MZB1 is a GRP94 cochaperone that enables proper immunoglobulin heavy chain biosynthesis upon ER stress

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MZB1 (pERp1) is a B-cell-specific and endoplasmic reticulum (ER)-localized protein implicated in antibody secretion and integrin-mediated cell adhesion. Here, we examine the role of MZB1 in vivo by conditional gene inactivation in the mouse germline and at different stages of B lymphopoiesis. Deletion of MZB1 impairs humoral immune responses and antibody secretion in plasma cells that naturally undergo ER stress. In addition, we found that experimental induction of ER stress by tunicamycin injections in mice results in a block of pro-B-cell to pre-B-cell differentiation specifically in Mzb1−/− mice. A similar developmental block was observed in Mzb1fl/flmb1Cre mice, whereby a Cre recombinase-induced genotoxic stress unmasks a role for MZB1 in the surface expression of immunoglobulin μ heavy chains (μHCs). MZB1 associates directly with the substrate-specific chaperone GRP94 (also called HSP90B1 or gp96) in an ATP-sensitive manner and is required for the interaction of GRP94 with μHCs upon ER stress. Thus, MZB1 seems to act as a substrate-specific cochaperone of GRP94 that enables proper biosynthesis of μHCs under conditions of ER stress.

[Keywords: MZB1; B lymphopoiesis; antibody secretion; ER stress; GRP94; cochaperone]

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B lymphopoiesis is a well-studied paradigm for a terminal differentiation process in which hematopoietic stem cells (HSCs) develop through multiple stages to finally generate highly specialized antibody-secreting plasma cells. Differentiation of murine HSCs via lineage-restricted common lymphoid progenitors (CLPs) generates pro-B cells that proliferate in response to interleukin-7 (IL-7) signaling and initiate the rearrangement of the immunoglobulin heavy chain (Igh) locus. Productive Igh rearrangement and assembly of the μ heavy chain (μHC) with the Ig surrogate light chains A5 and VpreB result in the expression of a functional pre-B-cell receptor (pre-BCR) and generation of pre-B cells that are still responsive to IL-7 signaling (von Boehmer and Melchers 2010; Herzog and Jumaa 2012). Signaling via the pre-BCR triggers several rounds of cell division and the rearrangement of Ig light chain genes, which leads to the surface expression of the IgM BCR and generation of immature B cells that migrate from the bone marrow to the spleen. In the periphery, immature B cells further differentiate via transitional B cell stages to mature B cells that respond to antigenic stimulation by terminal differentiation (Allman and Pillai 2008).

Surface expression and function of the pre-BCR require the endoplasmic reticulum (ER)-resident chaperones BiP (HSPA5) and GRP94 (also called HSP90B1 or gp96), which assist protein folding by recognizing exposed hydrophobic patches (Haas and Wabl 1983; Melnick et al. 1994; Meunier et al. 2002). Moreover, the folding of proteins with disulfide bonds, such as Iggs, requires the action of protein disulfide isomerases (PDIs) that control disulfide-linked IgM assembly by recognizing free cysteines and aberrant disulfide bonds (Lilie et al. 1994; Vavassori et al. 2013). Despite the function of elaborate protein-folding machineries in the ER, misfolded proteins can accumulate in the ER and result in a cellular stress, known as unfolded protein response (UPR) (Todd et al. 2008). The UPR results in the recruitment of BiP to unfolded proteins and dissociation of BiP from the ER.
transmembrane protein inositol-required enzyme 1 (IRE1) (Bertolotti et al. 2000). This dissociation of BiP and IRE1 leads to an unconventional mRNA processing and excision of 26 nucleotides (nt) from Xbp1 mRNA to generate spliced Xbp1s (Xbp1s), which encodes a nuclear transcription factor (Yoshida et al. 2001; Calon et al. 2002). The UPR also plays a major role in the terminal differentiation process of B cells into plasma cells, which is accompanied by the expansion of the ER and the up-regulation of proteins involved in folding and secretion (Iwakoshi et al. 2003; Shaffer et al. 2004). Stimulation of the BCR leads to the proteasomal degradation of BCL6 [Niu et al. 1998] and up-regulation of IRF4, Blimp1, and XBP1, which results in terminal differentiation of B cells, expansion of the ER, and the expression of genes related to the UPR, including BiP and GRP94 (van Anken et al. 2003; Shaffer et al. 2004; Taubenheim et al. 2012). Increased Ig production also drives a feed-forward circuit by triggering IRE1 activation and enhancing generation of Xbp1s (Reimold et al. 2001).

B cells in the periphery consist of multiple cell populations that differ in the phenotype and responsiveness to antigenic stimulation. In particular, cells residing in the marginal zone (MZ) of the spleen, termed MZ B cells, and B-1 cells found in the peritoneum quickly differentiate into antibody-secreting cells and produce polyreactive antibodies (Martin et al. 2001). In contrast to these cells, which have also been termed “innate-like” B cells, the majority of conventional B cells, termed follicular B (FoB) cells, produce specific antibodies with much slower kinetics. In an attempt to understand the phenotypic differences between peripheral B cell subsets, we and others have previously identified MZB1 (also referred to as pERp1 and PACAP) as an ER protein that is abundantly expressed in murine bone marrow (Martin et al. 2001). Therefore, we crossed Mzb1 knockout mice with mice expressing the FLPe recombinase under the control of the human actin promoter (Rodriguez et al. 2000). We crossed Mzb1+/−/Lox mice with expressing the FLPe recombinase under the control of the human actin promoter (Rodriguez et al. 2000). We crossed Mzb1+/−/Lox mice with mice expressing the FLPe recombinase under the control of the human actin promoter (Rodriguez et al. 2000). We crossed Mzb1+/−/Lox mice with mice expressing the FLPe recombinase under the control of the human actin promoter (Rodriguez et al. 2000). We crossed Mzb1+/−/Lox mice with mice expressing the FLPe recombinase under the control of the human actin promoter (Rodriguez et al. 2000). We crossed Mzb1+/−/Lox mice with mice expressing the FLPe recombinase under the control of the human actin promoter (Rodriguez et al. 2000). We crossed Mzb1+/−/Lox mice with mice expressing the FLPe recombinase under the control of the human actin promoter (Rodriguez et al. 2000). We crossed Mzb1+/−/Lox mice with mice expressing the FLPe recombinase under the control of the human actin promoter (Rodriguez et al. 2000). We crossed Mzb1+/−/Lox mice with mice expressing the FLPe recombinase under the control of the human actin promoter (Rodriguez et al. 2000). We crossed Mzb1+/−/Lox mice with mice expressing the FLPe recombinase under the control of the human actin promoter (Rodriguez et al. 2000). We crossed Mzb1+/−/Lox mice with mice expressing the FLPe recombinase under the control of the human actin promoter (Rodriguez et al. 2000).
however, the immune response of \( \text{Mzb}^{1/-} \) mice was significantly reduced compared with \( \text{Mzb}^{+/+} \) mice [Fig. 1E,F]. MZ B cells also generate significant levels of IgG3 during a TI-2 response [Guinamard et al. 2000; Zheng et al. 2013] and are capable of switching to IgG3 upon in vitro stimulation with LPS and IL-4 [Oliver et al. 1997]. Indeed, we detected reduced amounts of anti-TNP-specific antibodies of the IgG3 isotype in \( \text{Mzb}^{1/-} \) mice compared with \( \text{Mzb}^{+/+} \) mice throughout the TI-2 response [Fig. 1G].

To examine whether the impaired humoral immune response in \( \text{Mzb}^{1/-} \) mice is due to a B-cell-intrinsic defect, we crossed \( \text{Mzb}^{1/-} \) mice with the \( \text{CD}21^{+/-} \) transgenic strain in which the \text{Cre} recombine gene is expressed in transitional and mature B-cell stages in the spleen [Kraus et al. 2004]. Consistent with the expression of \( \text{CD}21^{+/-} \), MZB1 protein levels were found to be decreased at the CD19+AA4.1+IgMhi\( \text{CD}23^- \) transitional 1 [T1] B-cell stage, and MZB1 was undetectable in mature FoB and MZ B cells [Fig. 2A]. Flow cytometric analysis showed normal numbers of splenic B cells in \( \text{Mzb}^{1/-}\text{CD}21^{+/-} \) mice (Supplemental Fig. S2A). Moreover, we detected normal frequencies of transitional B cells [type 2 and 3], FoB cells, and MZ B cells [Fig. 2B,C]. In FoB and MZ B cells of \( \text{Mzb}^{1/-}\text{CD}21^{+/-} \) mice, we also observed virtually normal levels of surface IgM on gated MZ B cells. Numbers indicate the percentage of cells within the gated population.

Immunization of \( \text{Mzb}^{1/-}\text{CD}21^{+/-} \) mice and \( \text{Mzb}^{+/+}\text{CD}21^{+/-} \) control mice with the TD antigen TNP-KLH revealed a significantly reduced anti-TNP-specific immune response of the IgM isotype in \( \text{Mzb}^{1/-}\text{CD}21^{+/-} \) mice [Fig. 2E]. In \( \text{Mzb}^{1/-}\text{CD}21^{+/-} \) mice immunized with the TI-2 antigen TNP-Ficol, TNP-specific IgM antibody titers were not detected until day 6 and decreased afterward, whereas in \( \text{Mzb}^{1/-}\text{CD}21^{+/-} \) mice, the corresponding antibody titers increased between day 2 and day 4 and
IgG3 isotype in comparison with a reference sample. (*) Unpaired two-tailed Student’s t-test. Data are representative of triplicate assays. Statistical differences in the number of antibody-secreting cells and the spot intensity were assessed post-immunization with intravenously administered NP-Ficoll to detect NP-specific IgM-secreting cells and their secretion efficiency. Data are representative of five or more experiments. [E] Flow cytometric analysis of splenic CD19+/AA4.1+ immature B-cell stages gated for IgMloCD23– T1, IgMhiCD23+ T2, and IgMloCD23+ T3 B cells. [C] FACS profile of splenic CD19+/AA4.1+ gated mature B cells to detect the percentage of FoB and MZ B cells. Numbers in quadrants indicate the percentage of cells within the gated population. Data in B and C are representative of five or more experiments. [F] Flow cytometric analysis of Mzb1fl/flCD21-cre (red line) and Mzb1fl/flCD21-cre (black line) mice to detect IgM surface expression on gated FoB and MZ B cells. As a control, CD19– non-B cells (gray line) were gated for IgM expression. Data are representative of three or more experiments. [F] Primary immune responses to the TD antigen TNP-KLH (E) and Ti-2 antigen TNP-Ficoll (F). Following intraperitoneal injection of 150 μg of adsorbed TNP-KLH or intravenous injection of 25 μg of TNP-Ficoll, blood serum of Mzb1fl/flCD21-cre mice (n = 8; n = 5; black lines) and Mzb1fl/flCD21-cre mice (n = 4; n = 4; red lines) was analyzed by ELISA for the presence of anti-TNP IgM antibodies at the indicated time points. Each data point represents the mean of the respective cohort, whereas error bars indicate the SD of the mean. An unpaired two-tailed Student’s t-test was employed for statistical analysis of individual time points. (*) P < 0.05, (**) P < 0.005, (*** ) P < 0.001. [G] Analysis of IgG3 serum titers of Mzb1fl/flCD21-cre (n = 4; black line) and Mzb1fl/flCD21-cre (n = 4; red line) mice injected with 25 μg of TNP-Ficoll. Serum was collected on the indicated days and analyzed by an ELISA to detect anti-TNP antibodies of the IgG3 isotype in comparison with a reference sample. (*) P ≤ 0.05, (**) P ≤ 0.005, [H] ELSpot assay of B220+CD138+ plasma cells sorted 5 d post-immunization with intravenously administered NP-Ficoll to detect NP-specific IgM-secreting cells and their secretion efficiency. Data indicate the mean and SD of triplicates. Statistical differences in the number of antibody-secreting cells and the spot intensity were assessed with an unpaired two-tailed Student’s t-test. Data are representative of three biological replicates of each genotype.

peak at day 6 (Fig. 2F). Moreover, the amount of anti-TNP-specific antibodies of the IgG3 isotype was found to be reduced in Mzb1fl/flCD21-cre mice (Fig. 2G; Supplementary Fig. S2B). The impaired immune response could be ascribed to impaired plasma cell differentiation and/or altered efficiency of antibody secretion. To this end, we analyzed the frequency of B220+CD138+ plasma cells post-immunization and determined antibody secretion by ELISpot analysis. The percentage of plasma cells was not significantly altered in Mzb1-deficient mice (Supplemental Fig. S2C). However, the number of antibody-secreting cells within the plasma cell population and the amount of secreted antibody per cell, as determined by the number and intensity of spots, were approximately twofold reduced relative to wild-type controls (Fig. 2H; Supplementary Fig. S2D). In addition, we examined the amount of secreted IgM of LPS-stimulated MZO B cells and observed that the secreted IgM titer was approximately twofold reduced relative to the Mzb1fl/flCD21-cre control [Supplemental Fig. S2E]. Since LPS is sensed through Toll-like receptor 4 (TLR4), which subsequently initiates a signaling cascade resulting in B-cell activation and differentiation toward the plasma cell stage, we determined the cell surface
expression level of TLR4. Neither TLR4 nor the B-cell activation marker CD86 was affected by the absence of MZB1 (Supplemental Fig. S2F,G). Therefore, the inactivation of Mzb1 in immature B cells reduces humoral immune responses by affecting the number of antibody-secreting plasma cells and the efficiency of antibody secretion.

Pharmacologically induced ER stress synergizes with the Mzb1-null mutation

The normal surface IgM expression in unstimulated mature B cells raises the questions of whether MZB1 is only required for secretion of IgM and whether naturally occurring ER stress in antibody-secreting cells augments the requirement for MZB1. To this end, we intraperitoneally administered the UPR inducer tunicamycin in Mzb1−/− and Mzb1+/+ littermates and analyzed the B-cell populations in the bone marrow and spleen. We could not detect any difference in the frequency of mature splenic B cells between tunicamycin-treated Mzb1−/− and Mzb1+/+ mice (data not shown). However, in the bone marrow of tunicamycin-treated Mzb1−/− mice, we observed a significant reduction in the B220+CD43− pre-B-cell compartment relative to tunicamycin-treated Mzb1+/+ mice [Fig. 3A,B]. Moreover, in tunicamycin-treated Mzb1−/− mice, the percentage of B220+CD43− pro-B cells was increased, indicative of a block in pro-B-cell to pre-B-cell differentiation. In contrast, the frequency of B220+CD43− recirculating B cells was similar in tunicamycin-treated Mzb1−/− and Mzb1+/+ mice [Fig. 3A]. Semiquantitative RT–PCR analysis of mRNA of sorted pro-B cells, pre-B cells, and recirculating B cells for the presence of Xbp1 transcripts revealed a prominent Xbp1s band in B220+CD43− pro-B cells of vehicle-treated Mzb1+/+ and Mzb1−/− mice (Fig. 3C). However, the ratio of Xbp1s to total amount of Xbp1 was further increased in tunicamycin-treated mice, and the highest ratio of Xbp1s to total Xbp1 was detected in pro-B cells of tunicamycin-treated Mzb1−/− mice (Fig. 3C, D). In pro-B cells of Mzb1−/− mice, we also detected an increased expression of the UPR-induced gene Ddit3 [Chop], which has been implicated in ER stress-induced cell death [Fig. 3E] (Zinszner et al. 1998; Han et al. 2013). Tunicamycin treatment augmented the expression of Ddit3 in Mzb1−/− pro-B cells, suggesting that the combination of

Figure 3. Pharmacologically induced ER stress synergizes with the Mzb1-null mutation. (A) Flow cytometric analysis of Mzb1+/+ and Mzb1−/− mice treated for 12 h with 150 mM glucose [vehicle] or 10 μg of tunicamycin per gram of body weight in 150 mM glucose. Bone marrow cells of sacrificed mice were analyzed for the frequencies of B220+CD43− pro-B and early pre-B cells, B220+CD43− late pre-B and immature B cells, and B220+CD43− recirculating B cells within the living lymphocyte gate. Numbers in quadrants indicate the percentage of cells. Data are representative of four experiments. (B) Statistical analysis of the frequency of B220+CD43− pro-B cells of the indicated genotypes. A significant difference in the percentage of pre-B cells between Mzb1+/+ and Mzb1−/− mice was assessed by an unpaired two-tailed Student’s t-test. (*) P ≤ 0.05; (ns) not significant. (C) Semiquantitative RT–PCR to detect the transcript levels of unspliced Xbp1 [Xbp1u] and Xbp1s in the indicated sorted B-cell populations shown in A. Actin served as a loading control. (D) Quantification of the amount of Xbp1s to the total amount of Xbp1 in the sorted B220+CD43− pro-B-cell compartment of vehicle- and tunicamycin-treated Mzb1+/+ and Mzb1−/− mice using ImageJ. (E) Quantitative RT–PCR of the indicated sorted cell populations shown in A of vehicle- or tunicamycin-treated Mzb1+/+ and Mzb1−/− mice to detect the mRNA expression level of the UPR-induced gene Ddit3, normalized to the transcript levels of Actin. Error bars indicate the SD of the mean of technical triplicates. Data in C and E are representative of three independent experiments.
**MZB1 is required for the interaction of mHC with GRP94 under conditions of ER stress**

Previous reports indicated that MZB1 is coimmunoprecipitated with mHC in lysates of cross-linked plasmacytoma cells (Shimizu et al. 2009; van Anken et al. 2009). We confirmed an association of MZB1 with mHC in lysates of cross-linked B1–8 plasmacytoma cells (data not shown). However, in lysates of non-cross-linked B1–8 cells, the interaction between MZB1 and mHC is virtually undetectable and can be detected only upon treatment of cells with tunicamycin (Fig. 4A). Moreover, the association of MZB1 with the chaperone GRP94 could be detected only under conditions of tunicamycin-induced ER stress, which results in an up-regulation of GRP94 (Fig. 4B).

GRP94 is a substrate-specific chaperone implicated in the folding of secreted IgM (Melnick et al. 1994). Therefore, we examined whether the interaction of MZB1 with mHCs was increased under conditions of ER stress.

Figure 4. MZB1 is required for the interaction of mHCs with GRP94 under ER stress. 
(A, B) Co-IP approach using a monoclonal MZB1-specific antibody or anti-rat IgG2a isotype control cross-linked to sepharose beads to detect the association of MZB1 with mHCs (A) and GRP94 (B) in non-cross-linked B1–8 plasmacytoma cells. Cells were treated with the diluent DMSO or 5 μg/mL tunicamycin (Tm) for 8 h. Data are representative of three or more experiments. (C) Unglycosylated. (D) Co-IP approach to detect the interaction of GRP94 with mHCs in MACS-sorted CD19+ splenic B cells of Mzb1−/− and Mzb1+/+ mice that had been treated with 150 mM glucose (vehicle) or 10 μg of tunicamycin per gram of body weight in 150 mM glucose for 12 h. Immunoprecipitation of GRP94 from cell lysates was performed using a GRP94-specific polyclonal antibody or an isotype control. GRP94-associated mHCs were subsequently detected with an mHC-specific antibody. Data are representative of three experiments. (E) Co-IP approach of recombinant MZB1 and purified mouse liver GRP94 to detect a direct interaction of MZB1 and GRP94. Recombinant MZB1 was incubated with murine GRP94 and purified by an anti-MZB1 monoclonal antibody coupled to sepharose beads. Data are representative of three experiments. (F) ELISA-based assay to detect the interaction between GRP94 and MZB1. Recombinant MBP-GRP94 or MBP as a control was immobilized on an ELISA plate and incubated with recombinant MZB1 in the presence of increasing concentrations of ATP or its nonhydrolyzable form, AMP-PNP, in triplicates. GRP94-associated MZB1 was detected by a rat anti-MZB1 monoclonal antibody followed by an anti-rat HRP detection step. OD450 readings were normalized to background MBP values, each data point indicates the mean and the SD of the triplicates. Data are representative of four independent experiments. (G) Enzymatic assay to detect the ATPase activity of GRP94 in the absence (C) and presence (H) of a fourfold molar excess of recombinant MZB1. A reaction with geldanamycin (GA), which specifically blocks the ATPase activity of GRP94, was used to assess any residual contaminating background ATPase activity. The slope of the graph represents the actual ATPase activity.
GRP94 and/or μHC is important for binding of GRP94 to μHC in vivo. To this end, we performed coimmunoprecipitation (co-IP) experiments with lysates of splenic B cells that were sorted from vehicle-treated and tunicamycin-treated Mzb1+/− and Mzb1+/+ mice. In B cells from vehicle-treated Mzb1+/− and Mzb1+/+ mice, we detected an association of GRP94 with the μHC (Fig. 4C). However, this GRP94-μHC association was undetectable in B cells from tunicamycin-treated Mzb1+/− mice, although it was even increased in cells from tunicamycin-treated Mzb1+/+ mice.

Mzb1 shows homology with the Canopy family of proteins, including six conserved cysteines and the C-terminal ER retrieval signal (data not shown). One of the members of this protein family, CNPY3 [PRAT4A], has been shown to function as a cochaperone of GRP94 in the folding process of TLR4 [Liu et al. 2010]. Cochaperones interact directly with the chaperones, and therefore we examined the interaction of purified GRP94 and recombinant MZB1 by an in vitro immunopurification assay. MZB1 was able to directly associate with the chaperone GRP94 (Fig. 4D). The binding of MZB1 to GRP94 was sensitive to increasing concentrations of ATP (Fig. 4E,F), which is reminiscent of the ATP sensitivity of the interaction of GRP94 with the TLR4-specific cochaperone CNPY3 [Liu et al. 2010]. However, ATP hydrolysis is not required for abolishing the observed interaction, as the association of MZB1 and GRP94 could also be prevented in a dose-dependent manner by the nonhydrolyzable ATP analog AMP-PNP (Fig. 4E,F). Despite the competitive binding of MZB1 and ATP to the chaperone GRP94, we could not observe any obvious difference in the ATPase activity of GRP94 in the absence and presence of MZB1 (Fig. 4G,H). Thus, MZB1 potentiates the interaction of μHC with the substrate-specific chaperone GRP94 under conditions of UPR-induced ER stress and shares properties with another GRP94 co-chaperone.

Moreover, the frequency of peritoneal B cells was reduced in Mzb1fl/flmb1Cre mice (Supplemental Fig. S3D). Flow cytometric analysis of the bone marrow of Mzb1fl/flmb1Cre and Mzb1+/+mb1Cre mice indicated that Mzb1-deficient B-lineage cells did not efficiently differentiate beyond the B220+/CD43+ pre-B-cell stage (Fig. 5B). Consistent with the block at the transition from pro-B to pre-B cells, Mzb1fl/flmb1Cre mice showed a marked reduction in CD25+ pre-B cells and an increase in the percentage of B220+/CD19−μHC−CD25− late pro-B cells (Fig. 5C). The pro-B-cell to pre-B-cell transition is characterized by the complete rearrangement of the IgH gene and the assembly and expression of the pre-BCR.

To address the question of whether the developmental block in Mzb1fl/flmb1Cre mice can be rescued by the expression of a functionally rearranged μHC, we crossed Mzb1fl/flmb1Cre mice with IghB1−/−B1−/−Igk−/− mice that carry a productively rearranged Igh gene [Takeda et al. 1993; Sonoda et al. 1997]. However, flow cytometric analysis of bone marrow cells of Mzb1fl/flmb1CreIghB1−/− mice revealed that pro-B cells did not efficiently traverse to the B220+/CD43− pre-B-cell stage (Fig. 5D). Thus, other mechanisms associated with the function of the pre-BCR may account for the developmental block in Mzb1fl/flmb1Cre mice.

The developmental block of Mzb1fl/flmb1Cre mice resembles that found in tunicamycin-treated Mzb1+/− mice and coincides with the stage at which the pre-BCR complex provides a signal for further differentiation to generate immature B cells (Kitamura et al. 1991, 1992). Therefore, we examined the surface expression of the pre-BCR by flow cytometry and found that the frequency of cells positive for both μHC and the Ig surrogate light chain λ5 is reduced by a factor of three in Mzb1fl/flmb1Cre mice relative to Mzb1+/+mb1Cre mice (Fig. 5E,F). Cell surface expression of λ5, which can be detected in wild-type pro-B cells even in the absence of μHC [Karasuyama et al. 1994; Winkler et al. 1995], appeared to be normal. Thus, the impaired surface expression of the pre-BCR may reflect a defect in the surface expression of μHC. As the majority of pre-BCR complexes is retained in the intracellular compartment of the ER [Brouns et al. 1996; Mielenz et al. 2003], we crossed Mzb1fl/flmb1Cre mice onto a Slp65−/− background in which the surface expression of the pre-BCR complex is increased due to impaired pre-BCR receptor down-regulation [Jumaa et al. 1999; Flemming et al. 2003]. In the bone marrow of Mzb1fl/flmb1CreSlp65−/− mice, the frequency of μHC+λ5− cells was reduced 2.5-fold relative to Mzb1+/+mb1CreSlp65−/− littermate controls, whereas the percentage of cells expressing only λ5 was increased (Supplemental Fig. S3E,F). Consistent with these findings, primary bone marrow-derived IL-7 B-cell cultures of Mzb1fl/flmb1Cre mice showed a marked decrease in the number of cells expressing surface μHC, although the frequency of cells expressing intracellular μHC was similar to that found in Mzb1+/+mb1Cre IL-7 B-cell cultures (Fig. 5G,H). Both Mzb1+/+mb1Cre and Mzb1fl/flmb1Cre cell cultures contained surface μHC-positive cells that were also Igκ-positive, as evidenced by anti-κ staining and analysis of

Role of MZB1 in B lymphopoiesis

Mzb1Cre-induced genotoxic stress unmask a role for MZB1 in pro-B cells

Tunicamycin treatment of mice unmasked a specific role for MZB1 in pro-B-cell to pre-B-cell differentiation. In these experiments, we detected Xbp1s expression already in untreated B220+/CD43− pro-B cells but not in B220+/CD43− recirculating mature B cells (Fig. 3C). The UPR detected in untreated wild-type pro-B and pre-B cells may be linked to genotoxic stress by RAG-mediated recombinations of the Ig loci. Genotoxic stress can also be induced by expression of Cre recombinase [Loonstra et al. 2001; Pfeifer et al. 2001].

Analysis of Mzb1fl/flmb1Cre mice in which the Mzb1 allele was deleted by the expression of a codon-optimized Cre recombinase in pro-B cell [Supplemental Fig. S3A,B; Hobeika et al. 2006] indicated a reduced size of the spleens of Mzb1fl/flmb1Cre mice relative to Mzb1+/+mb1Cre control mice [Supplemental Fig. S3C]. Flow cytometric analysis of the splenic B-cell compartment showed a marked decrease in the number of CD19+ B cells [Fig. 5A].
Figure 5. Mzb1fl/fl mb1Cre mice show impaired B-cell differentiation and reduced surface expression of μHCs. (A) Flow cytometric profile of the splenic B-cell compartment in Mzb1+/+ mb1Cre and Mzb1fl/fl mb1Cre mice to analyze the frequency of CD19+ B cells in the living lymphocyte gate. (B) Flow cytometric profile of Mzb1+/+ mb1Cre and Mzb1fl/fl mb1Cre bone marrow cells to detect B220+CD43+ pro-B and early pre-B cells, B220+CD43+ late pre-B and immature B cells, and B220+CD43+ recirculating B cells within the living lymphocyte gate. (C) FACS profile of bone marrow B220+CD19+ B cells of Mzb1+/+ mb1Cre and Mzb1fl/fl mb1Cre mice gated into μHC+CD25− pre-B cells, CD25− pre-B cells, and μHC+CD25− immature and recirculating B cells. (D) Flow cytometric profile of bone marrow cells of Mzb1+/+ mb1CreIghB1–8/B1–8Igk−/− and Mzb1fl/fl mb1CreIghB1–8/B1–8Igk−/− mice to detect B220+CD43+ pro-B cells, B220+CD43+ immature and recirculating B cells within the living lymphocyte gate. Numbers in the FACS profiles show the percentage of cells within the gated population. Data in A–D are representative of five or more independent experiments. (E) Statistical analysis of the frequency of μHC+λ5+ cells in the B220+CD19+ bone marrow B-cell gate of the biological replicates in the FACS experiment shown in E. Long horizontal bars represent the mean, while short horizontal bars indicate the SD of the mean. Statistical significance between the indicated genotypes was assessed by a two-tailed unpaired Student’s t-test. Asterisks indicate the statistical significance value. (***) P < 0.0001. (G,H) FACS profiles of IL-7 cultured bone marrow-derived B cells of Mzb1+/+ mb1Cre and Mzb1fl/fl mb1Cre mice to examine the surface expression (G) and intracellular expression (H) of μHCs. The data shown are representative of two experiments.
Igk rearrangements (Supplemental Fig. S4A,B). These results suggest that these cells have matured; however, the frequency of μHC⁺Igk⁺ cells is reduced in Mzb1⁺/⁻/IL-7 B-cell cultures, consistent with the developmental block caused by the impaired surface expression of μHC. The phenotype of Mzb1⁺/⁻/mb1Cre mice raised the question of whether the mb1Cre allele synergizes with Mzb1 deficiency in pro-B cells. Indeed, flow cytometry of splenic B cells of Mzb1⁺/⁻/mb1Cre mice indicated that the number of CD19⁺ B cells in Mzb1⁺/⁻/mb1Cre mice is markedly reduced relative to Mzb1⁺/⁻/+ mice (Fig. 6A).

Moreover, we found reduced percentages of B220⁺CD43⁻ recirculating B cells and B220⁺CD43⁻ pre-B cells in the bone marrow of Mzb1⁺/⁻/mb1Cre mice (Fig. 6B). The mb1Cre deleter strain, the coding sequence of a mammalian codon-optimized humanized Cre recombinase (hCre) gene replaces the endogenous CD79a (mb1) gene, resulting in heterozygous mb1⁺/Cre mice (Shimshek et al. 2002; Hobeika et al. 2006). Heterozygous mb1⁺/Cre mice have a reduced expression of the mb1 gene product Igk (Fig. 6C). Therefore, we examined a potential effect of mb1 gene dosage by breeding Mzb1⁺/⁻/+ mice with mice in which...
differentiation and may be involved in the biosynthesis of tunicamycin-treated wild-type mice (Bruning et al. 2008), indicating that early stage B cells but not mature B cells undergo ER stress. The ER stress in normal pro-B and pre-B cells may synergize with Mzb1 deficiency and unmask a role for MZB1 in proper surface expression of μHC and the pre-BCR.

What could account for the discrepancy of pro-B-cell phenotypes in Mzb1fl/flmb1Cre and Mzb1fl/flmb1Cre mice? In principle, the heterozygosity of the mb1 allele and reduced expression of Igα could synergize with the mutant Mzb1fl/fl allele. However, the combination of a null allele of Mzb1 and a mutant mb1Cre allele did not result in a pro-B-cell block. Alternatively, augmented ER stress may underlie the mutant phenotype of Mzb1fl/flmb1Cre mice. Notably, we found significantly more Xbp1s transcripts in mb1Cre pro-B cells, raising the possibility that the mb1Cre allele enhances the ER stress normally detected in pro-B cells. Consistent with a potential synergy of the mutant Mzb1 allele and ER stress, tunicamycin treatment of Mzb1fl/mb1Cre mice was found to mimic the block in pro-B-cell differentiation. Therefore, we favor the view that pro-B cells may be able to cope with the naturally occurring ER stress at this stage of B-cell differentiation but may require the action of additional folding assistants, including GRP94 and MZB1, if any further increase in ER stress occurs. In support of this view, splenic B cells of tunicamycin-treated wild-type mice show an increased association of GRP94 with μHC, which is virtually abolished in the absence of MZB1. Interestingly, we could not detect Xbp1s transcripts in recirculating FoB cells of Mzb1fl/fl mice, suggesting that mature B cells do not experience ER stress and thus may not need MZB1 for proper folding of the membrane-bound IgM. However, differentiation of activated B cells into antibody-secreting plasma cells is accompanied by a natural UPR-mediated ER stress (Gass et al. 2002; Iwakoshi et al. 2003), which may result in enhanced expression of Cre and an augmented DNA damage response. Moreover, DNA damage induced by γ irradiation was found to result

Discussion

Here, we show that MZB1, a B-lineage-specific ER resident protein, is required for efficient humoral immune responses. Conditional inactivation of Mzb1 in the mouse germline or immature B cells revealed defects in the response to T-cell-independent antigens that either bind directly to MZ B cells or are scavenged by CD11c+ dendritic cells in the blood and are presented to MZ B cells (Guinamard et al. 2000; Balazs et al. 2002). Moreover, the TD response of FoB cells to the antigen TNP-KLH was found to be impaired in MZB1-deficient mice, suggesting a more general function of MZB1 in humoral immune responses. The reduced antibody titers in immunized Mzb1fl/flCD21-cre mice suggest a B-cell-intrinsic defect because CD21 is almost specifically expressed in B cells [Martin and Weis 1993; Kozono et al. 1998]. Moreover, the number of germinal center B cells was not altered in immunized Mzb1fl/flCD21-cre mice relative to Mzb1fl/+CD21-cre control mice [data not shown]. Therefore, the decrease in antigen-specific antibody titers in immunized Mzb1-deficient mice could reflect impaired plasma cell differentiation and/or defects in Ig secretion. The expression of transcriptional regulators of plasma cell differentiation, such as Prdm1 (Blimp1) and Xbp1, were found to be normal in immunized Mzb1-deficient mice [data not shown]. Thus, MZB1 may be dispensable for the early steps of plasma cell differentiation and may be involved in the biosynthesis and secretion of Igs in vivo. In particular, MZB1 regulates both the number of antibody-secreting plasma cells and the efficiency of antibody secretion per cell.

A role of MZB1 in early B lymphopoiesis was also observed in mice in which ER stress or genotoxic stress had been induced by tunicamycin treatment and Cre expression, respectively. Tunicamycin-treated Mzb1fl/fl mice and Mzb1fl/mb1Cre mice both show a block at the transition of pro-B to pre-B cells that is dependent on the expression of a functional pre-BCR. Indeed, we observed an impaired surface expression of μHC in pro-B cells of Mzb1fl/mb1Cre mice that cannot be accounted for by a defect in rearrangement of the Igα locus. Why does ER stress or genotoxic stress unmask a requirement of MZB1 for μHC surface expression in early but not late stage B cells? In concordance with previous studies, we detected Xbp1s transcripts specifically in B220+CD43+ pro-B cells of wild-type mice (Brünsing et al. 1998), suggesting that DNA damage induced by Cre expression or γ irradiation may also result in ER stress. Thus, naturally occurring ER stress in plasma cells and experimentally induced stress in pro-B cells require the function of MZB1 in enabling proper biosynthesis of μHC.
in an increase in the numbers of Xbp1s transcripts. However, the induction of Xbp1s by γ irradiation is modest relative to that induced by tunicamycin or Cre, suggesting that the Cre-mediated toxicity may not be solely via random DNA damage. The mechanism by which DNA damage induces ER stress is still unclear, and therefore it remains plausible that MZB1 has additional functions that are independent of its role in the biosynthesis of μHC.

How does MZB1 affect the biosynthesis of μHC? Previously, we and others found that MZB1 directly associates with the PDI ERp57 and the chaperones BiP and GRP94, suggesting that MZB1 does not act on its own but rather functions in collaboration with folding factors [Shimizu et al. 2009; van Anken et al. 2009; Flach et al. 2010]. GRP94 is a substrate-specific chaperone that serves a rather small subset of client proteins, including μHCs, TLRs, and integrins [Melnick et al. 1994; Staron et al. 2010; Luo et al. 2011]. Notably, we found that the associations of MZB1 with both GRP94 and μHC are markedly enhanced by induction of a UPR in tunicamycin-treated cells. Moreover, the association of GRP94 with its client μHC is critically dependent on the presence of MZB1, as this interaction is reduced and virtually undetectable in unstimulated and tunicamycin-treated MZB1-deficient splenic B cells, respectively. Therefore, we suggest that MZB1 can function as a cochaperone for GRP94 and facilitates its interaction with μHC, especially under conditions of ER stress. Although GRP94 is required for the folding and surface expression of TLRs [Staron et al. 2010], this process requires the action of the co-chaperone CNPY3, which forms a complex with GRP94 that is sensitive to the presence of ATP [Liu et al. 2010]. MZB1 shares with CNPY3 the ATP sensitivity of its interaction with GRP94 and the enhancement of substrate binding to GRP94. However, MZB1-deficient B cells show normal surface expression of TLR4 and LPS responsiveness, indicating that GRP94 may use distinct chaperonins for the folding of immunoglobulins and TLRs. Consistent with this view, knockout of Cnpy3 encoding a co-chaperone of GRP94 affects the surface expression of TLRs but does not alter the expression of Igs [Takahashi et al. 2007]. In contrast to the inactivation of Cnpy3, which abrogates TLR expression, the effects of Mzb1 mutation are less pronounced and are augmented by ER stress and genotoxic stress. This difference could reflect a partially redundant function of MZB1 with other members of the Canopy family of cochaperones.

In conclusion, our data show that MZB1 regulates humoral immune responses and the process of antibody secretion in vivo. In particular, MZB1 is needed for the proper biosynthesis of μHCs under conditions of natural and experimentally induced ER stress, including pharmacologically or genetically induced ER stress. MZB1 associates directly with the substrate-specific chaperone GRP94, and MZB1 is required for the interaction of μHCs with GRP94 under ER stress, suggesting that MZB1 acts as a substrate-specific co-chaperone of GRP94.

Materials and methods

Mice

All mouse experiments were carried out in accordance with the guidelines of the Federation of European Laboratory Animal Science Association [FELASA] and following legal approval of the Regierungspräsidium Freiburg. Mzb1+/+ (control) and Mzb1−/− chimeric mice were generated in the transgenic facility of the Max Planck Institute of Immunobiology and Epigenetics by Benoit Kanzler. ACT-FLPe mice [Susan Dymecki, Harvard Medical School] were ordered from Jackson Laboratories, while Stp63−/−, mbl1−/−, and mbl1−/−/fl mice were provided by the laboratory of Michael Roth (Albert-Ludwigs-University Freiburg). Igδ1−/−Iglk−/−, CMV-cre, and CD124-cre animals were obtained from the laboratory of Klaus Rajewsky (Max-Delbrück-Center for Molecular Medicine). Mouse strains were bred and maintained in the institute’s conventional animal care facility and genotyped using the appropriate primers. For experiments, animals were backcrossed for at least six generations onto a 129S2/SvPas or C57BL/6J [Jackson Laboratories] background. Unless otherwise stated, experiments were performed in a C57BL/6J background.

Flow cytometry

Cells were blocked with α-CD16/32 clone 93 [BD Pharmingen] and stained with the following fluorochrome-coupled antibodies purchased from either eBioscience or BD Pharmingen: α-AA4.1 clone AA4.1, α-B220 clone RA3-6B2, α-BPl clone BP-1, α-CD5 clone 53-7.3, α-CD16/32 clone 93, α-CD19 clone ID3, α-CD21 clone 7G6, α-CD23 clone B3B4, α-CD38 clone 90/CD38, α-CD43 clone S7, α-CD138 clone 281-2, α-HSA clone M1/69, α-Igα clone 24C2.5, α-IL-21 clone 187.1, NP-PE [Biosearch Technologies], streptavidin-PE [#554061, BD Pharmingen], streptavidin-APC [#554067, BD Pharmingen], α-IgM-APC [#115-175-075, Jackson ImmunoResearch Europe], and IgM-PE [#1020-09S, SouthernBiotech]. The stained cells were then subjected to FACS analysis on a special-order LSR II equipped with a UV, a violet, and a yellow-green laser (BD Biosciences) background. Unless otherwise stated, experiments were performed in a C57BL/6J background.

Immunizations and ELISA

TD antigen TNP(12)-KLH [Biosearch Technologies] was adsorbed overnight in a 1:1 ratio onto Alu-Gel-S (Serva). Mice were either intraperitoneally injected with 150 μg of adsorbed TNP(12)-KLH [Biosearch Technologies] or immunized with 25 μg of T-cell-independent antigen TNP[27]-Ficoll [Biosearch Technologies] or 0.5 μg of S. P. S. (American Type Culture Collection [ATCC]) by intravenous injection into the lateral tail vein. Blood was collected into SST Microtainer tubes (BD) and agglutinated. Pure serum was then obtained by a centrifugation step of 1500g. ELISA plates [Corning] were coated overnight with 10 μg/mL TNP-BSA [Biosearch Technologies], S. P. S. [ATCC], or α-mouse IgM [Thermo Fisher Scientific] in PBS. The next day, plates were washed under tap water and blocked for 1.5 h with PBS/0.5% BSA. After removal of the blocking solution, 1:100 dilutions of mouse sera were serially diluted in duplicate with an appropriate standard [mouse α-TNP-IgM; #55581, BD Pharmingen] or a reference sample. For primary B-cell ELISA, an undiluted supernatant

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of 2 μg/mL LPS-stimulated (Sigma) MZ B cells that had been counted after 48 h and plated out in triplicate overnight was employed, with unlabeled mouse IgM [SouthernBiotech] as a standard. The plates were incubated for 1 h at 37°C, washed under tap water, and incubated with a 1:1000 dilution of the appropriate biotinylated secondary antibody (SouthernBiotech) for 1 h at 37°C. Following three washing steps under tap water, plates were incubated with a 1:3000 dilution of streptavidin–alkaline phosphatase (SouthernBiotech) for 1 h at room temperature. Subsequently, plates were washed under tap water and developed by addition of ELISA substrate buffer (52.57 g of diethanolamine, 400 mg of MgCl2·6 H2O, 100 mg of NaN3 at pH 9.6, 500 mL of ddH2O). Enzymatic reaction was stopped by addition of 4N NaOH, and plates were read out using a Spectramax 250 ELISA reader [Molecular Devices].

ER stress induction

In vivo, ER stress was pharmacologically induced by intraperitoneal injection of 10 μg of tunicamycin per gram of body weight (Enzo Life Sciences) in 150 mM glucose for 12 h, while in cell culture, cells were treated with 5 μg/mL tunicamycin for 8 h.

γ Irradiation of cell culture cells

IL-7 cultured pro-B cells were irradiated with a dose of 20 Gy in a cell culture dish using a irradiator with a 137Cs source [Biobeam GM 8000, Gamma Service Medical GmbH] and incubated for 5 h at 37°C with 5% CO2. Following the purification of living cells by a Ficoll gradient, cells were subjected to RNA preparation.

mRNA preparation and (semi)quantitative RT–PCR

mRNA preparation was performed using Trizol reagent [Life Technologies], and cDNA was subsequently prepared using SuperScript II reverse transcriptase [Invitrogen, Life Technologies] according to the manufacturer’s protocol. One microliter of cDNA was mixed with 1× final concentration of Fast SYBR Green Master Mix [Applied Biosystems, Life Technologies] and 0.2 pmol/μL final concentration of respective forward and reverse primers in a total volume of 10 μL in an Applied Biosystems MicroAmp optical 96-well plate. The reaction was performed in an Applied Biosystems 7500 Fast real-time PCR machine. For semiquantitative RT–PCR, the reaction was performed in 0.2-mL PCR tubes [Biozym] using HT Master Taq polymerase, 0.2 mM dNTPs, MgCl-containing PCR buffer (Genaxxon Bioscience), and 1.5 μM forward and reverse primers.

Co-IP and immunoblot analysis

Cells were washed three times with 1 mL of cold PBS and resuspended in an appropriate cell number-dependent volume of immunoprecipitation buffer 1 containing 20 mM HEPES-KOH (pH 7.6), 2 mM MgCl2, 150 mM NaCl, 5% glycerol, 0.1% NP40, 1 mM Na2VO4, 1 mM PMSF, 1× homemade protease inhibitor mix, and 10 mM NaF. Following sonication in a precooled Branson 450 water bath sonifier, the cell lysate was centrifuged at 16,100g for 10 min at 4°C. The protein concentration of the supernatant was measured by a Bradford assay, and 1 mg of total protein was then mixed with α-MZB1 or α-rat IgG2a isotype control [Southern Biotech] linked to sepharose beads [GE Healthcare] or with α-GRP94 ab13509 [Abcam] or rabbit IgG control [Bethyl Laboratories] together with equilibrated Protein G sepharose beads [GE Healthcare] and rotated end over end for 4 h at 4°C. Subsequently, beads were washed three times with immunoprecipitation buffer 1 and once with immunoprecipitation buffer 1 containing 500 mM NaCl. Associated proteins were eluted by addition of 2× sample buffer and a boiling step of 10 min at 95°C. Following separation of the proteins by SDS-PAGE, proteins were detected by the following antibodies: α-MZB1 clone 2F9 [Flach et al. 2010], α-GAPDH clone 6C5 [Calbiochem, Merck Millipore], α-GRP94 #2104S, Cell Signaling, peroxidase-conjugated α-mouse-IgM, α-mouse-IgG, α-rabbit-IgG, and α-rat-IgG [Jackson ImmunoResearch Europe]. Blots were developed with SuperSignal WestPico or WestFemto (Thermo Fisher Scientific).

For the in vitro immunopurification assay, murine GRP94 was purified from mouse livers as previously described [Li and Srivastava 1993] and incubated end over end with recombinantly purified MZB1 [Flach et al. 2010] and increasing concentrations of ATP or AMP-PNP [Sigma] for 2 h at 4°C in the presence of anti-MZB1 monoclonal antibody coupled to sepharose beads. Following three washes with immunoprecipitation buffer 1, proteins were eluted and analyzed by SDS-PAGE and immunoblotting.

Statistical analysis

Microsoft Excel or GraphPad Prism 6.0b software was used to calculate mean and standard deviation of the mean, while the GraphPad Prism 6.0b software was employed for testing statistical significance by an unpaired two-tailed Student’s t-test with a significance level of α = 0.05. For quantification of semiquantitative RT–PCR, the ImageJ program (version 1.47) was employed.

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