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Incomplete block of B cell development and immunoglobulin production in mice carrying the μMT mutation on the BALB/c background

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The expression of the preB cell receptor (preBCR), composed of the μ chain, surrogate light chains and the Igα/Igβ signal transduction unit, permits further differentiation of B cell precursors. C57BL/6 mice homozygous for an inactivating mutation of the membrane exon of the μ chain gene (C57BL/6^{μMT/μMT}) cannot form a preBCR and are, consequently, devoid of mature B lymphocytes. Here we present evidence that the block of B cell development by the μMT mutation is incomplete in BALB/c mice. Unlike C57BL/6^{μMT/μMT}, BALB/c^{μMT/μMT} mice generate small numbers of mature B cells, accumulate plasma cells and produce high levels of all immunoglobulin isotypes, except IgM. The observed phenomenon seems to be controlled by a single genetic locus that is not linked to IgH.

Key words: μ chain / Immunoglobulin / Isotype switch / Plasma cell / Immune response

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1 Introduction

During their differentiation, B cells pass through several stages marked by successive steps in the rearrangement and expression of immunoglobulin genes, as well as ordered changes in the expression of surface receptors and stage-specific genes [1, 2]. One of the key checkpoints in developing B cells is the stage of the large preB cell, at which the cells express the preB cell receptor (preBCR), composed of immunoglobulin μ chain, λ5 and VpreB, and the Igα/β heterodimer whose signaling enables their further development [3–9]. The essential role of membrane-bound μ chains for the expression of the preBCR and continuation of B cell differentiation was documented in mice homozygous for an inactivating mutation of the membrane exon of their immunoglobulin μ chain gene (μMT). Since no functional preBCR can be formed in these mice, B cell development is arrested at the proB cell stage [10].

Here we show that, in contrast to the situation in C57BL/6^{μMT/μMT} mice, the block of B cell development is leaky on the BALB/c background.

2 Results

2.1 BALB/c^{μMT/μMT} mice fail to generate IgM, but produce all other immunoglobulin isotypes

After transfer of the μMT mutation from C57BL/6 to the BALB/c background, the offspring of homozygous breeding (BALB/c^{μMT/μMT}) were screened for the presence of immunoglobulins in their sera. Consistent with previous findings on μMT/μMT mice on a mixed C57BL/6/129 background [10], the sera were devoid of IgM, but, unexpectedly, positive for other immunoglobulin classes and subclasses (Fig. 1 A). Indeed, the average concentrations of IgG₁, IgG_{2a}, IgG_{2b}, or IgA did not significantly differ between control BALB/c and BALB/c^{μMT/μMT} sera. In contrast, BALB/c^{μMT/μMT} sera contained significantly less IgG₃ than the controls ($p < 0.05$), but detectable levels of IgD, which were not seen in BALB/c sera. In most of the mutant animals serum IgE was elevated. Sera of C57BL/6^{μMT/μMT} mice were found negative for all immunoglobulin isotypes (Fig. 1 A). Interestingly, the average κ:λ light chain ratio in BALB/c^{μMT/μMT} sera was 2.6, *i.e.* almost ten times lower than in sera of BALB/c mice (Fig. 1 B). Immunoprecipitation and separation by PAGE under reducing and nonreducing conditions were used to verify the identity of immunoglobulins in the sera of BALB/c^{μMT/μMT} mice. Electrophoresis under reducing conditions separated proteins of the molecular weights of immunoglobulin heavy and light chains, whereas separation under nonreducing conditions showed the molecular weight of complete immunoglobulin mole-

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M. H. and B. P. equally contributed to this work.

cules (Fig. 1 C). Western blotting, followed by staining with anti-mouse Ig (H+L) (Fig. 1 C) and antibodies specific to mouse IgM, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃ and IgA provided evidence for the identity of the immunoprecipitated immunoglobulins (data not shown).

2.2 Rescue of B lymphopoiesis in BALB/c^{μMT/μMT} mice is controlled by a single genetic locus

What is the genetic basis for the observed phenomenon? Is the leakiness a consequence of some modification of the IgH locus that might have happened during the backcross or is it controlled by other genetic loci differing between the two backgrounds? We performed PCR and Southern blot analysis to verify the presence of the original μMT mutation in BALB/c^{μMT/μMT} mice. Amplification of the DNA fragment spanning the *neo*^r cassette and neighboring intron of the mutated μ chain gene gave

rise to identical products in BALB/c^{μMT/μMT} and C57BL/6^{μMT/μMT} mice (data not shown). In addition, like in C57BL/6^{μMT/μMT} mice, EcoRI-digested genomic BALB/c^{μMT/μMT} DNA showed a specific band of 3.2 kb when hybridized with a probe specific for the inserted *neo*^r cassette (Fig. 2 A). To assess the identity of the μMT mutation on the two genetic backgrounds, BALB/c^{μMT/μMT} mice were backcrossed to C57BL/6, the offspring intercrossed and selected for homozygous μMT (serum IgM⁻) mutants. Of six such animals one did not produce serum IgG, whereas the others exhibited the BALB/c^{μMT/μMT} phenotype. When one of these latter animals was again backcrossed to C57BL/6 and the offspring intercrossed, 3/7 μMT homozygous (IgM⁻) mice produced serum IgG, whereas the others did not (Fig. 2 B). IgM⁻ mice with serum titers of IgG < 1:200 were considered IgG⁻ (with low levels of maternally transferred IgG), since serum titers of IgG-producing littermates (and positive controls) were much higher (≥ 1:1,600). In addition, spleens and

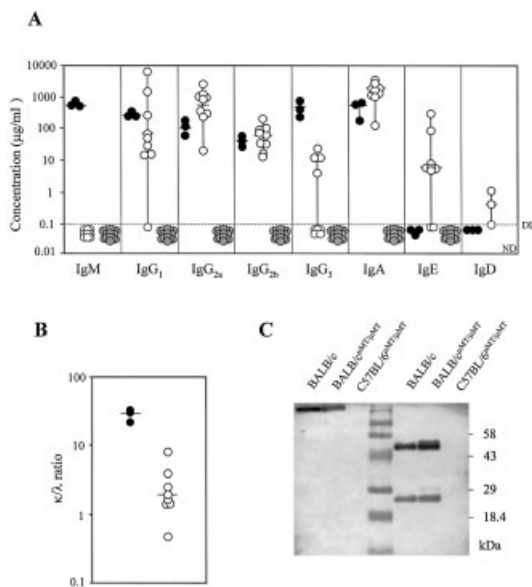


Fig. 1. Serum immunoglobulins of BALB/c^{μMT/μMT} mice. (A) The data represent concentrations of immunoglobulin classes and subclasses in sera of individual BALB/c (●), BALB/c^{μMT/μMT} (○) and C57BL/6^{μMT/μMT} (●) mice, determined by ELISA. Horizontal lines, mean values. DL, detection limit. ND, not determined. (B) Symbols represent ratios between κ and λ chain-bearing antibodies in individual BALB/c (●) and BALB/c^{μMT/μMT} (○) sera. Horizontal lines, mean values. (C) Serum immunoglobulins of BALB/c, BALB/c^{μMT/μMT} and C57BL/6^{μMT/μMT} mice were purified on protein A-Sepharose and separated by PAGE under nonreducing (left) and reducing (right) conditions. After Western blotting, immunoglobulins were stained by anti-mouse Ig-POD/DAB. The origin of the samples (top) and sizes of the marker bands (right) are indicated.

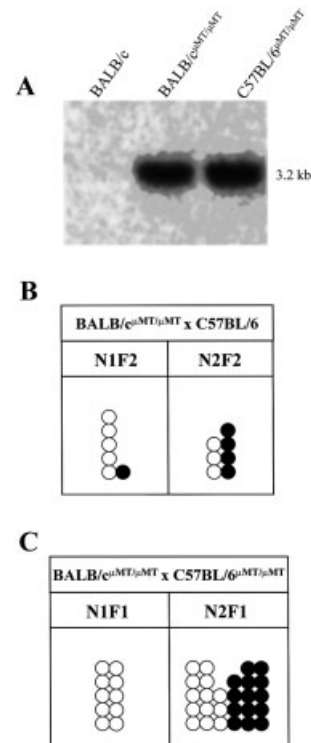


Fig. 2. A single gene controls leakiness of the block caused by the μMT mutation in BALB/c^{μMT/μMT} mice. (A) Genomic DNA was digested with BamHI and hybridized with a *neo*^r probe. The band of 3.2 kb indicates the presence of *neo*^r cassette in the first exon of the μ-chain gene. (B) Backcross of the μMT mutation from the BALB/c to the C57BL/6 background. For explanation see text. Symbols represent serum from IgG⁺ (○) and IgG⁻ mice (●). (C) Backcross of BALB/c^{μMT/μMT} to C57BL/6^{μMT/μMT} mice. For explanation see text. Symbols represent serum from IgG⁺ (○) and IgG⁻ mice (●).

guts of IgG⁻ mice were also tested by immunohistology for the presence of Ig-producing cells and none of these mice were found positive (data not shown). These results demonstrate that the μMT allele transferred onto the BALB/c genetic background has retained its functional identity and that its leaky phenotype is controlled by a gene or genes unlinked to the IgH locus. A further series of crosses indicated that a single dominant genetic locus exerts this control: intercrossing male BALB/c^{μMT/μMT} and female C57BL/6^{μMT/μMT} mice produced offspring all of which produced IgG. Backcross of these animals to C57BL/6^{μMT/μMT} led to equal proportions of IgG⁺ and IgG⁻ offspring (Fig. 2 C).

Does the transfer of the μMT mutation to mouse strains other than BALB/c result in a similar phenomenon? We backcrossed C57BL/6^{μMT/μMT} to CBA/J and found neither Ig⁺ cells in the spleen and the small intestine, nor immunoglobulins in the sera of μMT homozygous mice obtained by intercrossing the second backcross generation (data not shown). Although the significance of this analysis is limited by the small number of mice analyzed ($n = 5$), the leakiness of the μMT mutation may be a peculiarity of the BALB/c background.

2.3 Immunoglobulin positive (Ig⁺) cells are scattered throughout the lymphatic organs of BALB/c^{μMT/μMT} mice

In an attempt to localize the cells that produce immunoglobulins in BALB/c^{μMT/μMT} mice, lymphatic organs and small intestine were immunohistochemically stained for the presence of Ig⁺ (Fig. 3) and IgA⁺ cells (data not shown). Sections from BALB/c and C57BL/6^{μMT/μMT} mice were used as positive and negative controls, respectively. Similar to C57BL/6^{μMT/μMT}, BALB/c^{μMT/μMT} mice were devoid of B cell areas in periarteriolar lymphatic sheath of the spleen (Fig. 3, upper panel) and lymph nodes (data not shown). However, Ig⁺ cells could be detected, scattered throughout the parenchyma (upper panel). The vast majority of the Ig⁺ cells showed cytoplasmic staining, and the cells were surrounded by diffuse (non-cell associated) staining, which might indicate embedding of immunoglobulins into the surrounding tissue. Whereas small numbers of Ig⁺ cells were detected in the spleen, analysis of the lamina propria of the intestinal mucosa revealed the presence of many Ig⁺ (Fig. 3, middle panel) cells in BALB/c^{μMT/μMT} animals, comparable in numbers and distribution to those of control BALB/c mice. Cyto-

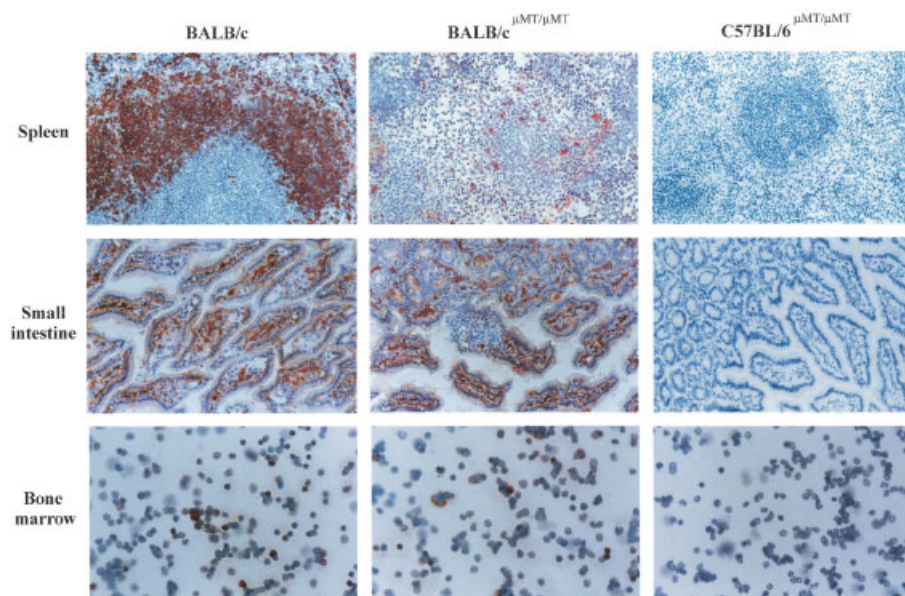


Fig. 3. Ig⁺ cells in different tissues of BALB/c^{μMT/μMT} mice. Immunohistology was performed using anti-mouse Ig-POD/AEC on frozen sections of the spleen and small intestine, or cytopsin smears of the bone marrow. Spleen sections are shown in the upper row. BALB/c^{μMT/μMT} mice (middle panel) lack lymphatic follicles, but small numbers of Ig⁺ cells are scattered throughout the parenchyma. Ig⁺ cells were not observed in C57BL/6^{μMT/μMT} mice (right panel). The middle row presents Ig⁺ cells in the mucosa of the small intestine. The intensity of Ig staining is comparable in BALB/c (left panel) and BALB/c^{μMT/μMT} mice (middle panel). The small intestine of C57BL/6^{μMT/μMT} mice was negative for Ig staining (right panel). In the lower row cytopsin smears of the bone marrow are shown, containing Ig⁺ cells in BALB/c^{μMT/μMT} (middle panel), and BALB/c mice (left panel). The Ig⁺ cells show either membrane or intracellular staining. In the bone marrow of C57BL/6^{μMT/μMT} mice (right panel) no Ig⁺ cells were observed. Magnification, 200 X.

spin preparations of BALB/c^{μMT/μMT} bone marrow showed the presence of both membrane and cytoplasmic Ig⁺ cells (Fig. 3, lower panels). Sections of the spleen and the small intestine, as well as cytopsin smears of the bone marrow of C57BL/6^{μMT/μMT} mice were all negative (20/20) for Ig⁺ cells (Fig. 3). In view of the recent data of MacPhearson et al. [11], who found IgA⁺ cells in the intestinal tissue of C57BL/6^{μMT/μMT} mice, we additionally stained the spleen and the small intestine with anti-IgA antibodies, but could not detect IgA⁺ cells in any (0/14) of the tested animals. IgA⁺ cells were detectable in spleen and small intestine of BALB/c and BALB/c^{μMT/μMT} mice, with a distribution pattern similar to that of the Ig⁺ cells (data not shown).

Do the cytoplasmic Ig⁺ cells in bone marrow and spleen of BALB/c^{μMT/μMT} mice represent plasma cells? Spleen cells taken from 2-, 6- and 13-week-old BALB/c, BALB/c^{μMT/μMT} and C57BL/6^{μMT/μMT} mice were analyzed by ELISPOT in order to detect and quantify Ig-producing cells. As shown in Fig. 4, plasma cells were indeed detected in the spleens of BALB/c mice homozygous for the μMT mutation and their numbers increased with age. Whereas only 100–200 plasma cells were found in the spleen of 2-week-old animals, their numbers reached the level of control BALB/c animals at the age of 13 weeks. In addition, the numbers of bone marrow and lymph node plasma cells were comparable in adult animals of both strains (data not shown). As expected, no plasma cells were found in spleen, bone marrow and lymph nodes of C57BL/6^{μMT/μMT} mice (Fig. 4, data not shown).

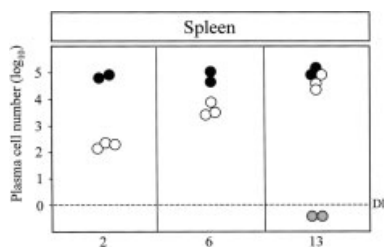


Fig. 4. Quantification of plasma cells in the spleen of BALB/c^{μMT/μMT} mice. Spleens of BALB/c (●), BALB/c^{μMT/μMT} (○) and C57BL/6^{μMT/μMT} (●) mice were analyzed for the presence of Ig-producing cells by ELISPOT assay. Symbols represent numbers of plasma cells in individual spleens of 2-, 6- and 13-week-old animals. DL, detection limits.

2.4 Expression of the immunoglobulin δ chain is involved in B cell differentiation in BALB/c^{μMT/μMT} mice

In order to compare B lymphopoiesis in the bone marrow of BALB/c^{μMT/μMT} and control mice, bone marrow cells were stained for the presence of CD19⁺CD43⁺ cells. As shown in Fig. 5 A, the population of CD19⁺CD43⁺ (proB) cells was larger in the bone marrow of BALB/c^{μMT/μMT} (59 %) than of C57BL/6^{μMT/μMT} mice (37 %), whereas BALB/c mice had a very small population of proB cells (3 %). Staining with the PB493 antibody that recognizes immature B cells [12] gave similar results. The proportion of PB493⁺ B cells was roughly three times larger in the bone marrow of BALB/c^{μMT/μMT} than that of C57BL/6^{μMT/μMT} mice (data not shown).

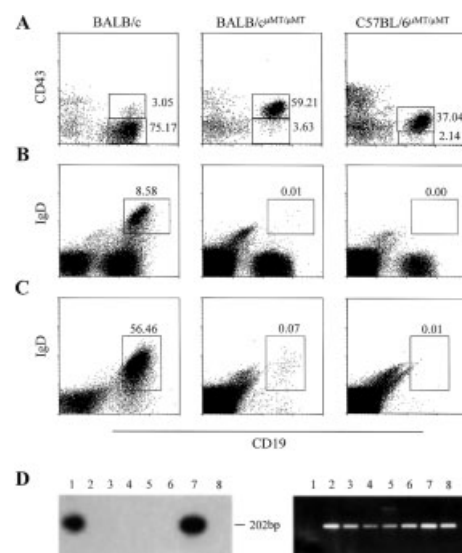


Fig. 5. Expression of IgD and a potential for premature isotype switching in BALB/c^{μMT/μMT} mice. Bone marrow (A and B) and spleen (C) cells of BALB/c (left panels), BALB/c^{μMT/μMT} (middle panels) or C57BL/6^{μMT/μMT} mice (right panels) were analyzed by flow cytometry for the surface expression of CD19/CD43 (A) and CD19/IgD (B and C). (D) CD19⁺CD43⁺IgD⁻ bone marrow cells were sorted and analyzed for the presence of “sterile” γ1 transcripts by RT PCR. The PCR product (202 bp) was visualized by Southern blot hybridization. (1) positive control, (2) negative control, (3) C57BL/6 proB/preBI, (4) BALB/c proB/preBI, (5) C57BL/6^{μMT/μMT} proB/preBI, (6) C57BL/6^{μMT/μMT} CD19⁺CD43⁺IgD⁻, (7) BALB/c^{μMT/μMT} proB/preBI, and (8) BALB/c^{μMT/μMT} CD19⁺CD43⁻IgD⁻ cells (left panel). Each sample was tested for the presence of cDNA by amplification of GAPDH cDNA. (1) genomic DNA, (2) C57BL/6^{μMT/μMT} proB/preBI, (3) BALB/c^{μMT/μMT} proB/preBI, (4) C57BL/6^{μMT/μMT} CD19⁺CD43⁺IgD⁻, (5) BALB/c^{μMT/μMT} CD19⁺CD43⁻IgD⁻, (6) C57BL/6 proB/preBI, (7) BALB/c proB/preBI and (8) positive control (right panel).

Earlier work has shown that IgD can take over the role of IgM during the development of B cells [13]. Although the μ MT mutation completely prevents expression of the δ chain in C57BL/6^{μMT/μMT} mice [10], this might not be the case on the BALB/c background. A small population of CD19⁺IgD⁺ bone marrow (0.01 %) (Fig. 5 B) and splenic B cells (≤ 0.1 %) (Fig. 5 C) was indeed detected in BALB/c^{μMT/μMT} mice. CD19⁺IgD⁺ cells in the spleen represented a mature population of B cells since the cells were positive for CD21 (Fig. 6 A) and CD23 (data not shown). Furthermore, we found a small, but reproducible, population of CD19⁺IgG₁⁺ cells (≤ 0.01 %) in the spleen (Fig. 6 B), but not in the bone marrow (data not shown) of these mice. Since we could not detect CD19⁺CD5⁺ cells in the peritoneal cavity of BALB/c^{μMT/μMT} (data not shown), B1 cells may not contribute to immunoglobulin production in these mice.

It has been reported that isotype switching can occur already at the stage of proB cells [14]. We therefore investigated whether early isotype switching might play a role in B cell development of BALB/c^{μMT/μMT} mice. CD43⁺CD19⁺IgD⁻ (proB/preB1) cells from the bone marrow BALB/c^{μMT/μMT} and C57BL/6^{μMT/μMT} mice were sorted and analyzed by RT-PCR for the presence of germ-line C γ 1 transcripts which precede switching to IgG₁ expression [15, 16]. RNA isolated from B cells stimulated with LPS and IL-4 *in vitro* served as a positive control. The presence of the RNA in various samples was verified by RT-PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fig. 5 D, right panel). The finding of “sterile” C γ 1 transcripts in proB/preB1 cells (Fig. 5 D, left panel) of BALB/c^{μMT/μMT}, but not of C57BL/6^{μMT/μMT} or control mice raises the possibility that besides δ chain expression, isotype switching to further downstream C region genes

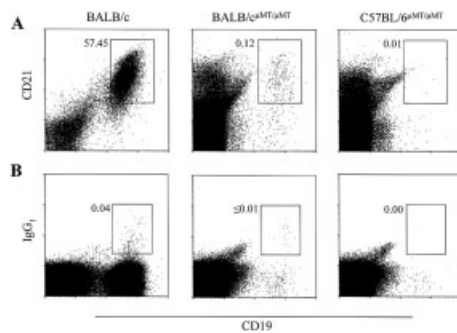


Fig. 6. Isotype switching in BALB/c^{μMT/μMT} mice. Splensens of BALB/c (left panel), BALB/c^{μMT/μMT} (middle panel) and C57BL/6^{μMT/μMT} (right panel) mice were analyzed for the presence of CD19⁺CD21⁺ (A) or CD19⁺IgG₁⁺ (B) cells. Small populations of CD21⁺ (0.12 %) and IgG₁⁺ cells (0.01 %) were observed in BALB/c^{μMT/μMT} mice.

might contribute to B cell development in the former mice.

2.5 BALB/c^{μMT/μMT} mice are not uniformly able to mount specific antibody responses

The ability of BALB/c^{μMT/μMT} mice to respond to T cell-independent antigens was tested by immunization with TNP-LPS and TNP-Ficoll. Sera were collected on days 0 and 14 post immunization. Most of BALB/c^{μMT/μMT} mice did not generate TNP-specific antibodies after immunization with either TNP-LPS or TNP-Ficoll (Fig. 7 A). However, immunoglobulins of some BALB/c^{μMT/μMT} mice bound TNP, regardless of the immunization, and some animals produced such immunoglobulins upon immunization. As expected, C57BL/6^{μMT/μMT} mice were unable to generate specific anti-TNP antibodies (data not shown).

The response to a T cell-dependent antigen was tested by infection of the mice with mouse cytomegalovirus

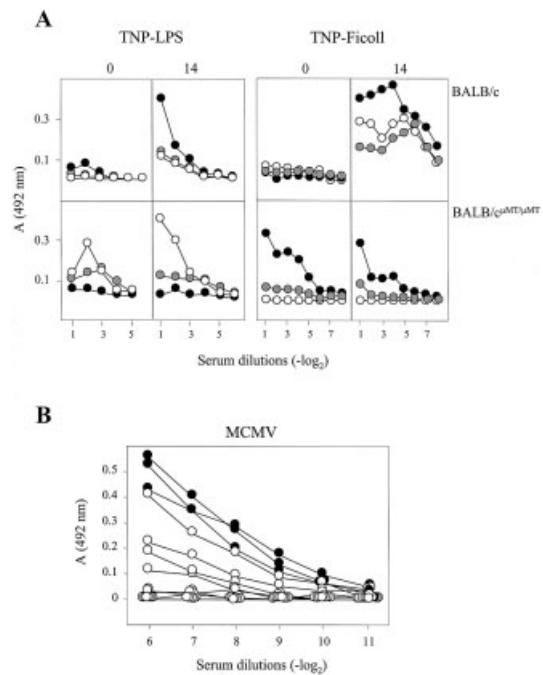


Fig. 7. Specific antibody response in BALB/c^{μMT/μMT} mice. (A) Mice were immunized with TNP-LPS (left panels) or TNP-Ficoll (right panels) and sera were analyzed on days 0 and 14 post-immunization. Symbols represent absorbance values of individual BALB/c^{μMT/μMT} (lower panel) and BALB/c (upper panel) sera. (B) BALB/c^{μMT/μMT} (○) and control BALB/c (●) mice were infected with MCMV and serum levels of anti-viral antibodies were determined 6 months after infection. Symbols represent absorbance value obtained for individual mouse sera.

(MCMV). Similar to the data described above, the ability of the infected BALB/c^{μMT/μMT} mice to respond specifically to MCMV was restricted to a fraction of the animals. Even in those, the serum levels of specific anti-viral antibodies were variable and lower compared to BALB/c controls (Fig. 7 B). In accordance with published data, no MCMV-specific antibodies were found in the sera of MCMV-infected C57BL/6^{μMT/μMT} mice [17].

Absence of significant reactivity against single-strand DNA, double-strand DNA or lysate of syngeneic fibroblasts indicated that the immunoglobulins detected in the sera of BALB/c^{μMT/μMT} mice were not self-reactive antibodies (data not shown).

3 Discussion

The data reported in this study show that the block of B lymphopoiesis, caused by the μMT mutation, is leaky in BALB/c mice. Although homozygous for the μMT mutation and expressing no surface IgM, BALB/c mice do produce some mature B cells, many plasma cells and large amounts of immunoglobulins. The partial rescue of B lymphopoiesis in these mice could be due to membrane expression of other immunoglobulin heavy chains that take over the role of the μ chain in B cell development. It is known from earlier work that Ig heavy chains such as δ, γ2b and γ1 can support B cell development in the absence of μ chains, although in the case of γ2b the situation is not entirely clear [13, 18–20]. Indeed, we found a small population of IgD⁺ mature B cells in the spleen of BALB/c^{μMT/μMT} mice, and small numbers of such cells could also be detected in the bone marrow. IgD was also present in low concentration in the serum of the animals in contrast to sera from control animals, whereas IgM was absent (Fig. 1). The latter results suggest that the IgD-expressing cells detectable in the mutants had switched to IgD expression through Cμ deletion, a process known to occur during B cell differentiation in mice at low frequency [21, 22]. Premature switching to IgG1 (and perhaps other isotypes; c.f. the elevated IgE levels in the blood) could also be involved in the partial rescue of B cell development in the mutants, as we detected “sterile” Cγ1 transcripts in the preB cell compartment of the mutants as well as small numbers of IgG1⁺ cells in the spleen. However, the latter cells could also be derived from IgD⁺ precursors. Whether the IgD⁺ cells themselves are generated in the bone marrow or from preB cells that have escaped into the periphery remains to be established, since IgD⁺ cells detected in the bone marrow could be immigrants from the periphery.

The genetic evidence indicates that the partial rescue of B cell development in BALB/c^{μMT/μMT} mice is under the

control of a single genetic locus (our group of linked loci) that is not linked to IgH and whose allelic counterpart in C57BL/6 does not permit B cell development. This locus could control the developmental progression from the expression of other isotypes, by regulating the accessibility of the IgM locus to class switch recombination or the switch recombination machinery itself. Alternatively, the control could be at a level unrelated to isotype switching, such as the life span or proliferative capacity of cells in the proB cell compartment. This latter possibility is suggested by the higher preB cell number in BALB/c^{μMT/μMT} mice compared to their C57BL/6 counterparts. This could increase the probability of premature isotype switching in the former. The high frequency of λ chain-bearing antibodies in BALB/c^{μMT/μMT} mice can be interpreted along the same lines, namely that the B cells generated in these animals have sufficient time in development to rearrange their Igλ loci at high frequency, in contrast to the situation in wild-type animals in which B cell development is not blocked at the proB cell stage and the inefficiency of Igλ vs. Igκ rearrangement becomes evident [23–26].

That a prolonged life span of proB cells may contribute to overcoming the developmental block imposed by the μMT mutation is consistent with earlier work in which the μMT mutation was combined with either the bcl-2 transgene [27] or mutations in the genes encoding Fas or Fas ligand [28], on the C57BL/6 background. In both instances substantial rescue of B cell development was observed, even more pronounced in terms of B cell numbers than in the BALB/c^{μMT/μMT} mice described in this report. The rescued B cells expressed Ig isotypes other than IgM on the surface (IgG and IgA [28]; IgD [27]). In the case of the bcl-2 transgene this correlated with an increase of the numbers of B cell progenitors in the bone marrow, while the role of the Fas signaling pathway in the generation and maintenance of proB cells remains elusive. The rescue of B cells in Fas-deficient C57BL/6^{μMT/μMT} mice may therefore relate (in part?) to a later developmental stage, which may be reflected by the fact that spontaneous autoantibody production was observed by Melamed et al. [28], but not in the present work.

The discrepancy between the high levels of serum immunoglobulins and the very small population of mature B cells in BALB/c^{μMT/μMT} mice can be ascribed to the accumulation of plasma cells over time (Fig. 4). This is in accord with results of earlier work showing that under conditions of limiting B lymphopoiesis in the bone marrow, the emerging B cells are preferentially drawn into the compartment of antibody-secreting cells [29, 30]. While the mechanism of this selection in which autoantigens may be involved is not clearly understood, it is

hardly surprising that the few and possibly strongly selected B cells in BALB/c^{μMT/μMT} mice are unable to mount efficient antibody responses upon deliberate immunization.

The recent observation by Macpherson et al. [11] that C57BL/6^{μMT/μMT} mice are able to produce IgA⁺ B cells in the intestinal tract could not be confirmed in the present study. The reasons for this discrepancy are unclear at this point and may relate either to differences in the microbial environments in which the animals are maintained or to an alteration of IgH locus carrying the μMT mutation. Alternatively, the phenomenon observed by Macpherson et al. could be due to another allelic form of the gene allowing Ig production in BALB/c^{μMT/μMT} mice, present in some C57BL/6 substrains.

In conclusion, we have shown that the block in B cell development caused by the μMT mutation is incomplete on the BALB/c genetic background and that this phenomenon is likely controlled by a single genetic locus that is unlinked to IgH. This should be taken into account when mouse B cell deficiency models are constructed, for which also deletion mutants of the JH region are available [31, 32].

4 Materials and methods

4.1 Mice

The C57BL/6^{μMT/μMT} mice from Institute for Genetics, Cologne, Germany [10] were backcrossed to C57BL/6 background (N > 8). BALB/c^{μMT/μMT} mice were obtained by transferring the μ chain gene mutation (μMT) from C57BL/6 onto BALB/c background (N ≥ 6). The homozygosity of the animals was confirmed by the absence of serum IgM. The μMT mice were kept in homozygous breeding. BALB/c (H-2^d), C57BL/6 (H-2^b), DBA.2J (H-2^d), CBA/J (H-2^k), C57BL/6^{μMT/μMT} and BALB/c^{μMT/μMT} mice were bred at the Central Animal Facility, Medical Faculty, University of Rijeka, Croatia.

4.2 Immunization procedures

Eleven- to twelve-week-old mice were immunized by intraperitoneal (i.p.) injection of 50 μg TNP-LPS (Sigma) or 25 μg TNP-AECM-Ficoll (Biosearch Technologies, Inc.) and bled from the tail vein on days 0, 7, 14 and 21 post-infection. Anti-viral immunization was performed by i.p. injection of 10⁵ PFU of mouse cytomegalovirus (MCMV) and blood was collected 22 days and 6 months post-infection.

4.3 Detection of immunoglobulins in mouse sera by ELISA

We screened mouse sera for the presence of IgM and IgG antibodies by ELISA assay [33], starting at initial serum concentrations of 1:50 and using 96-well plates (Greiner) coated with goat anti-mouse μ chain (10 μg/ml, Sigma) and goat anti-mouse γ chain antibodies (10 μg/ml, Southern Biotechnology Associates Inc. SBA), respectively. As secondary reagents we used peroxidase (POD)-conjugated antibodies specific for IgM (goat anti-mouse μ chain, Sigma) or IgG (sheep anti-mouse γ chains, Boehringer). Plates coated with TNP-BSA (10 μg/ml, Biosearch Technologies Inc.) were used for detection of TNP-specific antibodies in mouse sera, whereas the presence of anti-MCMV antibodies was tested on the plates coated with sonicated non-infected or MCMV-infected fibroblasts, as described previously [34]. Plates coated with sonicated murine embryonic fibroblasts, plasmid DNA or denaturated salmon sperm DNA were used for the detection of autoreactive antibodies. In this assay serum from lpr/lpr mouse we used as a positive control. POD-conjugated anti-mouse Ig (H+L) (Boehringer) we used as a secondary antibody. After incubation with the substrate (o-phenylenediamin, OPD), the absorbance was measured at 492 nm.

Serum immunoglobulin concentrations were determined by a quantitative ELISA, using plates coated with isotype-specific anti-mouse antibodies to: IgA (goat serum, Sigma), IgG₁ (goat serum, Sigma), IgG_{2a} (Nordic), IgG_{2b} (R14-50), IgG₃ (2E.6), IgM (R33-24-12), IgE (95.3), IgD (3,5), κ (187.1), or λ chain (goat anti-mouse, Institute for Genetics, Cologne). Biotin-conjugated antibodies directed against: IgA, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃ (goat anti-mouse, Southern Biotechnologies), IgM (R33-60), IgE (PharMingen), IgD (goat anti-mouse, Institute for Genetics, Cologne), κ- (R33-18.10.1) or λ- (goat anti-mouse, Southern Biotechnologies) chains were used as secondary antibodies. After incubation with streptavidin-alkaline phosphatase (Boehringer), p-nitrophenyl phosphate (Boehringer) substrate was added and absorbance measured at 405 nm. The following mAb were used as standards: IgA (233.1.3), IgG₁ (18-1-16), IgG_{2a} (41.2), IgG_{2b} (D313F1), IgG₃ (S24/63/63), IgM (267.7 μ), IgE (B1-8), IgD (267.7), κ (S8) and λ (N1G9).

4.4 Immunoprecipitation and Western blot

Immunoprecipitation was performed with protein A-Sepharose and the samples were separated by PAGE under reducing or nonreducing conditions according to standard protocols. PAGE was performed in duplicate during 12 h, on 11 % gel at 80V. One gel was stained with Coomassie blue, whereas proteins from the other gel were blotted on a membrane, incubated with anti-mouse Ig (H+L)-POD (Boehringer) during 2 h at room temperature and visualized with 3,3'-diaminobenzidine.

4.5 PCR, Southern blot and RT-PCR

The presence of the μ MT mutation was verified by PCR using primers and conditions as described previously [10]. In addition, genomic DNA of BALB/c, BALB/c ^{μ MT/ μ MT} and C57BL/6 ^{μ MT/ μ MT} mice was digested with EcoRI and the fragments were separated on agarose gels by electrophoresis. After Southern blotting on a membrane (HybondTM-N+, Amersham Pharmacia Biotech), the DNA was hybridized with a ³²P-labeled *neo* probe (pMMneoflox8, PstI-RsrII fragment).

Bone marrow cells were stained with anti-mouse CD19-PE (PharMingen), anti-mouse IgD-FITC (1-3.5, Institute for Genetics, Cologne) and anti-mouse CD43-biotin (S7, Institute for Genetics, Cologne). CD19⁺ CD43⁺ IgD⁻ cells were sorted on a FACStar (Becton Dickinson). Total RNA was isolated using TRIzol (GIBCO-BRL) and reverse transcribed by Superscript RNase-Reverse Transcriptase (GIBCO-BRL) according to the manufacturer's instructions. PCR amplification of "sterile" γ 1 transcripts was performed in 50- μ l samples, containing 4 μ l of cDNA, 0.05 mM dNTP, 2.5 U Taq DNA polymerase (GIBCO-BRL), 3 mM MgCl₂ and 25 pmol of forward (5' CCTCCTAGACAAGCACAGGCATGTAGA3') and reverse (5' ACCATGGAGTTAGTTTGGGCAGCAG3') exon-spanning (I γ 1 and C γ 1, respectively) primers. The reaction included 35 cycles: 94 °C 40 s, 57 °C 40 s and 72 °C 40 s. cDNA obtained from BALB/c B cells activated with IL-4 and LPS *in vitro* was used as a positive control. The amplification products were separated by gel electrophoresis and blotted onto a membrane. Hybridization was performed using the radioactively labeled (³²P) PCR product (202 bp) of the positive control. The presence of cDNA in each sample was confirmed by amplification of the housekeeping gene, GAPDH using exon-spanning primers 5'-TGTCAGCAATGCATCCTGCA-3' (forward) and 5'-CCGTTCCAGCTCTGGGATGAC-3' (reverse). Reaction conditions were as follows: 94 °C 60 s, 66 °C 60 s, 72 °C 60 s (35 cycles).

4.6 Immunohistology and determination of plasma cell numbers (ELISPOT)

Cytospin smears of bone marrow specimens (8 × 10⁵ cells/samples) obtained after centrifugation at 500 rpm (Cytospin 3[®], Shandon) and frozen sections of the spleen and of the small intestine (4 μ m thick) were air-dried and fixed in cold acetone. Endogenous POD activity was blocked with 30 % hydrogen peroxide in methanol-PBS (1:1). After saturation of nonspecific binding sites, the tissues were labeled with biotinylated sheep anti-mouse Ig (H+L) [F(ab')₂ fragments, Boehringer] or goat anti-mouse IgM (Medac), followed by staining with streptavidin-POD complex (Boehringer). 3-amino-9-ethyl carbazole (AEC) (DAKO) was used as a chromogen. All washes were carried out for 1 min with phosphate-buffered saline (PBS; pH 7.2) and all antibodies were diluted in TBS containing 1 % bovine serum albumin

(BSA). For detection of IgA⁺ cells in the tissues we used rabbit anti-mouse IgA and HRP-conjugated goat anti-rabbit IgG (H+L) (BioRad). Counterstaining was performed with hematoxylin (Shandon).

For the ELISPOT assay 96-well ELISA plates were coated with goat anti-mouse Ig (H+L) (10 μ g/mL) (Boehringer) and excess binding sites were saturated with 1 % BSA in PBS. Cell suspensions obtained from the spleen, the bone marrow and lymph nodes were resuspended in 10 % RPMI 1640 and subjected to lysis of erythrocytes. The samples were plated in eight dilutions, starting from 5 × 10⁵ cells/well. Plates were incubated for 3 h at 37 °C and 5 % CO₂ and washed eight times with PBS/0.01 % Tween 20. They were then incubated with sheep anti-mouse Ig (H+L)-biotin antibodies (F(ab')₂ fragments, Boehringer), followed by streptavidin-AP conjugate (Boehringer). After three additional washes in PBS, 5-bromo-4-chloro-3-indolyl phosphate (Sigma), diluted in 2-amino-2-methyl-1-propanol/0.6 % agarose buffer (pH 10.25) was added, the plates were incubated in darkness at room temperature for 10 h and the spots counted under a dissecting microscope.

4.7 Flow cytometry

Cells obtained from the spleen or the bone marrow were resuspended in 0.1 % NaN₃/PBS/1 % BSA (PBS-BSA). After the lysis of erythrocytes, cells were washed in PBS-BSA solution and nonspecific binding sites were saturated with rat anti-mouse CD16/32 (2G4-2) for 15 min. Staining was performed with PB493-biotin [12] or with anti-mouse IgG₁-DIG (kindly provided by Miltenyi Biotec), CD19-PE or -FITC (PharMingen), CD21-biotin (7G6, Institute for Genetics, Cologne), CD43-FITC (Institute of Genetics, Cologne), CD23-FITC (PharMingen), CD5-biotin (Becton Dickinson) and IgD-FITC (1-3.5, Institute of Genetics, Cologne) antibodies. Secondary reagents were anti-DIG-FITC (Boehringer), Streptavidin-FITC (PharMingen) and Streptavidin-Cychrome (PharMingen). The samples were analyzed on a FACS Calibur (Becton Dickinson).

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