Reduction of marginal zone B cells in CD22-deficient mice

Tatjana Samardzic[1], Dragan Marinkovic[1], Claus-Peter Danzer[2], Judith Gerlach[2], Lars Nitschke[2] and Thomas Wirth[1]

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

CD22 is a B cell-specific member of the immunoglobulin superfamily and binds to sialic acid. CD22 inhibits B cell receptor signaling. Mice deficient for CD22 show a largely normal B cell development. Here, we have performed a detailed analysis of the splenic B cell population and found that the subset of marginal zone (MZ) B cells was selectively reduced in CD22-deficient mice. CD22-deficient mice showed a lack of TNP-ficoll capturing cells in the MZ and a reduced response to TNP-ficoll, particularly when the antigen was applied intravenously. CD22-deficient B cells showed both enhanced motility as well as enhanced chemotaxis to certain chemokines. The altered chemokine responsiveness or the higher signaling capacity of CD22-deficient B cells may lead to the compromised MZ B cell compartment, as both processes have previously been shown to affect MZ composition.

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1 Introduction

CD22 is a B cell specific transmembrane protein. It is a member of the siglec (sialic acid binding immunoglobulin-like lectins) family of adhesion receptors that bind specifically to sialic acids [1]. CD22 acts as a negative regulator of B cell receptor (BCR) signaling [2, 3]. Upon engagement of the BCR CD22 is phosphorylated on tyrosines on its intracellular tail [4, 5]. Tyrosine phosphorylation of the immunoreceptor tyrosine-based inhibition motifs (ITIM) of CD22 leads to recruitment and activation of SHP-1 which is the most important factor binding to the CD22 tail [4]. The negative role of CD22 in BCR signaling has been clarified by generation of CD22-deficient mice. When B cells of these mice were stimulated with anti-IgM they showed a highly increased Ca^{2+} response [2, 3, 6–8]. B cell development in CD22-deficient mice was relatively normal with the exceptions of a mildly preactivated B cell phenotype in the spleen and a homing defect of mature recirculating B cells to the bone marrow [3, 6–8]. When CD22-deficient mice were challenged with a T cell-dependent antigen they showed a normal response, accompanied by normal germinal center formation [3, 7]. However, the thymus-independent type II (TI-2) response to TNP-ficoll was reduced [3, 7]. This seemed to be in contradiction to the enlarged Ca^{2+} response of CD22-deficient B cells. Impairment of the TI-2 response could be caused by either a functional alteration of CD22$^{-/-}$ B cells or by the lack of the cellular population responsible for this response. TI-2 responses are mainly performed by B1 B cells and marginal zone (MZ) B cells [9, 10]. Since B1 B cells occur in normal numbers in CD22-deficient mice we therefore analyzed here their MZ B cell population.

MZ B cells represent a distinctive subset of B lymphocytes. They are exclusively located in the periphery of the splenic periarteriolar lymphoid sheath (PALS), at the border of white and red pulp [11–13]. In addition to this specific localization, they can be distinguished from follicular, recirculating B cells by the characteristic expression of cell surface markers. MZ B cells are described as IgM^{-} ^{high}IgD^{low}CD21^{high}CD23$^{low/neg}$CD1^{high}, while follicular B cells are mainly IgM^{low}IgD^{high}CD21^{med}CD23^{high} CD1^{neg} [10, 12, 14–16]. These cells have a preactivated phenotype. They express high levels of B7–1 and B7–2 and upon stimulation with polyclonal activators they differentiate into plasma cells in a matter of hours [16]. This preactivated state and their localization adjacent to the marginal sinuses are critical for the proposed function of these cells. MZ B cells are among the first population of cells to "see" blood-borne antigens and recent experiments revealed that they play a critical role in host defense against bacterial pathogens [13].

The mechanisms governing the differentiation of MZ B cells have only recently been investigated. Several gene-

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The first two authors contributed equally to this work.

Abbreviations: MZ: Marginal zone **BCR:** B cell receptor **TI-2:** T-independent type II

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deficient mice, such as Pyk-2$^{-/-}$ mice, Lsc$^{-/-}$ mice, Aiolos$^{-/-}$ mice, NF-κB p50$^{-/-}$ and RelB$^{-/-}$ mice, have a reduced MZ B cell compartment [17–21]. Other gene-deficient mice such as BTK$^{-/-}$ or CD21$^{-/-}$ mice have proportionally increased MZ B cell numbers [18, 22]. One of the hypotheses derived from the analysis of these different genetically modified mice was that depending on the strength of the signal elicited at the BCR, differentiation to follicular or MZ B cells is affected. This model suggests that a strong signal will favor follicular B cell development (and perhaps B1 B cell development), whereas a weak signal will promote differentiation towards the MZ B cell direction [18, 22–24]. Transgenic systems have also shown that the specificity of the expressed BCR can bias B cell differentiation into the B2, B1 or MZ lineage [13, 23]. Here we have analyzed MZ B cells in CD22-deficient mice and find that their numbers are reduced which fits to the proposed signal strength model.

2 Results

2.1 CD22-deficient mice show an impaired TI-2 response

To extend our previous findings, CD22$^{-/-}$ mice and wild-type control mice were immunized with TI-2 antigen TNP-ficoll in two different routes. CD22$^{-/-}$ mice show a 2-fold lower response to TNP-ficoll, as compared to wild type mice, when the antigen was injected i.p. (Fig. 1), as previously shown [3, 7]. However, when the same antigen dose was applied i.v., the response was more impaired (three- to fourfold lower than control mice; Fig. 1). Similarly, the response of CD22$^{-/-}$ mice to i.p injected Pneumovax, a different TI-2 antigen, was also reduced two-fold compared to wild-type mice (not shown). Since MZ B cells are involved in early B cell responses, particularly to blood-borne antigens, this result suggested that the MZ B cell compartment could be affected in CD22$^{-/-}$ mice.

2.2 The number of MZ B lymphocytes is reduced in CD22$^{-/-}$ mice

To investigate whether CD22 deficiency affects the size of the MZ B cell compartment, we performed flow cytometry analyses of splenocytes isolated from wild-type and CD22$^{-/-}$ mice. Analysis of cell surface expression of CD21 and CD23 on the B cell population (B220/CD45R^{+} cells) allowed us to discriminate between newly formed (or transitional, T1) B cells (CD21^{neg}/CD23^{neg}), follicular, recirculating B cells (CD21^{med}/CD23^{high}) and MZ B cells (CD21^{high}/CD23$^{low/neg}$) (Fig. 2A). While proportions of newly formed and follicular B cells appeared similar in

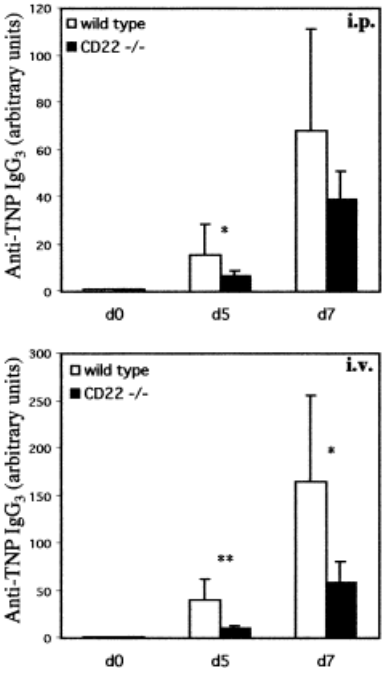

Fig. 1. CD22-deficient mice show an impaired response to i.p. or i.v. injected TNP-Ficoll. Five wild-type and five CD22-deficient mice (CD22$^{-/-}$) were immunized with 10 μg TNP-Ficoll, either i.p. (top) or i.v. (bottom). TNP-specific IgG_3 of day 5 and day 7 (d5, d7) was measured in ELISA and is shown here because it is the typical TI-2 IgG isotype with the highest induction. Anti-TNP IgM was also reduced, however, less pronounced (not shown). Means with standard deviation are given. *, $p<0.05$; **, $p<0.01$ in Student's *t*-test. One typical result of three experiments is shown.

both wild type and CD22$^{-/-}$ splenocytes, significant reduction of MZ B cells in CD22$^{-/-}$ animals was observed (Fig. 2A). Independent examination of ten animals of both wild-type and CD22$^{-/-}$ genotype confirmed that CD22 deficiency leads to reduction of MZ B cells in the range of three- to fourfold. This phenotypic defect was further confirmed by demonstrating the absence of CD21^{high} and CD1^{high} cells within the IgM^{high}/IgD^{low} population in CD22$^{-/-}$ animals (Fig. 2B). We checked the expression of activation markers in the remaining MZ B cell population as well as in the follicular B cells in CD22-deficient mice. In both B cell populations expression levels of MHC class II, B7.2 and CD69 were identical between wild-type and CD22$^{-/-}$ mice (data not shown). This shows that there is not a gross difference in the activation state of remaining MZ B cells in the CD22 deficient animals.

Histological examination of wild-type and CD22$^{-/-}$ mice showed a clear difference in splenic architecture. In wild-type mice a characteristic rim of IgM positive cells was

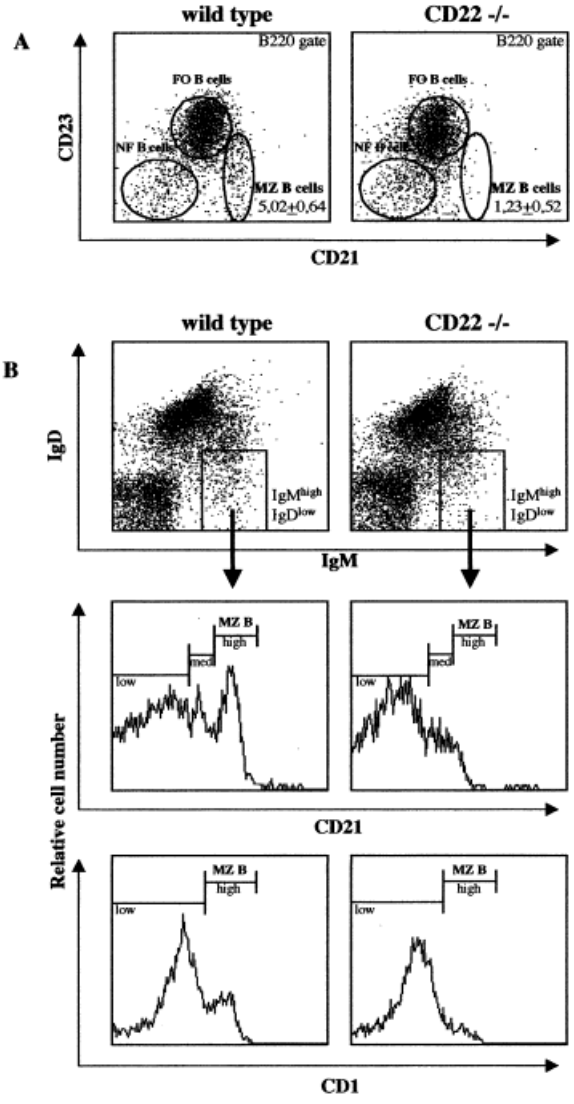

Fig. 2. CD22 deficiency impairs marginal zone B cells development. (A) Splenocytes isolated from wild-type and CD22$^{-/-}$ mice were stained with a combination of anti-B220, anti-CD21 and anti-CD23 antibodies. Within the B220^{+} cell gate, marginal zone (MZ), follicular (FO) and newly formed (NF) B cells are indicated. Mean percentages of MZ B cells with standard deviation out of ten analyzed animals of both genotypes are given. (B) Wild-type and CD22$^{-/-}$ splenocytes were stained with anti-IgM, anti-IgD and anti-CD21 or anti-CD1 antibodies. Histograms show CD21 or CD1 staining of IgM^{high}IgD^{low} gated splenocytes. CD21 high and CD1 high cells from this gate are MZ B cells.

located at the periphery of the follicles and separated by metallophilic (MOMA-1 positive) macrophages. These B cells are MZ B cells. This B cell population was significantly reduced in the spleens of CD22$^{-/-}$ animals (Fig. 3). These data confirm *in situ* the physical disappearance of MZ B cells, which was also observed in cytometric analysis.

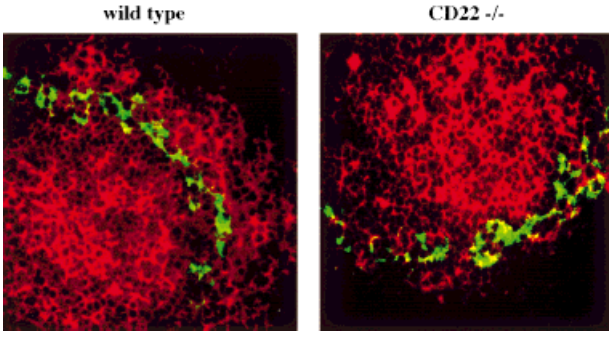

Fig. 3. Alteration of spleen architecture in CD22$^{-/-}$ mice. Spleen sections (4 μm thick) from wild-type and CD22$^{-/-}$ mice were stained with combination of PE-labeled anti-IgM (red) and FITC-labeled anti-MOMA-1 (green) antibodies. Representative results obtained from spleen sections of seven animals in each group are shown. Sections were viewed under 400x magnification.

2.3 TNP-Ficoll capturing is reduced in CD22$^{-/-}$ mice

It has been shown that intravenously injected TNP-Ficoll is initially specifically captured by marginal zone but not follicular B cells [9, 17, 20]. Wild-type and CD22$^{-/-}$ mice were injected i.v. with TNP-Ficoll and after 30 min spleens were examined for the presence of antigen using TNP-specific antibody. In agreement with previously reported data in wild-type mice binding of anti-TNP antibody was associated with the CD21^{high}/CD23$^{low/neg}$ (MZ) population of splenic B cells (Fig. 4A) and localized outside of the metallophilic macrophage rim (Fig. 4B). In the gate of follicular and newly formed B cells no significant capturing of antigen was observed (data not shown). In contrast to wild-type mice, capture of TNP-Ficoll was significantly reduced in CD22$^{-/-}$ mice as established by cytometric (Fig. 4A) as well as by histological examination (Fig. 4B).

2.4 CD22$^{-/-}$ splenocytes show altered migration

Recent data suggest that chemotactic responses play an important role in B cells compartmentalization [19–21, 25–28]. To address the question whether CD22 deficiency affects the ability of B cells to migrate, we compared the migration of wild type and CD22$^{-/-}$ B cells in the presence or absence of chemokines. This analysis revealed that residual MZ B cells have increased migration ability, although the number of MZ B cells is reduced in CD22-deficient mice. The percentage of CD22$^{-/-}$ MZ B cells that migrated in the absence of chemokines was significantly higher compared to wild-type cells (Fig. 5). The migration pattern differences between wild type and

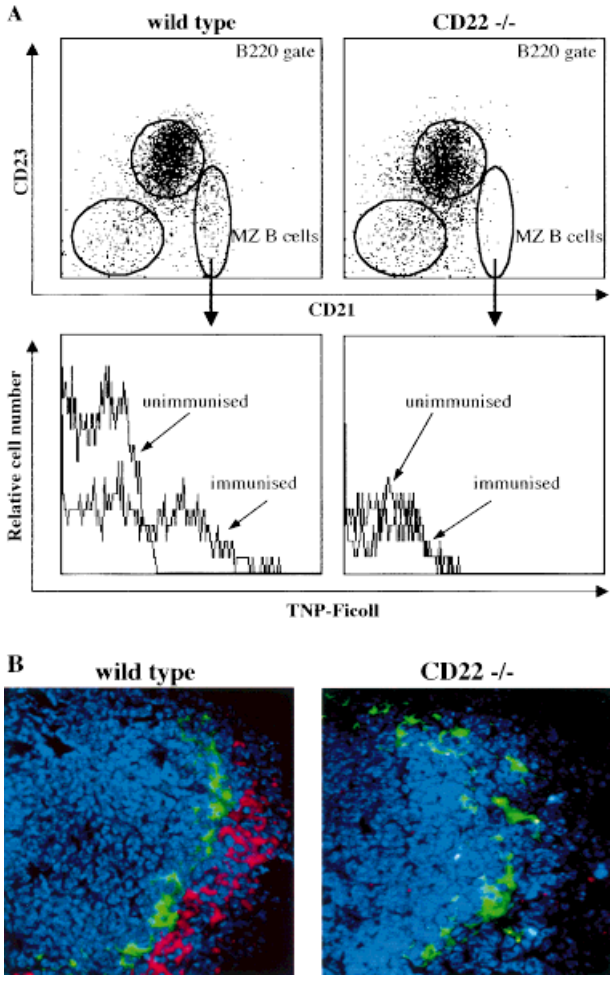

Fig. 4. T cell-independent type II antigen capturing in wild-type and CD22$^{-/-}$ mice. TNP-Ficoll (100 μg) was injected intravenously in wild-type and CD22$^{-/-}$ mice. After 30 min, animals were sacrificed and spleens were used for FACS and immunohistological examination. As a control we used unimmunized animals of the corespondent genotypes. (A) Splenocytes were stained with combination of anti-B220, anti-CD21, anti-CD23 and anti-TNP Ab. Marginal zone B cells (B220^{+}CD21^{high}CD23^{low}) were gated and histograms presenting anti-TNP staining of immunized and unimmunized animals are shown. Data are representative out of four sets of experiments. (B) Spleen sections from TNP-Ficoll immunized animals were stained with biotin-labeled anti-TNP (red) and FITC-labeled anti-MOMA-1 (green) antibodies. Biotinylated antibody was revealed with streptavidin-Cy-Chrome. Sections were mounted with Moviol containing DNA-specific fluorescent dye DAPI (blue), which stained all nucleated cells. Sections were viewed under 400x magnification.

CD22$^{-/-}$ B cells were further potentiated in the presence of MIP-3β and SDF-1α, while in the case of BLC-stimulated cells no further difference was observed. A similar migration pattern was observed for follicular and newly formed B cells (Fig. 5), indicating that generally CD22 deficient B cells have a higher migration property.

3 Discussion

In this study we have demonstrated for the first time that CD22-deficient mice have a specific defect in marginal zone B cell numbers. Flow cytometric analysis revealed that proportion of CD21^{high}CD23$^{low/neg}$CD1^{high}IgM^{high}IgD^{low} cells (MZ B cells) is three- to fourfold reduced in CD22$^{-/-}$ mice, while other splenic compartments (follicular and newly formed) appear normal. The obtained results can not be attributed to the alteration in surface marker expression since histological analysis show physical loss of IgM^{+} cells in the PALS. Furthermore, examination based on T-independent antigen (TNP-Ficoll) binding and humoral response to the same prove impaired marginal zone B cells function in CD22$^{-/-}$ mice.

The obvious question is the mechanism by which CD22 deficiency affects marginal zone B cell composition. Several recent papers suggested that the strength of the signal elicited at the B cell receptor affects MZ B cells development [17, 22–24]. This hypothesis predicts that weak signals favor the differentiation of MZ B cells, medium signals give rise to follicular B cells and strong signals induce differentiation of B1 cells. It was shown that B cells in Aiolos$^{-/-}$ mice, which have reduced MZ B cells, have an increased calcium influx upon BCR stimulation [18]. In contrast, marginal zone B cell numbers are increased in BTK-deficient mice, where BCR signal strength is reduced [18, 22]. It is well-documented that stimulation of CD22-deficient B cells results in an enhanced calcium influx [2, 3, 6–8]. Regarding these data, the observed reduction of MZ B cells in CD22$^{-/-}$ mice support the hypothesis that increased signal strength upon stimulation leads to impaired MZ B cell compartment.

A reduced MZ B cell compartment could be caused by defective development of MZ B cells, defective migration or defective retention at the correct microenvironmental site. There is good evidence that proper B cell compartmentalization depends on cell motility and chemotactic response [19–21, 25–28]. MZ B cells show a highly motile behavior, as was shown by *in vivo* application of the Gαi inhibitor pertussis toxin. This substance which inhibits all known chemokine receptor signals depleted MZ B cells within 48 h without affecting follicular cells [20]. Recently it has been shown that impaired MZ B cell development in Pyk–2$^{-/-}$, Lsc$^{-/-}$ and Dock2$^{-/-}$ mice is associated with altered motility [19, 20, 29]. Therefore, it was reasonable to assume that CD22 deficiency may

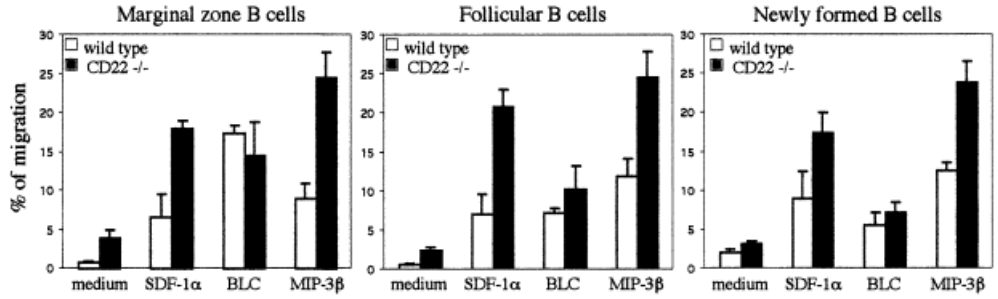

Fig. 5. Altered migration of CD22$^{-/-}$ splenocytes. Migration of lympholyte-M purified splenocytes (10^6) from wild-type and CD22$^{-/-}$ mice were analyzed. Cells placed in 5-μm pore-size transwell insert were incubated 4 h at 37°C in the gradient of 0.1 μg SDF-1α, 1 μg/ml BLC or 0.5 μg/ml MIP-3β. After incubation, migrated cells were harvested, stained with anti-B220, anti-CD21 and anti-CD23 antibodies, counted and analyzed by flow cytometry. Data present mean percentage of migrated cells from indicated B cell compartments obtained by analysis of three animals/group.

also lead to altered cell migration. Indeed, we observed that basal motility of CD22-deficient B cells is significantly increased compared to wild-type B cells. Surprisingly, while examination of B cell migration in the presence of MIP-3β and SDF-1α showed increase in CD22$^{-/-}$ mice, no additional increase in motility was observed in response to BLC.

How the lack of CD22 influences chemokinesis is not known. Principally, CD22 could act indirectly on chemokine signaling by changing the signaling threshold. It was shown that BCR engagement changes the chemokine responsiveness [30]. Since CD22$^{-/-}$ B cells are in a preactivated state this may easily account for altered responsiveness, for example by a changed chemokine receptor expression pattern. In this respect it is relevant that deficiency of lyn, the kinase responsible for CD22 phosphorylation and SHP-1 recruitment, causes loss of MZ B cells and shows also, when crossed to an Ig transgenic mouse, a higher responsiveness to MIP3β [31].

Alternatively, CD22 could directly inhibit chemokine signaling by a yet undefined mechanism. Engagement of chemokine receptors has been shown to activate Pyk-2, an intracellular tyrosine kinase that is important for MZ B cells [20, 32, 33]. It was demonstrated that by activating the chemokine receptor CCR5, SHP-1 is activated and binds to Pyk-2 [32]. Also, SHP-1 deficient hematopoietic cells from motheaten mice show enhanced chemotactic responses [34]. Since CD22 is a major activator of SHP-1 in B cells after BCR engagement, this activation may directly inhibit chemokine receptor signaling. However, specific experiments are needed to test these possibilities.

Since CD22 is an adhesion molecule and has an important role in bone marrow homing of mature lymphocytes it is also possible that the reduction of MZ B cells in CD22$^{-/-}$ mice is due to impaired homing to the MZ. We previously analyzed histological sections of various organs for expression of α2,6-linked sialic acid, the ligand of CD22. We observed specific expression on the endothelium only in the bone marrow, but not on endothelial cells of the spleen or lymph node [35]. Therefore, we consider a homing defect to the MZ as a less likely explanation for the loss of MZ B cells in CD22-deficient mice.

There is good evidence that thymus-independent immune responses of the TI-2 type are made by B1 cells, the class of B cells found mainly in the peritoneum, and by MZ B cells of the spleen [9, 13]. These cells are the main cells which generate a primary IgM and IgG_3 response to bacterial antigens. The role of MZ versus B1 cells in the TI-2 response has recently been studied by a transgenic model in which MZ or B1 cells could be followed histochemically or in FACS by use of anti-idiotypic antibodies [13]. This study has demonstrated that the route of antigen application as well as the BCR specificity determines the relative contribution of these two B cell compartments to the response. MZ B cells are particularly involved in response to blood borne antigens, which is to be expected because of their location next to the marginal sinus in the spleen. The knockout of various genes which are expressed in B cells has led to impaired TI-2 responses of the deficient mice. Some of these, such as BTK$^{-/-}$ or Vav1$^{-/-}$ have a reduced B1 compartment, while others, such as Pyk2$^{-/-}$, Lsc$^{-/-}$ or Dock2$^{-/-}$ mice have a compromised MZ B cell compartment. However, since some of these mice also show impaired cellular signaling, it is not clear to what extend just the loss of a certain B cell population can serve as an explanation for the impaired immune response.

CD22 deficiency does not lead to impaired B cell signaling, but rather to an enhanced response. This together

with the presence of normal [3, 7] or even increased [6, 8] B1 cell numbers suggests that the strong reduction of MZ B cells in CD22-deficient mice may explain their impaired TI-2 response. Our findings that the i.v. route of antigen application leads to stronger reduction in response than the i.p. route does, could be taken as evidence for this mechanism. However, alternative explanations for the impaired TI-2 response, such as a stronger apoptosis induction in CD22-deficient B cells by antigens such as TNP-ficoll, can not be fully excluded.

In summary, we have shown that CD22-deficient mice have a reduced MZ B cell compartment. Their B cells are less able to bind TNP-ficoll and to trigger TI-2 immune responses. Also, $CD22^{-/-}$ B cells show enhanced chemotaxis which may affect the recruitment or retention in the MZ compartment.

4 Materials and methods

4.1 Mice

C57BL/6 wild-type and CD22-deficient mice on the same genetic background were obtained from our breeding facility. Mice were analyzed between 8–10 weeks after birth.

4.2 Immunizations

$CD22^{-/-}$ or wild-type control mice were immunized with 10 μg TNP-Ficoll (Biosearch Technologies, Novato, CA) and bled on day 0, day 5 and day 7. The TNP-specific antibodies in the sera were determined by ELISA with TNP-BSA-coated polysorb plates (Nalge Nunc, Rochester, NY). Sera were applied in serial dilutions on the plates and IgM or IgG_3 was measured by use of goat-anti IgM or anti-IgG_3 alkaline phosphatase-labeled antibodies (Southern Biotechnologies) and the substrate *p*-nitrophenyl phosphate (Sigma). One pool of sera, applied to all plates, served as an internal standard defining arbitrary units. Alternatively mice were injected intravenously with 100 μg of TNP-Ficoll. At 30 min after injection, mice were sacrificed and spleens were taken out. One half of the spleen was used for histological examinations while the other half was used for FACS analysis.

4.3 Flow cytometry analysis

Single-cell suspensions of Lympholyte-M (Cederlane, 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-biotin, anti-CD21-FITC, anti-CD23-PE, anti-IgM-PE, anti-IgD-biotin, anti-CD1-FITC, anti-MHC class II-FITC, anti-CD69-biotin, anti-B7.2-FITC and anti-TNP-biotin (all produced by Pharmingen, San Diego, CA). Stainings were performed in PBS containing 0.1% BSA (Roche Diagnostics, Mannheim, Germany), 0.1 % Na-azide and saturating concentration of anti-CD16/CD32 (Pharmingen) to block Fcγ III/II receptors. Biotin-labeled antibodies were revealed by Streptavidin-Cy-Chrom (Pharmingen). Cell surface marker expression was analyzed using a four-color flow cytometer (FACScalibur) and Cell Quest Software (Becton Dickinson, Heidelberg, Germany).

4.4 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 incubated 45 min with staining buffer (PBS containing 0.1% BSA, 0.1% Na-azide and anti-CD16/CD32 followed by staining for 45 min with 1:100 dilution of both anti-IgM-PE and MOMA-1-FITC (Serotec, GB). Sections from TNP-Ficoll-treated animals were stained with combination of anti-TNP-biotin and MOMA-1-FITC antibodies. Biotinylated antibodies were revealed with streptavidin-Cy-Chrom (1:100) and slides were mounted in Moviol containing DAPI (0.1 μg/ml) (Roche). Slides were analyzed using a Leica microscope (DMIRB/E) and OpenLab software (version 2.2.5). The excitation wavelength for FITC was 494 nm, for PE 576 nm, for Cy 581 nm, and for DAPI excitation wavelength was 359 nm.

4.5 Migration assay

Lympholyte-M purified splenocytes (10^6) were assayed for transmigration using 5-μm pore-size transwell culture inserts (Costar, GB). The medium used for the experiments was RPMI 1640 supplemented with 0.25 % fatty-acid free BSA (Sigma). To assay chemotaxis, the bottom chambers were filled with 1 μg/ml of MIP-3β, 0.5 μg/ml of BLC or 0.1 μg/ml of SDF-1α (all from R&D). The migration occurred at 37°C in a humidified atmosphere with 5% CO_2 over 4 h. After incubation, cells which had migrated to the lower chamber were harvested, stained with anti-B220-PerCP (Pharmingen), anti-CD21-FITC, and anti-CD23-PE, and counted by FACScalibur and analyzed by CellQuest software.

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