the co-activator could be induced much faster and to a higher extent in CD22-deficient B cells upon stimulation with LPS. An indication for the enhanced proliferation of CD22$^{-/-}$ B cells was the simultaneous probing of the blot with a Cyclin A antibody, since Cyclin A is required for activating the DNA replication machinery during the interphase of the cell cycle (Fig. 7).

2.7 Proliferation of LPS stimulated BOB.1/OBF.1×CD22 double-deficient B cells

T cell depleted splenic B cells of BOB.1/OBF.1$^{-/-}$, CD22$^{-/-}$, double-deficient, and control mice were stimulated with the indicated concentrations of LPS for 24 h (Fig. 8). BOB.1/OBF.1$^{-/-}$ B cells showed a similar response as wild-type B cells to LPS treatment, whereas CD22$^{-/-}$ B cells proliferated about twice as high as control cells, even at a concentration as low as 0.5 μg/ml LPS. Surprisingly, double-deficient B cells proliferated at least as well as CD22$^{-/-}$ B cells (Fig. 8). We can conclude from these results that the increased BOB.1/OBF.1 expression is not crucial for the increased proliferative response of CD22$^{-/-}$ B cells upon stimulation with LPS.

3 Discussion

As has recently been described, B cells in BOB.1/OBF.1$^{-/-}$ mice have a developmental block at the transitional B cell stage [1]. In the present study, a potential candidate causing the developmental block could be identified, namely the adhesion molecule CD22. The expression level of CD22 was increased on B-lineage cells in the bone marrow of BOB.1/OBF.1$^{-/-}$ mice compared to wild-type bone marrow cells. When analyzing bone marrow B cells of BOB.1/OBF.1×CD22 double-deficient mice, we found that development was rescued resulting in normal numbers of transitional B cells. Furthermore, this increased cell number contributed to an almost wild-type ratio of B to T cells in the spleen of double-deficient mice. More mature IgM^{low}IgD^{high} B cells were found in the spleen, but did not reach the number found in control mice. Subsequently, the capacity of BOB.1/OBF.1$^{-/-}$ and double-deficient splenic B cells to initiate BCR signaling was determined by measuring Ca^{2+} mobilization upon BCR cross-linking. BOB.1/OBF.1$^{-/-}$ splenic B cells were unable to induce Ca^{2+} signals, whereas double-deficient B cells revealed an at least wild-type Ca^{2+} flux. However, upon immunization with antigen inducing a T cell-dependent immune response, no Ag-specific IgG_1 antibodies or germinal center formation was detectable in double-deficient mice. In CD22-deficient B cells, we observed an earlier onset and an increased level of BOB.1/OBF.1 protein upon stimulation with LPS. To narrow down the role of BOB.1/OBF.1 in LPS-induced signaling, BOB.1/OBF.1×CD22$^{-/-}$ B cells were subjected to proliferation assays. The transcriptional co-activator appeared not to be a crucial downstream target in the LPS pathway, since in BOB.1/OBF.1$^{-/-}$ and in BOB.1/OBF.1×CD22 double-deficient B cells the LPS response occurred independently of BOB.1/OBF.1.

BOB.1/OBF.1 deficiency seems to modify the expression level of CD22, as we could show by the increased surface expression of CD22 on B lineage cells. CD22 can be detected on the surface of wild-type bone marrow B cells beginning at the pre-B cells stage and steadily increasing during developmental progression [16, 17]. So far, the function of CD22 during B cell development is not clear, because B cell maturation in CD22-deficient mice occurs normal. Since CD22 is a negative regulator of the BCR signaling, an increased CD22 expression may suppress BCR-dependent differentiation in the bone marrow. Thus, the block in development to the transitional B cell stage in BOB.1/OBF.1$^{-/-}$ mice may be mechanistically explained by the higher CD22 expression pattern of the immature B cell population. Additionally, CD22 may already suppress differentiation at the pre-B cell stage by inhibiting the pre-BCR signal. Pre-B cells require a pre-BCR signal to expand and enter the next differentiation stage [24]. However, it is not known whether CD22 can modulate the pre-BCR signal.

The development of BOB.1/OBF.1$^{-/-}$ B cells in the bone marrow can be rescued by the addition of the CD22 deficiency, therefore, we expected to find expanded B cell populations in the periphery. This was confirmed by an almost wild-type B to T cell ratio and an increase of IgM^{low}IgD^{high} mature, long-lived B cells in the spleen of BOB.1/OBF.1×CD22 double-deficient mice. We speculated that this would result from a stronger BCR signal which forces immature B cells to proceed in development, as has been described for CD22-deficient B cells [16, 19–21]. To analyze the signaling capacity of splenic B cells, Ca^{2+} mobilization was measured upon BCR engagement. In BOB.1/OBF.1$^{-/-}$ B cells almost no Ca^{2+} mobilization can be detected, whereas in BOB.1/OBF.1×CD22 double-deficient B cells the Ca^{2+} level is clearly increased. These results show for the first time that the BCR-initiated Ca^{2+} signal is strongly affected in BOB.1/OBF.1$^{-/-}$ B cells. Supposedly, the co-activator is essential for regulating the expression of so-far-unidentified signaling molecules, which are involved in Ca^{2+} mobilization. Additionally, the increased CD22 expression level of BOB.1/OBF.1 $^{-/-}$ B cells probably also contributes to the decrease of Ca^{2+} mobilization. When BOB.1/OBF.1 and CD22 deficiencies were combined, the inability to initiate a Ca^{2+} signal can be corrected. We have demonstrated that the CD22 deficiency can compensate the defective Ca^{2+} signaling of BOB.1/OBF.1$^{-/-}$ B cells. Whether or not the BOB.1/OBF.1 and the CD22 signaling pathways are coupled, we cannot conclude from our data. However, the Ca^{2+} mobilization initiated in double-deficient mice might not only be important for maturation in the periphery, but might also cause the developmental rescue in the bone marrow. Supposedly, BCR signal-dependent developmental checkpoints are rescued by the higher Ca^{2+} signal in the bone marrow of BOB.1/OBF.1×CD22 double-deficient B cells.

As just discussed for the rescue of B cell differentiation in double-deficient mice, the pathways initiated by the BCR might explain the defect of BOB.1/OBF.1$^{-/-}$ B cells. Stimulation of BOB.1/OBF.1-deficient B cells with anti-IgM antibodies leads to a fourfold reduction in proliferation, whereas the addition of T cell-derived signals like IL-4 or CD40 improve the proliferative response [10]. T cell-dependent immune responses do not exclusively depend on the BCR signal, but also on cytokines such as IL-4, co-stimulatory molecules like CD40 and B7, as well as on chemokines. Therefore, we wondered, whether a T cell-dependent immune response could be induced in BOB.1/OBF.1×CD22$^{-/-}$ mice. However, neither the production of TNP-specific IgG_1 antibodies, nor germinal center formation were improved. This was not all that surprising, since in CD22$^{-/-}$ mice no increased T cell-dependent response can be found, suggesting that CD22 is not crucial for the T cell-dependent immune response. In contrast, BOB.1/OBF.1$^{-/-}$ mice lack the formation of germinal centers, which indicates the requirement of the transcriptional co-activator. One explanation for the inability to form germinal centers could be that BOB.1/OBF.1 is critical for the transcription of the chemokine receptor BLR1, which guides B cells into B cell follicles [15, 25].

Finally, we also checked the responsiveness of BOB.1/OBF.1$^{-/-}$ splenic B cells upon stimulation with LPS. These cells proliferate at a comparable level to wild-type B cells, demonstrating that the activation pathway initiated by LPS is not affected by the BOB.1/OBF.1 deficiency. When CD22$^{-/-}$ B cells are treated with LPS, they show a higher proliferation rate and increased BOB.1/OBF.1 induction compared to wild-type B cells. We therefore speculated, that the up-regulation of BOB.1/OBF.1 may be mechanistically involved in the increased proliferation of CD22-deficient B cells. However, stimulating BOB.1/OBF.1×CD22 double-deficient splenic B cells with LPS revealed that those B cells proliferate at least as strong as CD22-deficient B cells. This finding indicates that BOB.1/OBF.1 does not have an essential role in LPS-induced proliferation. Thus, the mechanism by which LPS-induced proliferation is increased in CD22-deficient B cells is still not solved.

Overall we can conclude that by deleting CD22 in BOB.1/OBF.1$^{-/-}$ mice the B cell development in the bone marrow is rescued. Furthermore, the number of mature B cells in the periphery increases, but does not reach wild-type level. The reason for this improvement seems to be the Ca^{2+} mobilization in double-deficient splenic B cells that is comparable to that of wild-type B cells. In addition

to the impairment of BOB.1/OBF.1$^{-/-}$ B cells to initiate BCR signals, other mechanisms are also affected as can be seen by the lack of T cell-dependent immune responses, which can not be rescued by crossing in CD22 deficiency. In addition, we showed that BOB.1/OBF.1 is not essential in LPS-induced proliferation in CD22-deficient B cells.

4 Materials and methods

4.1 Mice

C57BL/6 wild-type, BOB.1/OBF.1$^{-/-}$, CD22$^{-/-}$ and BOB.1/OBF.1×CD22 double-deficient mice on the same genetic background were obtained from our breeding facility. Mice were analyzed 8–12 weeks after birth. We tried to use littermates for each experiment.

4.2 Flow cytometry analysis

Single-cell suspensions of Lympholyte-M (Cedarlane, Laboratories Ltd., Ontario, Canada) purified splenocytes (5×10^5) were incubated for 30 min at +4°C with different combinations (as indicated in the results section) of the following antibodies: anti-B220-FITC, anti-IgM-PE, anti-IgD-biotin, anti-CD3-PE and anti-CD22-PE (all produced by PharMingen, San Diego, CA). Suspensions of bone marrow cells obtained by conventional method were stained with combinations of anti-B220-FITC, anti-IgM-PE or anti-IgM-biotin, anti-CD22-PE, anti-CD25-biotin, anti-HSA-biotin and anti-BP1-biotin. Stainings were performed in PBS containing 0.1 % BSA (Roche Diagnostics, Mannheim, Germany), 0.1% sodium azide and saturating concentration of anti-CD16/CD32 (PharMingen) to block FcγIII/II receptors. Biotin-labeled antibody was revealed by streptavidin-CyChrom (PharMingen). Cell surface marker expression was analyzed using a four-color flow cytometer (FACScalibur) and CellQuest Software (Becton Dickinson, Heidelberg, Germany).

4.3 Measurement of Ca^{2+} movement

Splenic B cells from indicated mice were loaded with 4.5 μM Indo-1 plus 0.003% pluronic F-127 (both Molecular Probes, Eugene, OR) in RPMI containing 1% fetal calf serum for 45 min at 37°C. Staining was performed on ice for 30 min with biotinylated antibodies against CD11b (MAC.1) and Thy1.2, followed by streptavidin-PE (all PharMingen). Ca^{2+} movement was measured upon stimulation with 10 μg/ml B7–6 monoclonal anti-IgM antibody on gated MAC.1- and Thy1.2-negative B cells.

4.4 Immunization

BOB.1/OBF.1$^{-/-}$, CD22$^{-/-}$, double-deficient and control mice of 10–12 weeks age were immunized i.p. with 100 μg TNP_{13}-OVA (Biosearch Technologies, Novato, CA) preincubated in Alu-Gel-S (Serva, Boehringer Ingelheim, Heidelberg, Germany) at day 0 and boosted at day 14. Mice were bleed at days 0, 7, 14 and 21. TNP-specific antibody levels were determined by ELISA with TNP_{17}-BSA (Biosearch Technologies) coated polysorb plates (Nalge Nunc, Rochester, NY). Sera were applied in serial dilutions onto the plates. Concentration of IgM and IgG_1 levels were determined using goat anti-IgM or anti-IgG_1 alkaline phosphatase-linked antibodies and the substrate p-nitrophenyl phosphate. As an internal standard for each plate, independent sera were pooled and diluted as described. This served as an arbitrary standard defining arbitrary units.

4.5 Proliferation assay

Splenocytes from the indicated animals were T cell depleted by pretreating the cells with anti-CD4 and anti-CD8 IgM antibodies on ice and incubating them with baby rabbit complement (Cedarlane, Ontario) for 45 min at 37°C afterwards. For stimulation 1×10^5 B cells in RPMI containing 5% fetal calf serum were supplemented with different concentration of LPS (Calbiochem, Novabiochem, Darmstadt) for 24 h. Proliferation was measured by [^{3}H]thymidine incorporation (1 μCi/well) during the last 10 h of culture.

4.6 Immunohistochemistry

Spleens were embedded in Tissue-Tek O.C.T. compound (Sakura, The Netherlands), snap frozen in liquid nitrogen and stored at –80°C. Cryostat sections of 4 μm were prepared, air dried, and fixed in acetone (10 min at room temperature). Slides were stained 30 min with 20 μg/ml of PNA-biotin (Vector, CA) in 150 mM NaCl, 10 mM Hepes, pH 7.5. After washing, slides were incubated with 1:100 dilution of streptavidin-biotinylated horseradish peroxidase (HRP; Amersham, GB). Staining is visualized using AEC compound (Sigma), followed by counterstaining with hematoxylin (Sigma). Slides were mounted with Kaiser's glycerol gelatin (Merck, Germany) and analyzed using a Leica microscope (DMIRB/E) and OpenLab software (version 2.2.5).

4.7 Western blot

T cell-depleted splenocytes from wild-type and CD22$^{-/-}$ mice were stimulated with 15 μg/ml LPS (Calbiochem, Novabiochem, Darmstadt) for 12, 24, 36 and 48 h. Buffer used for cell lysis consisted of 20 mM Tris pH 7.5, 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate and 0.1% SDS. Proteins were resolved on a 12.5% SDS-polyacrylamide gel and transferred on PVDF membrane (Millipore). For immunoblotting of BOB.1/OBF.1 polyclonal rabbit anti-BOB.1/OBF.1 antibody was used. For detection of Rel A and Cyclin A antibodies from Santa Cruz were purchased. Proteins were visualized using donkey anti-

rabbit HRP-conjugated antibody (Santa Cruz) and ECL detection system (Amersham).

Acknowledgements: We thank Astrid Heiter for technical help. This work is supported by grant (SFB 497/C5) to Thomas Wirth and by a grant (SFB 465/B8) to Lars Nitschke.

References

1 **Hess, J., Nielsen, P. J., Fischer, K. D., Bujard, H. and Wirth, T.,** The B lymphocyte-specific coactivator BOB. 1/OBF. 1 is required at multiple stages of B cell development. *Mol. Cell. Biol.* 2001. **21**: 1531–1539.

2 **Gstaiger, M., Georgiev, O., van Leeuwen, H., van der Vliet, P. and Schaffner, W.,** The B cell coactivator Bob1 shows DNA sequence-dependent complex formation with the Oct-1/Oct-2 factors, leading to differential promoter activation. *EMBO J.* 1996. **15**: 2781–2790.

3 **Gstaiger, M., Knoepfel, L., Georgiev, O., Schaffner, W. and Hovens, C. M.,** A B cell coactivator of octamer-binding transcription factors. *Nature* 1995. **373**: 360–362.

4 **Luo, Y., Fujii, H., Gerster, T. and Roeder, R. G.,** A novel B cell-derived coactivator potentiates the activation of immunoglobulin promoters by octamer-binding transcription factors. *Cell* 1992. **71**: 231–241.

5 **Luo, Y., Ge, H., Stevens, S., Xiao, H. and Roeder, R. G.,** Coactivation by OCA-B: definition of critical regions and synergism with general cofactors. *Mol. Cell. Biol.* 1998. **18**: 3803–3810.

6 **Pfisterer, P., Annweiler, A., Ullmer, C., Corcoran, L. and Wirth, T.,** Differential transactivation potential of Oct1 and Oct2 is determined by additional B cell-specific activities. *EMBO J.* 1994. **13:** 1654–1663.

7 **Schubart, D. B., Rolink, A., Kosco-Vilbois, M. H., Botteri, F. and Matthias, P.,** B cell-specific coactivator OBF-1/OCA-B/Bob1 required for immune response and germinal center formation. *Nature* 1996. **383**: 538–542.

8 **Strubin, M., Newell, J. W. and Matthias, P.,** OBF-1, a novel B cell-specific coactivator that stimulates immunoglobulin promoter activity through association with octamer proteins. *Cell* 1995. **80**: 497–506.

9 **Nielsen, P. J., Georgiev, O., Lorenz, B. and Schaffner, W.,** B lymphocytes are impaired in mice lacking the transcriptional coactivator Bob1/OCA-B/OBF1. *Eur. J. Immunol.* 1996. **26:** 3214–3218.

10 **Kim, U., Qin, F. -F., Gong, S., Stevens, S., Luo, Y., Nussenzweig, M. and Roeder, R. G.,** The B cell-specific transcription coactivator OCA-B/OBF-1/Bob-1 is essential for normal production of immunoglobulin isotypes. *Nature* 1996. **383**: 542–547.

11 **Greiner, A., Müller, K., Hess, J., Pfeffer, K., Müller-Hermelink, K. H. and Wirth, T.,** BOB. 1/OBF. 1 expression is upregulated in normal germinal center B cells and germinal center derived B cell lymphomas. *Am. J. Pathol.* 2000. **156**: 501–507.

12 **Qin, X. F., Reichlin, A., Luo, Y., Roeder, R. G. and Nussenzweig, M. C.,** OCA-B integrates B cell antigen receptor-, CD40L- and IL 4-mediated signals for the germinal center pathway of B cell development. *EMBO J.* 1998. **17**: 5066–5075.

13 **Boehm, J., He, Y., Greiner, A., Staudt, L. and Wirth, T.,** Regulation of BOB. 1/OBF. 1 stability by SIAH. *EMBO J.* 2001. **20:** 4153–4162.

14 **Tiedt, R., Bartholdy, B. A., Matthias, G., Newell, J. W. and Matthias, P.,** The RING finger protein Siah-1 regulates the level of the transcriptional coactivator OBF-1. *EMBO J.* 2001. **20:** 4143–4152.

15 **Wolf, I., Pevzner, V., Kaiser, E., Bernhardt, G., Claudio, E., Siebenlist, U., Forster, R. and Lipp, M.,** Downstream activation of a TATA-less promoter by Oct-2, Bob1, and NF-κB directs expression of the homing receptor BLR1 to mature B cells. *J. Biol. Chem.* 1998. **273**: 28831–28836.

16 **Nitschke, L., Carsetti, R., Ocker, B., Kohler, G. and Lamers, M. C.,** CD22 is a negative regulator of B cell receptor signaling. *Curr. Biol.* 1997. **7**: 133–143.

17 **Stoddart, A., Ray, R. J. and Paige, C. J.,** Analysis of murine CD22 during B cell development: CD22 is expressed on B cell progenitors prior to IgM. *Int. Immunol.* 1997. **9**: 1571–1579.

18 **Nitschke, L., Floyd, H., Ferguson, D. J. and Crocker, P. R.,** Identification of CD22 ligands on bone marrow sinusoidal endothelium implicated in CD22-dependent homing of recirculating B cells. *J. Exp. Med.* 1999. **189**: 1513–1518.

19 **O'Keefe, T. L., Williams, G. T., Davies, S. L. and Neuberger, M. S.,** Hyperresponsive B cells in CD22-deficient mice. *Science* 1996. **274**: 798–801.

20 **Otipoby, K. L., Andersson, K. B., Draves, K. E., Klaus, S. J., Farr, A. G., Kerner, J. D., Perlmutter, R. M., Law, C. L. and Clark, E. A.,** CD22 regulates thymus-independent responses and the lifespan of B cells. *Nature* 1996. **384**: 634–637.

21 **Sato, S., Miller, A. S., Inaoki, M., Bock, C. B., Jansen, P. J., Tang, M. L. and Tedder, T. F.,** CD22 is both a positive and negative regulator of B lymphocyte antigen receptor signal transduction: altered signaling in CD22-deficient mice. *Immunity* 1996. **5:** 551–562.

22 **Doody, G. M., Justement, L. B., Delibrias, C. C., Matthews, R. J., Lin, J., Thomas, M. L. and Fearon, D. T.,** A role in B cell activation for CD22 and the protein tyrosine phosphatase SHP. *Science* 1995. **269**: 242–244.

23 **Cornall, R. J., Cyster, J. G., Hibbs, M. L., Dunn, A. R., Otipoby, K. L., Clark, E. A. and Goodnow, C. C.,** Polygenic autoimmune traits: Lyn, CD22, and SHP-1 are limiting elements of a biochemical pathway regulating BCR signaling and selection. *Immunity* 1998. **8**: 497–508.

24 **Rajewsky, K.,** Clonal selection and learning in the antibody system. *Nature* 1996. **381**: 751–758.

25 **Forster, R., Mattis, A. E., Kremmer, E., Wolf, E., Brem, G. and Lipp, M.,** A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell* 1996. **87**: 1037–1047.

26 **Loder, F., Mutschler, B., Ray, R. J., Paige, C. J., Sideras, P., Torres, R., Lamers, M. C. and Carsetti, R.,** B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. *J. Exp. Med.* 1999. **190**: 75–89.

Correspondence: Thomas Wirth, Department of Physiological Chemistry, Ulm University, Albert-Einstein-Allee 11, D-89081 Ulm, Germany
Fax: +49-731-502-2892
e-mail: thomas.wirth@medizin.uni-ulm.de