Mannose 6 phosphorylation of lysosomal enzymes controls B cell functions

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Antigen processing and presentation and cytotoxic targeting depend on the activities of several lysosomal enzymes that require mannose 6-phosphate (M6P) sorting signals for efficient intracellular transport and localization. In this paper, we show that mice deficient in the formation of M6P residues exhibit significant loss of cathepsin proteases in B cells, leading to lysosomal dysfunction with accumulation of storage material, impaired antigen processing and presentation, and subsequent defects in B cell maturation and antibody production. The targeting of lysosomal and granular enzymes lacking M6P residues is less affected in dendritic cells and T cells and sufficient for maintenance of degradative and lytic functions. M6P deficiency also impairs serum immunoglobulin levels and antibody responses to vaccination in patients. Our data demonstrate the critical role of M6P-dependent transport routes for B cell functions in vivo and humoral immunity in mice and human.

Introduction

Lysosomes function in the degradation of macromolecules delivered by the biosynthetic, endocytic, or autophagic pathway and depend on the concerted action of ~60 lysosomal enzymes at low pH (Saftig and Klumperman, 2009; Schröder et al., 2010). Newly synthesized lysosomal hydrodrolases are modified on their N-linked oligosaccharides with mannose 6-phosphate (M6P) residues, which can be recognized by M6P-specific receptors in late Golgi compartments mediating their segregation from the secretory pathway and delivery to endosomal/lysosomal structures (Braulke and Bonifacino, 2009). The key enzyme in the formation of M6P residues is the N-acetylgalcosamine-1-phosphotransferase complex consisting of three subunits that are encoded by two genes, GNPTAB and GNPTG (Reitman et al., 1981; Waheed et al., 1981; Bao et al., 1996; Raas-Rothschild et al., 2000; Kudo et al., 2005; Tiede et al., 2005). The loss of phosphotransferase activity in individuals with mucolipidosis II (MLII or I-cell disease), a rare lysosomal storage disease with an incidence of 1:650,000, prevents the formation of the M6P recognition marker, which subsequently leads to missorting and hypersecretion of multiple lysosomal enzymes associated with lysosomal dysfunction and accumulation of nondegraded material (Braulke et al., 2013). However, in certain cell types in MLII patients such as hepatocytes, Kupffer cells, or cytolytic lymphocytes, the absence of lysosomal storage material and nearly normal level of selected lysosomal enzymes were observed, suggesting the existence of alternate M6P-independent targeting pathways (Owada and Neufeld, 1982; Waheed et al., 1982; Griffiths and Isaaz, 1993; Glickman and Kornfeld, 1993). Data on the direct consequences of variable targeting efficiency of nonphosphorylated lysosomal enzymes on cell functions in vivo are lacking.

Previous mouse studies have demonstrated that in antigen-presenting cells several lysosomal enzymes, in particular cathepsin proteases, are implicated in the limited degradation of proteins destined for the major histocompatibility complex (MHC) class II processing pathway. Furthermore, cathepsins have been shown to be involved in the stepwise proteolytic removal of CD74 (invariant chain), which regulates the assembly, production, and localization of class II MHC molecules on the cell surface. In humans, absence of MHC class II peptide loading through the loss of cathepsins or HLA-DM negatively affects humoral immunity in mice and human.

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peptide loading, and export of MHC II molecules for recognition by CD4 T cells (Riese et al., 1998; Driessen et al., 1999; Nakagawa et al., 1998, 1999; Honey and Rudensky, 2003).

To examine the significance of variable targeting efficiencies of lysosomal enzymes in the absence of phosphotransferase activity on cells of the immune system in vivo, Gna1ab knock-in mice (MLII mice) were analyzed. These mice mimic the clinical symptoms of MLII patients (Kollmann et al., 2012, 2013) and we find that the levels of lysosomal proteases are severely decreased in MLII B cells and impair the proliferation, differentiation, and antigen presentation as well as their interaction with T helper cells, resulting in reduced immunoglobulin production. Compared with MLII B cells, MLII T and dendritic cells (DCs) maintained higher lysosomal protease activities, and their cell functions were only moderately affected. Importantly, defective humoral immunity was also observed in MLII patients.

Results and discussion

Missorting of lysosomal proteases causes accumulation of storage material in B cells

In B cells of MLII mice, a profound and specific reduction (<10% of wild-type [WT]) of lysosomal protease activities, namely of cathepsin B (CtsB) and CtsL (Fig. 1 A), and a complete loss of immunoreactive CtsZ and CtsS were observed (Fig. 1 B). In contrast, activities of β-hexosaminidase (βHex), β-galactosidase (βGal), α-fucosidase (αFuc), and α-mannosidase (αMan), all involved in lysosomal degradation of oligosaccharides, were not or only moderately reduced in B cells of MLII mice (Fig. 1 A) or in B lymphoblasts of MLII patients (Little et al., 1987; Tsuji et al., 1988; Glickman and Kornfeld, 1993). The higher amounts of the lysosome-associated membrane protein 1 (Lamp1) in MLII B cells indicated an increased number and/or size of lysosomes most likely because of storage material (Karageorgos et al., 1997; Kollmann et al., 2012). Indeed, ultrastructural analysis showed a high number of both electron-lucent vacuoles and multi-lamellar bodies representing heterogenous accumulation of storage material in 42% of MLII B cells, which was absent in WT B cells (Fig. 1 C). Compared with MLII B cells, MLII T cells showed higher residual enzyme activities of CtsB and CtsL (~20% of WT), and CtsZ immunoreactivity was still detectable (Fig. 1, D and E). Activities of lysosomal glycosidases βGal, αFuc, and αMan were only marginally reduced in MLII T cells (>50% residual activity compared with WT T cells), and βHex was not changed at all (Fig. 1 D). No lysosomal storage vacuoles could be detected by transmission EM in splenic CD4 (Fig. 1 F) or CD8 (Fig. 1 G) MLII T cells. The partial loss of lysosomal enzymes did not reveal any statistically significant effects on the splenic immune cell composition in MLII mice (Fig. S1). In previous studies on cytotoxic lymphocytes of MLII patients a reduction of βHex activity to 30% of control has been observed (Griffiths and Isaaq, 1993). Our data on MLII T cells are in agreement with numerous other studies that 5–10% of normal activity of single lysosomal enzymes is sufficient to maintain their function in lysosomes and prevent the accumulation of storage material (Fratantoni et al., 1969; Sawkar et al., 2006). Furthermore, these data suggest the existence of M6P-independent transport routes for distinct lysosomal/granule functions, which appear to be sufficient for maintaining degradative lysosomal and lytic granule functions, e.g., in MLII T cells.

Antigen fragmentation and presentation is affected in M6P-deficient B cells

In professional antigen-presenting cells, lysosomal proteases are required for the degradation of antigenic proteins in the endosomal pathway and processing of CD74 (invariant chain), which prevents in its intact form premature loading of peptides to MHC II molecules (Honey and Rudensky, 2003). The antigen–MHC II complexes are subsequently transported to the cell surface and presented to CD4 T cells. DC–T cell interactions are essential for T cell priming, and B cell–T cell interactions are crucial for B cell differentiation after activation. We undertook several experimental approaches to examine the impact of variable missorting of lysosomal enzymes in MLII B cells and DCs on antigen presentation to CD4 T cells. First, we investigated antigen uptake by B cells and DCs. Cells were incubated with fluorochrome-conjugated ovalbumin (Ova) protein and subsequently analyzed by flow cytometry. After 20-min incubation with Alexa Fluor 647–Ova, similar intracellular fluorescence intensities were observed in WT and MLII B cells and DCs (Fig. 2 A), indicating comparable rates of antigen uptake. Next, we studied the antigen degradation by incubating cells with DQ-Ova, which releases the DQ-fluorophore upon lysosomal proteolysis of Ova. After the 20-min loading period, a linear increase in the fluorescence intensity was observed in WT B cells reaching a plateau after 90 min (Fig. 2 B). In contrast, no increase in fluorescence intensity was detectable in MLII B cells. Compared with B cells, both WT and MLII DCs display a similar time course of increasing fluorescence intensity with slightly reduced rates of Ova proteolysis in MLII DCs (Fig. 2 B). Thus, MLII and WT antigen-presenting cells were similarly efficient in protein uptake. However, MLII B cells, but not MLII DCs, were profoundly impaired in proteolytic degradation of the endocytosed Ova protein.

In both MLII B cells and DCs, we observed an accumulation of intermediate CD74 degradation fragments (Fig. 2 C), which most likely prevent MHC II loading with antigenic peptides (Roche and Cresswell, 1991). Proteolytic cleavage of CD74 depends on CtsS. At steady state, MLII DCs maintained higher amounts of CtsS protein (~38% of WT DCs) than MLII B cells (~5% of WT B cells), which might restrict accumulation of CD74 degradation intermediates in MLII DCs. Furthermore, the total level of MHC II molecules at the surface of activated B cells from MLII mice was reduced to 58% of WT B cells (Fig. 2 D). This might be the consequence of ubiquitination with chains of distinct length, which is a prerequisite for the subsequent degradation of internalized surface MHC II (Ma et al., 2012; Furuta et al., 2013) in MLII B cells. EM analysis of MLII DCs revealed no changes in morphology (Fig. 2 E), and the lack of lysosomal storage material suggests that residual lysosomal enzyme activities are sufficient for maintaining cellular homeostasis. Furthermore, MLII DCs fail to exhibit severe antigen degradation defects. This might be explained by...
The relatively high residual expression of CtsS and most likely other acid proteases, as compared with B cells, which allow rather normal degradation of the indicator antigen DQ-Ova and partial proteolytic processing of CD74.

To directly measure antigen presentation to CD4 T cells in vitro, activated B cells were incubated with Ova protein for 2 h, washed, and then cultured for 4 d with Ova-specific CD4 T cells obtained from Ova-specific T cell receptor transgenic mice.
Incubation of Ova-loaded WT B cells with OT-II T cells also caused further maturation and the acquisition of the plasma cell marker CD138 by WT B cells (Fig. 3, C and D). In contrast, there was reduced expression of CD138 both in terms of frequency of positive cells and expression level per cell (mean fluorescence intensity [MFI]) on the surface of MLII B cells, indicating that defects in antigen presentation in MLII B cells result in restricted cooperation with T cells and lead to reduced B cell maturation. Defects in antigen degradation and presentation can be easily explained by the low abundance of the lysosomal proteases CtsL, CtsS, and CtsZ, and most likely also of the disulfide bond cleaving γ-interferon–inducible lysosomal thiolreductase in MLII B cells, because mice deficient in these enzymes show impaired MHC II–restricted antigen presentation (Nakagawa et al., 1998, 1999; Driessen et al., 1999; Maric et al., 2001; Honey and
or even slightly higher than that by WT DCs (Fig. 3 J), indicating that the DCs are not generally deficient in activation of T cells. It has been shown that DCs and B cells require a closely defined range of protease activities that, on one hand, generate peptides for presentation and, on the other hand, do not lead to the complete degradation of antigens (Delamarre et al., 2005, 2006). Our data suggest that the lysosomal proteolytic capacity of MLII DCs is still within this range and therefore allows generation of antigenic peptides, although at reduced levels.

Rudensky, 2003). The presence of lysosomal storage material, however, might also contribute to abnormal B cell functions, similarly to dysfunctional MLII osteoblasts and chondrocytes (Kollmann et al., 2013).

MLII DCs were also tested for their capacity to present antigen to CD4 T cells. MLII DCs displayed some reduction in Ova protein presentation to T cells (Fig. 3 I); however, this impairment was far less profound than the defect observed in MLII B cells. Peptide presentation by MLII DCs was similar or even slightly higher than that by WT DCs (Fig. 3 J), indicating that the DCs are not generally deficient in activation of T cells. It has been shown that DCs and B cells require a closely defined range of protease activities that, on one hand, generate peptides for presentation and, on the other hand, do not lead to the complete degradation of antigens (Delamarre et al., 2005, 2006). Our data suggest that the lysosomal proteolytic capacity of MLII DCs is still within this range and therefore allows generation of antigenic peptides, although at reduced levels.
incubated with phorbol 12-myristate 13-acetate and ionomycin. Secretion of cytokines was blocked with brefeldin A (Donaldson et al., 1992) and the intracellular accumulation of TNF and IFN-γ in CD4, CD8, and γδ T cells was analyzed by flow cytometry. The extent of TNF and IFN-γ response after activation was similar in WT and MLII T cell populations (Fig. 4 A). In addition, when spleen cells were stimulated with anti-CD3 mAb, similar amounts of IL-6 were secreted by WT and MLII cells (Fig. 4 B).

To examine the function of cytotoxic T cells derived from MLII mice, spleen cells were stimulated with anti-CD3 mAb for 3 d and the accumulation of granzyme B in CD8 T cells was analyzed by flow cytometry. Similar amounts of granzyme B were found in MLII and WT CD8 T cells (Fig. 4 C). When CD8 T cells from WT and MLII mice were incubated with allogeneic spleen cells, a comparable killing ability of allogeneic target cells was observed for WT and MLII cytotoxic T cells (Fig. 4 D).

These findings are in agreement with conserved cytotoxicity of T cells from MLII patients, although these cells showed diminished levels of granzyme A and B when compared with control levels (Griffiths and Isaaz, 1993). The data suggest that the loss of M6P residues on lysosomal and granular enzymes and their subsequent missorting in MLII T cells can be partially compensated by alternative lysosomal targeting pathways that allow maintenance of central T cell functions such as cytokine production and cytotoxicity.

Impaired antibody response in MLII

To examine the biological significance of impaired homeostasis of lysosomes in B cells, the immune status of MLII mice in vivo was studied. Sera from MLII mice showed normal
serum levels of IgM but significantly reduced IgG (Fig 5 A). Upon subcutaneous immunization with Ova in complete Freund’s adjuvant (Fig. 5 B), we found similar serum levels of Ova-specific IgM in MLII and WT mice 10 d after immunization (Fig. 5 C). However, Ova-specific total IgG, IgG1, and IgG2 were significantly reduced in MLII mice at days 10 and 18 after immunization (Fig. 5 D). Histochemically, no differences in splenic germinal center formation could be observed between WT and MLII mice.

To determine whether alterations in B cell functions in MLII mice are relevant for human disease, the immune status of four MLII patients was analyzed. Normal blood cell counts, including the numbers of leukocytes, erythrocytes, and platelets, have been reported in the medical records of four MLII patients. However, when we analyzed fresh blood samples from two patients for the distribution of different lymphocyte subsets by flow cytometry, we noticed a decreased percentage of B cells and an increased percentage of T cells compared with the age-matched reference range (Table S1; Comans-Bitter et al., 1997). When the B cell subset composition of the 2.4-yr-old female MLII patient 3 was analyzed further by flow cytometry, she exhibited a high percentage of naive B cells (identified as CD27–IgD+), whereas the relative proportion of memory B cells (CD27+), including the cells that underwent class switching, were reduced in comparison with age-matched reference ranges (Table S1; Comans-Bitter et al., 1997). Examination of serum immunoglobulins revealed low concentrations of total IgG, IgA, and IgM in MLII patients compared with the age-matched reference range (Table S2; Stiehm and Fudenberg, 1966), with the exception of patients 1 and 2 who showed normal IgG levels caused by intravenous injection of immunoglobulin preparations to prevent common infections. Most importantly, the specific antibody response to vaccination was poor or not detectable in all MLII patients (Table S2), which is consistent with MLII mouse data. The low level of memory B cells in the patient analysis indicates impaired B cell maturation in response to antigen and defective immunoglobulin class switch, altogether preventing an effective response against pathogens.

There are only few studies that report on B cell function in lysosomal storage disorders. Kojima et al. (1979) described an MLII patient with a low number of B cells, but normal immunoglobulin concentrations; however, they did not provide data on the underlying mechanisms. Patients with α-mannosidosis also showed low IgG and weak response to vaccinations (Desnick et al., 1976; Malm et al., 2000). Interestingly, lymphocytes from α-mannosidosis patients were filled with storage material that could impair antigen processing and presentation by these cells. The cause of the defects in B cell response is unclear and might be different to MLII because we observe normal αMan activity in MLII B cells. Studies on mice defective in the lysosomal degradation of glycosaminoglycans have led to the speculation that the accumulating negatively charged glycosaminoglycans affect lysosomal function or directly impact lysosomal enzyme activities or peptide loading to MHC II molecules (Daly et al., 2000). The identity of storage material in MLII B cells is unknown; however, our results do not exclude that accumulated storage materials observed in MLII B cells could affect general lysosomal functions and primarily or secondarily impair antigen presentation as well as antibody production. Although airway obstruction caused by skeletal deformities, enlarged tongue, thickened mucosa, and larynx alterations are believed to be responsible for the recurrent infections in MLII (Peters et al., 1985), the data shown here indicate that the functionally impaired immune system contributes to the high predisposition to infections in MLII patients.

The data presented here demonstrated the biological significance of M6P-independent targeting pathways in vivo allowing the transport of lysosomal hydrolases lacking M6P residues in sufficient amounts to maintain almost normal DC and T cell functions. In contrast, the missorting of subsets of non-M6P–modified enzymes, in particular cathepsin proteases, severely impairs antigen fragmentation and presentation in B cells and DCs as well as B cell maturation and the subsequent immune status in mice and patients with MLII.
Materials and methods

Human

MLII patients' data were collected from their medical record under the written informed parent's consent according to the Declaration of Helsinki. Patient 1 and 2 were of Japanese, patient 3 of German, and patient 4 of Portuguese origin. The study was approved by the medical ethics committee of the Ärztekammer Hamburg. Genomic DNA was extracted from patients' skin fibroblasts or lymphocytes, and individual exons and adjacent intronic regions of GNPTAB were amplified with primers previously published (Tiede et al., 2005; Otomo et al., 2009) followed by standard BigDye sequencing protocol (Applied Biosystems).

Mouse strains

A knock-in mouse model for MLII was generated by the insertion of a cytotoxic at position c.3082 in exon 16 of the mouse Gntpad gene (Gnpptpd3082loxP) that caused a premature translational termination leading to a truncated phosphotransferase protein (p.G1028Rfs) that caused a premature translational termination leading to a truncated phosphotransferase protein (p.G1028Rfs). This mutation corresponds to a mutation, c.3145insC, identified in an MLII patient (Tiede et al., 2005). For the experiments, age- (between 6 and 15 wk) and gender-matched mice were used. WT littersmates served as controls. OTII mice were transgenic for an αβ T cell receptor specific for chicken Ova in the context of MHC class II (I-Aq; Banerd et al., 1998). MLII mice and OTII mice were bred under specific-pathogen-free conditions at the animal facility of the University Medical Center Hamburg-Eppendorf. Experiments were conducted according to German animal protection law.

Isolation and culture of immune cells from mouse

Immune cells were isolated from spleen by passing through a 200-µm metal sieve and 70-µm cell strainer into 10 mM PBS, pH 7.4. Subsequently, red blood cells were lysed with ACK lysis buffer (155 mM NH4Cl, 10 mM KC104, and 100 µM EDTA, pH 7.2). The remaining immune cells were used for further analysis including flow cytometry, activation, and culture. Cells were cultured with complete medium (RPMI 1640 medium supplemented with 5% heat-inactivated FCS, 2 mM l-glutamine, 50 µM β-mercaptoethanol, and 50 µg/ml gentamicin) in 10-cm dishes with or without stimulation with 10 µg/ml LPS (Sigma-Aldrich) or hamster anti–mouse CD3ε (EM902; Carl Zeiss). Pictures were taken with a MegaView III digital camera (Visitron Systems). For ultrastructural analysis, B cell blasts obtained with LPS stimulation for 3 d or BM-DCs were fixed with 4% PFA and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2–7.4, three times in 0.1 M sodium cacodylate buffer, pH 7.2–7.4, and were postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.2–7.4. After rinsing with distilled water, samples were dried using a critical point dryer (Polaron; Quorum Technologies) and sputtered with a thin layer of gold to improve the resolution in low-molecular mass range (Schägger, 2006). For the Lamp1 and CtsZ immunoblot, 10 µg of cellular protein were solubilized and separated by 12.5% Tris-tricine SDS-PAGE to improve the resolution in low-molecular mass range (Schägger, 2006). For the Lamp1 and CtsZ immunoblot, 10 µg of cellular protein were solubilized and separated by 12.5% Tris-SDS-PAGE to improve the resolution in low-molecular mass range (Schägger, 2006).

B and T cell blasts and DCs were sonicated in 25 mM TBS, pH 7.4, containing 1% Triton X-100. Protein concentration was determined by Bradford protein assay. Specific enzyme activities for CtsB and CtsL were measured by incubating 4 µg of cellular protein with Z-Phe-Arg-AMC substrate (50 µM; Bachem) in reaction buffer (50 mM sodium acetate, 8 mM EDTA, and 8 mM DTT, pH 6.0) for 15 min at 37°C, followed by determination of fluorescence (excitation 380 nm; emission 442 nm). In parallel, incubation was performed in the presence of specific CtsL inhibitor (SID26681509; 1 µM) or CtsB inhibitor (CA-074Me, 5 µM), and the subtracted values were calculated as CtsB or CtsL activities, respectively. The activities of granzyme B, granzyme A, and granzyme M were measured by incubation with 25 µg/ml Ova-conjugated anti–mouse CD3ε antibody and intracellular staining with Alexa Fluor 647–conjugated Ova (200 µg/ml and 100 µg/ml, respectively; Life Technologies) and DQ-conjugated Ova (200 µg/ml and 100 µg/ml, respectively; Life Technologies). After incubation with HRP-conjugated secondary antibodies, immunoreactive bands were detected by enhanced chemiluminescence. Stripping of antibodies from membranes was performed with 0.2 M NaOH for 20 min at RT.

Microscope image acquisition

For ultrastructural analysis, B cell blasts obtained with LPS stimulation for 3 d or BM-DCs were fixed with 4% PFA and 1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Thereafter, they were rinsed and spun down at 1,000 g three times in 0.1 M sodium cacodylate buffer, pH 7.2–7.4, and osmicated with 1% osmium tetroxide (Science Services) in the same buffer. After osmication, the cells were dehydrated using ascending ethyl alcohol concentration steps, followed by two rinses in propylene oxide. Infiltration of the embedding medium was performed by immersing the pellets in a 1:1 mixture of propylene oxide and Epon and finally in neat Epon. Ultrathin sections (60 nm) were examined in an electron microscope (EM902; Carl Zeiss). Pictures were taken with a MegaView III digital camera and image software (TRS). For quantification of cells containing lysosomal storage material, each 100 WT and MLII B cells were evaluated for the presence of both electron-lucent vacuoles and multi-lamellar bodies.

Antigen uptake and degradation assay

Purified splenic B cells stimulated with IPS for 2 d and DCs were incubated with Alexa Fluor 647–conjugated Ova (200 µg/ml and 100 µg/ml, respectively; Life Technologies) and DQ-conjugated Ova (200 µg/ml and 100 µg/ml, respectively; Life Technologies). After 20-min incubation, Ova-loaded cells were washed twice with PBS and chased with Ova-free complete medium. At each time point, cells were immediately moved on ice, washed twice with PBS, and fixed with PBS with 1% PFA. Intracellular fluorescence was measured by flow cytometry.

Flow cytometry for mouse cells

After preparation, surface staining of the immune cells was performed with fluorochrome or biotin-conjugated mAb for 20 min on ice. Dead cells were stained with Pacific orange succinimidyl ester (Life Technologies). Cells were fixed with PBS with 2% PFA for 20 min at RT. Intracellular staining was performed with fluorochrome-conjugated mAb for 20 min at RT in permeabilization buffer (PBS, 0.3% saponin, and 0.1% BSA). Intracellular staining of FoxP3 was performed using FoxP3 fixation/permeabilization reagents (eBioscience) according to the manufacturer’s protocol with fluorochrome-conjugated anti-FoxP3 antibody. After staining, cells were measured on a Canto II flow cytometer (BD) and data were analyzed using the FACS Diva software (BD) and FlowJo (Tree Star).

Flow cytometry for human blood

50 µl of freshly drawn blood were stained with cocktails of antibodies designed to identify the main leukocyte subsets and the B cell subpopulations. After 15-min incubation with the antibody cocktails, erythrocytes were lysed using the BD Lysing Solution (BD) and the remaining cells were washed, resuspended in FACs buffer, and analyzed using a FACs Canto (BD). Data were acquired and analyzed using the FACs Diva software.

Biochemical analysis

B and T cell blasts and DCs were sonicated in 25 mM TBS, pH 7.4, containing 1% Triton X-100. Protein concentration was determined by Bradford protein assay. Specific enzyme activities for CtsB and CtsL were measured by incubating 4 µg of cellular protein with Z-Phe-Arg-AMC substrate (50 µM; Bachem) in reaction buffer (50 mM sodium acetate, 8 mM EDTA, and 8 mM DTT, pH 6.0) for 15 min at 37°C, followed by determination of fluorescence (excitation 380 nm; emission 442 nm). In parallel, incubation was performed in the presence of specific CtsL inhibitor (SID26681509; 1 µM) or CtsB inhibitor (CA-074Me, 5 µM), and the subtracted values were calculated as CtsB or CtsL activities, respectively. The activities of granzyme B, granzyme A, and granzyme M were measured by incubation with HRP-conjugated secondary antibodies, immunoreactive bands were detected by enhanced chemiluminescence. Stripping of antibodies from membranes was performed with 0.2 M NaOH for 20 min at RT.
Antigen presentation assay

Purified splenic B cells stimulated with LPS for 2 d and DCs were incubated with 5, 1, 0.2, and 0.04 mg/ml of chicken Ova or 10⁻⁵, 10⁻⁶, 10⁻⁷, and 10⁻⁸ M of Ova324-340 peptide for 2 h at 37°C and then washed three times. Ova-specific CD4 T cells were purified from OT-II mice and stained with carboxyfluorescin succinimidyl ester (CFSE). 10³, 0.3 x 10³, 0.1 x 10³, and 0.03 x 10³ Ova-loaded B cells or DCs (from WT or MLII mice) and 10³ Ova-specific CD4 T cells (from OT-II mice) were coincubated for 4 d on a 96-well plate and investigated with flow cytometry after staining with surface markers. Proliferation of CD4 T cells was measured either by determining the number of CD4 T cells in flow cytometry with the help of count beads or by assessing the decrease of CFSE staining on CD4 T cells. Both measurements gave comparable results. In the same samples, CD19 and CD138 expression of B cells were determined by flow cytometry.

T cell stimulation and cytokine determination

TNF and IFN-γ production by T cells was determined after stimulation with 50 ng/ml phorbol 12-myristate 13-acetate and 1 µM ionomycin in the presence of 10 µg/ml brefeldin A for 4 h at 37°C. After fixation and permeabilization, intracellular TNF and IFN-γ were quantified by flow cytometry.

Immunoglobulin quantification

ELISA for total IgM and IgG in mouse serum were performed by sandwich ELISA. 96-well plates were coated with goat anti-mouse IgG (H+L) antibody (1.3 µg/ml; Dianova) followed by blocking with 1% BSA. Sequentially diluted mouse sera (1:1,000–1:150,000) were applied to the plate. Plates were washed and Ig binding was detected by HRP-conjugated antibodies: goat anti-mouse IgM µ-chain (EMD Millipore) and goat anti-mouse IgG γ-chain (EMD Millipore). ELISA for Ova-specific antibodies in mouse serum was performed by indirect ELISA. Plates were coated with Ova (5 µg/ml) in 0.1 M NaHCO₃, pH 8.2 followed by blocking with 1% BSA. Diluted serum samples (1:4,000) were applied to the plate and detected by HRP-conjugated antibodies: goat anti-mouse IgM µ-chain (EMD Millipore); goat anti-mouse IgG γ-chain (EMD Millipore); goat anti-mouse IgG1 γ-chain (SouthernBiotech); and rabbit anti-mouse IgG2b γ-chain (Invitrogen). Enzyme reaction was done with 3,3′,5,5′-Tetramethylbenzidine substrate (Sigma-Aldrich) and stopped with 0.5 M H₂SO₄, and absorbance was measured by plate reader (VICTOR3; Perkin Elmer) at 450 nm.

IL-6 quantification

Spleen cells (4 x 10⁵ cells in 200 µl of medium per well) from mice were measured by plate reader (VICTOR3; Perkin Elmer) at 450 nm.

Purified splenic B cells stimulated with LPS for 2 d and DCs were incubated with 50 ng/ml phorbol 12-myristate 13-acetate and 1 µM ionomycin in the presence of 10 µg/ml brefeldin A for 4 h at 37°C. After fixation and permeabilization, intracellular TNF and IFN-γ were quantified by flow cytometry.

Statistical analysis

Data from at least three independent experiments were used for statistical analysis. Statistical significance was calculated by Student’s t test. Error bars represent SEM.

Online supplemental material

Fig. S1 shows the composition of immune cells in the spleen of WT and MLII mice. Table S1 contains data of flow cytometric subset analysis of lymphocytes in MLII patients. Table S2 contains data on mutations in the GNPTAB gene; antibody responses to vaccination; and basal IgG, IgA, and IgM concentrations in serum of four MLII patients. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201407077/DC1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201407077.dv.

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