

The Bcl10–Malt1 complex segregates FcεRI-mediated nuclear factor κB activation and cytokine production from mast cell degranulation

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Mast cells are pivotal effector cells in IgE-mediated allergic inflammatory diseases. Central for mast cell activation are signals from the IgE receptor FcεRI, which induce cell degranulation with the release of preformed mediators and de novo synthesis of proinflammatory leukotrienes and cytokines. How these individual mast cell responses are differentially controlled is still unresolved. We identify B cell lymphoma 10 (Bcl10) and mucosa-associated lymphoid tissue 1 (Malt1) as novel key regulators of mast cell signaling. Mice deficient for either protein display severely impaired IgE-dependent late phase anaphylactic reactions. Mast cells from these animals neither activate nuclear factor κB (NF-κB) nor produce tumor necrosis factor α or interleukin 6 upon FcεRI ligation even though proximal signaling, degranulation, and leukotriene secretion are normal. Thus, Bcl10 and Malt1 are essential positive mediators of FcεRI-dependent mast cell activation that selectively uncouple NF-κB-induced proinflammatory cytokine production from degranulation and leukotriene synthesis.

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Abbreviations used: Bcl10, B cell lymphoma 10; BCR, B cell receptor; BMDC, bone marrow-derived mast cell; DNFB, dinitrofluorobenzene; DNP, dinitrophenyl; Erk, extracellular signal-regulated kinase; IκB, inhibitor of κB; IKK, IκB kinase; Iono, ionomycin; Jnk, c-Jun NH₂-terminal kinase; Malt1, mucosa-associated lymphoid tissue 1; MAP, mitogen-activated protein; PCA, passive cutaneous anaphylaxis; PKC, protein kinase C; SCF, stem cell factor.

Mast cells are derived from myeloid progenitor cells and widely distributed throughout vascularized tissues. They participate in innate and adaptive immune defenses against bacteria and parasites and play a key role in IgE-mediated allergic diseases such as atopy and asthma, which are responsible for increasing global health problems (1). Moreover, mast cells contribute to autoimmunity and are involved in pathological tissue remodeling processes that are associated with chronic inflammation. All these biological and pathological functions are triggered by mast cell-derived proinflammatory mediators such as histamine, arachidonic acid metabolites, and cytokines, which are released upon mast cell activation.

The major stimulus for mast cell activation is the aggregation of the high-affinity receptor

for IgE, FcεRI (2, 3). Cross-linking of FcεRI-bound IgE with multivalent antigen or allergen triggers a series of biochemical events that culminate in mast cell effector function. Signaling is initiated through the phosphorylation of immunoreceptor tyrosine-based activation motifs in the tails of the FcεRI β and γ subunits by Src family protein tyrosine kinases (2). The tyrosine-phosphorylated immunoreceptor tyrosine-based activation motifs recruit the kinase Syk, which, together with the activated receptor-proximal Src protein tyrosine kinases, mediates phosphorylation and consequent reorganization of adaptor and scaffolding proteins at the activated FcεRI complex. Collectively, early signaling induces the activation of downstream enzymes such as phosphatidylinositol 3-kinase and phospholipase C, the generation

of second messengers (e.g., inositol-1,4,5-triphosphate, 1,2-diacylglycerol, and free calcium), and activation of protein kinase C (PKC) isoforms.

Ultimately, FcεRI aggregation activates several downstream pathways that initiate the allergic inflammatory process by eliciting mast cell degranulation with a rapid release of preformed vasoactive amines such as histamine and serotonin and by triggering the de novo synthesis of proinflammatory arachidonic acid metabolites and potent cytokines like TNF-α or IL-6 (1). In addition, signals from the FcεRI activate genetic survival programs that block cell death after IgE stimulation (4, 5). Crucial for immediate-type allergic reactions is the instant degranulation, whereas mast cell-mediated late phase reactions and IgE-induced chronic allergic inflammatory processes are mainly dependent on the production of cytokines and the initiation of leukocyte effector cascades (1, 6). Major questions in mast cell biology are how early signaling events after FcεRI aggregation are integrated and how selected mast cell responses—such as the immediate degranulation or the delayed cytokine production—are individually controlled, because the identification of molecules that regulate specific mast cell effector functions selectively would provide novel targets for rational therapies of mast cell-mediated diseases (3).

NF-κB is a master transcription factor that controls the expression of proinflammatory gene products in cells of many different lineages (7). The predominant NF-κB dimer in many cell types, including mast cells, is a p50/RelA heterodimer (7, 8). The activity of NF-κB is tightly controlled by inhibitor of κB (IκB) proteins that can bind to NF-κB dimers and retain them in an inactive state in the cytoplasm. NF-κB can be activated through either the canonical or the alternative pathway (7). The canonical pathway is responsible for the activation of p50/RelA dimers and involves the activation of the multisubunit IκB kinase (IKK) that phosphorylates IκB proteins on conserved serine residues to target them to ubiquitin-dependent degradation. This process frees NF-κB and allows its translocation into the nucleus and transactivation of target genes. Many of the proinflammatory cytokine genes that are expressed in activated mast cells are regulated by NF-κB (7–14). In particular, the production of TNF-α and IL-6 in response to FcεRI ligation is strictly dependent on IKK and NF-κB activity (10, 14). Both cytokines play key roles in mast cell-mediated inflammatory responses. Yet, the signaling intermediates that connect FcεRI-proximal events to IKK activation are unknown.

Recently, the caspase recruitment domain protein B cell lymphoma 10 (Bcl10) and the paracaspase mucosa-associated lymphoid tissue 1 (Malt1) were identified as key regulators of T cell and B cell antigen receptor signaling (15). Bcl10 and Malt1 can directly bind to each other, and the two proteins cooperate in the assembly of a cellular complex that can mediate signal-specific activation of IKK. Both Bcl10 and Malt1 additionally regulate the c-Jun NH₂-terminal kinase (Jnk) and p38 mitogen-activated protein (MAP) kinase pathways in lymphocytes, and Bcl10 also has a Malt1-independent role

during neurodevelopment (16–20). Immunological functions of Bcl10 and Malt1 in nonlymphoid cells are still largely undefined, and it is as of yet unknown whether they play any role in mast cells.

In this paper, we show that Bcl10 and Malt1 are essential for normal mast cell function in vivo and in vitro. Although mice deficient for either molecule exhibit normal numbers of skin mast cells and regular IgE-mediated immediate phase anaphylactic reactions, late phase anaphylactic reactions are severely blunted in these animals. In vitro bone marrow-derived mast cells from Bcl10- or Malt1-deficient mice neither produce TNF-α nor IL-6 in response to FcεRI stimulation, whereas the capacity to produce leukotrienes and degranulation is normal. On a molecular level, we find that FcεRI-proximal signaling (the activation of the MAP kinases extracellular signal-regulated kinase [Erk], Jnk, and p38) and the activation of Akt are intact in Bcl10- or Malt1-deficient mast cells. However, NF-κB cannot be activated in response to FcεRI aggregation. Thus, we identify Bcl10 and Malt1 as crucial positive regulators of FcεRI-dependent NF-κB activation that selectively uncouple proinflammatory cytokine production from degranulation and lipid mediator synthesis.

RESULTS

Mast cell development is not affected by Bcl10 or Malt1 deficiency

To study potential roles for Bcl10 and Malt1 in mast cells, we first assessed the effects of genetic *Bcl10* or *Malt1* disruptions in mice on several aspects of mast cell development in vivo and in vitro (Fig. 1). Mast cells in the skin of WT, Bcl10-deficient (Bcl10^{-/-}), and Malt1-deficient (Malt1^{-/-}) mice were comparable in their morphology and anatomical distribution (Fig. 1 A). The frequencies of these cells in the dermis of the ear, neck, and groin were similar in all three genotypes as determined after toluidine blue staining (Fig. 1 B).

In vitro culture of WT, Bcl10^{-/-}, and Malt1^{-/-} bone marrow cell suspensions in the presence of IL-3 and stem cell factor (SCF) revealed highly pure mast cell populations in all three genotypes (bone marrow-derived mast cells [BMMCs]). These cells were indistinguishable in morphology when stained with toluidine blue or with alcian blue (unpublished data). The growth rate and total cell numbers in these cultures, as well as the frequency of BMMCs (constantly >95%) as revealed by flow cytometric analysis of the surface expression of c-kit and FcεRI, were also equal (Fig. 1 C). Importantly, the FcεRI expression level was not influenced by either the *Bcl10* or *Malt1* disruption. Western blot analysis demonstrated that WT mast cells express both Bcl10 and Malt1 (Fig. 1 D). As expected, Bcl10^{-/-} mast cells do not produce the Bcl10 protein, whereas Malt1^{-/-} mast cells do not contain the Malt1 protein. Collectively, these findings demonstrate that, although Bcl10 and Malt1 are expressed in normal mast cells, their absence affects neither skin mast cell development in vivo nor BMMC differentiation in vitro. Interestingly, the expression level of Bcl10 is reduced in

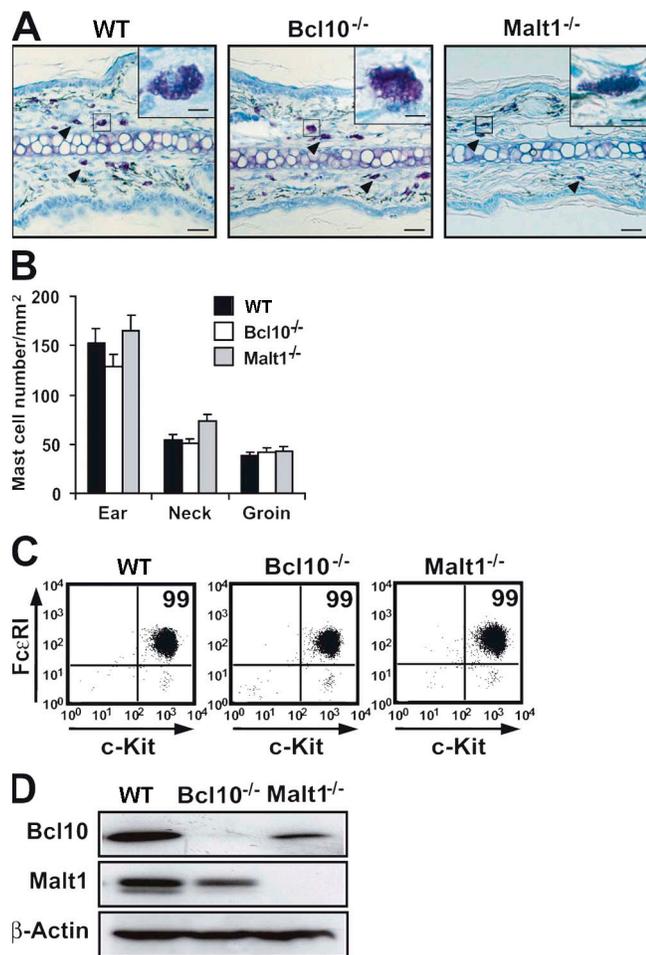


Figure 1. Mast cell development in the absence of Bcl10 or Malt1. (A and B) Skin biopsies from WT, Bcl10^{-/-}, and Malt1^{-/-} mice. (A) Representative areas from the ear skin show the presence of toluidine blue-positive mast cells in all three genotypes (arrowheads) at 20 \times . Insets at 100 \times show the boxed mast cells from each section. Bars: (full images) 10 μ m; (insets) 2 μ m. (B) Mast cell frequencies in skin sections from the ear, neck, and groin ($n = 3$ mice/genotype). There was no important difference among the three genotypes. Data are means \pm SEM. (C) Flow cytometric analysis of BMMCs from WT, Bcl10^{-/-}, and Malt1^{-/-} mice. Percentages of Fc ϵ RI and c-kit double-positive mast cells are indicated. Results are representative of five different experiments. (D) Bcl10 and Malt1 expression levels in BMMCs. Western blots of WT, Bcl10^{-/-}, and Malt1^{-/-} BMMCs with antibodies against Bcl10, Malt1, and β -actin. Results are representative of five independent experiments.

Malt1^{-/-} mast cells and the Malt1 protein concentration is smaller in Bcl10^{-/-} BMMCs compared with the WT, suggesting that cellular levels of each of the two binding partners depend on the presence of the other.

Bcl10 and Malt1 are required for normal mast cell function in vivo

To test whether Bcl10 or Malt1 play a functional role in mast cells in vivo, we performed two types of Fc ϵ RI-mediated passive cutaneous anaphylaxis (PCA) experiments (21, 22).

We addressed the immediate and the late phase PCA responses separately (Fig. 2).

For early phase PCA reactions, mice were primed by intradermal injection of monoclonal anti-dinitrophenyl (anti-DNP) IgE antibody into the ear. 24 h later, the animals were i.v. co-injected with Evans blue dye and the antigen DNP coupled to HSA (DNP-HSA). The extravasation of Evans blue dye during the first hour of the PCA reaction is mainly dependent on the degranulation of activated mast cells with rapid histamine and serotonin release resulting in locally increased blood vessel permeability (21). The Evans blue dye extravasation was monitored by inspection and quantified 60 min after antigen challenge (Fig. 2, A and B). Neither the extravasation kinetics nor the total amount of Evans blue dye in the ear were substantially different in the three genotypes, indicating that the mast cell-mediated immediate phase PCA reaction does not require Bcl10 or Malt1.

We next examined the late phase PCA response that is promoted by mast cell-derived proinflammatory cytokines (1, 23, 24). Mice were sensitized i.v. with anti-DNP IgE antibody. 24 h later, the hapten dinitrofluorobenzene (DNFB; 0.2% wt/vol) was epicutaneously applied to both sides of the ears. Although WT mice exhibited the anticipated prominent edema as early as 6 h and reached a maximum 24 h after antigen stimulation, both Bcl10^{-/-} and Malt1^{-/-} mice showed only a minor response (Fig. 2 C). Even 24 and 48 h after antigen challenge, the increase in ear thickness in the mutant animals reached <20% of that observed in WT mice. Therefore, we conclude that both Bcl10 and Malt1 are essentially required for regular IgE-dependent late phase PCA reactions in vivo.

Bcl10 and Malt1 differentially regulate Fc ϵ RI-mediated degranulation, lipid mediator secretion, and cytokine production

To provide a basis for the defective PCA reactions in Bcl10- and Malt1-deficient mice, we examined the capacities of Bcl10^{-/-}, Malt1^{-/-}, and WT mast cells to degranulate, synthesize, and secrete lipid mediators and to produce cytokines (Fig. 3 and see Fig. 4). These and all subsequent analyses were performed on BMMCs in vitro.

WT, Bcl10^{-/-}, and Malt1^{-/-} BMMCs were loaded with antigen-specific IgE and subsequently activated by Fc ϵ RI cross-linking with increasing doses of multivalent antigen to induce cell degranulation (Fig. 3 A). Mast cells release the enzyme β -hexosaminidase from intracellular granules whose activity in the culture supernatant can be quantified to determine the efficiency of mast cell degranulation (25). Consistent with the normal Evans blue dye extravasation during the immediate phase PCA reaction in vivo, mast cells derived from Bcl10- or Malt1-deficient mice released similar amounts of β -hexosaminidase as WT BMMCs in response to Fc ϵ RI ligation. We also stimulated cells with PMA together with the calcium ionophore ionomycin (Iono). These pharmacological agents bypass Fc ϵ RI-proximal signaling events and stimulate cells directly by mobilizing free calcium ions and

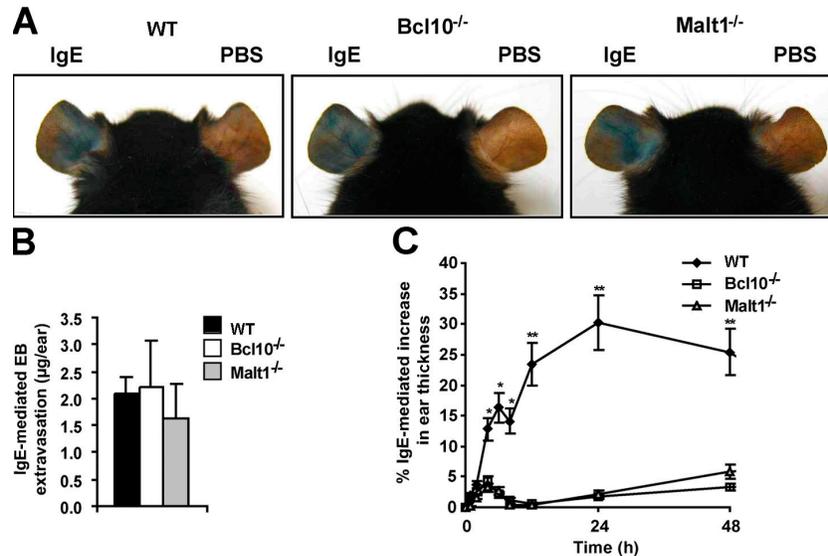


Figure 2. Normal immediate but defective late phase PCA in Bcl10^{-/-} and Malt1^{-/-} mice. (A and B) Immediate phase PCA reactions. Mice were passively sensitized by intradermal injection of anti-DNP IgE into the left ear. The right ear was injected with PBS. Subsequently, mice were challenged by i.v. injection of antigen in PBS/Evans blue. (A) Ears from WT, Bcl10^{-/-}, and Malt1^{-/-} mice 1 h after antigen challenge. (B) The IgE-mediated Evans blue extravasation was calculated as the difference in the amount of extravasated dye in the IgE-sensitized and the nonsensitized control ears. Differences between the three genotypes were not

statistically significant ($P \geq 0.05$). (C) Late phase PCA reactions. WT, Bcl10^{-/-}, and Malt1^{-/-} mice were passively sensitized by i.v. injection of anti-DNP IgE. 24 h later, they were challenged by epicuta-neous application of DNFB to both ears, and ear thickness was measured over time. The increase in ear thickness was calculated as described in Material and methods. Results are representative of three independent experiments. Data are presented as means \pm SEM. * and **, $P \leq 0.05$ and $P \leq 0.02$ between WT and Bcl10^{-/-} or WT and Malt1^{-/-} mice, respectively.

activating PKC enzymes. Again, no substantial differences in β -hexosaminidase activity were detected in the supernatants of BMMCs of the three genotypes (Fig. 3 A).

Furthermore, we investigated the production of proinflammatory lipid mediators in WT, Bcl10^{-/-}, and Malt1^{-/-} BMMCs in response to Fc ϵ RI ligation using an enzyme-linked immunoassay (Fig. 3 B). Mast cells of the three genotypes produced equal amounts of the leukotrienes C₄, D₄, and E₄ (LTC₄, LTD₄, and LTE₄). BMMC activation with PMA/Iono also induces robust leukotriene production. Again, no differences were found between WT, Bcl10^{-/-}, and Malt1^{-/-} BMMCs (Fig. 3 B). Collectively, these first sets of in vitro experiments show that the signaling proteins Bcl10 and Malt1 are dispensable for mast cell degranulation or leukotriene synthesis in response to Fc ϵ RI ligation or PMA/Iono treatment.

It is known that the late phase PCA response in vivo is at least in part induced by proinflammatory cytokines, in particular TNF- α , released from activated mast cells (1, 23, 24). Therefore, we were especially interested in measuring the transcriptional induction, production, and release of cytokines by Bcl10^{-/-} and Malt1^{-/-} mast cells in response to Fc ϵ RI stimulation in vitro. To this end, we first performed time course experiments and analyzed the concentrations of TNF- α and IL-6 in the supernatant of stimulated cells by ELISA. WT mast cells produced both mediators readily (Fig. 4 A). The maximal concentrations were detected after 2 h

of stimulation. In sharp contrast, Bcl10^{-/-} and Malt1^{-/-} BMMCs produced only minute amounts of TNF- α or IL-6 even at later time points. To further characterize the defects in TNF- α and IL-6 production in Bcl10^{-/-} and Malt1^{-/-} BMMCs, we analyzed cytokine mRNA levels before and 30 min after Fc ϵ RI ligation using semiquantitative RT-PCR. Unstimulated and stimulated populations of all three cell lines contained equal amounts of β -actin transcripts, indicating appropriate normalization (Fig. 4 B). In WT BMMCs, both TNF- α and IL-6 mRNA levels increased substantially upon Fc ϵ RI stimulation. However, in the absence of Bcl10 or Malt1 the induction of these cytokine transcripts was either absent or largely reduced compared with the WT. Thus, Bcl10 and Malt1 are critically required for Fc ϵ RI-mediated TNF- α and IL-6 gene transcription and subsequent protein production. These results indicate that the defective PCA reactions observed in Bcl10^{-/-} or Malt1^{-/-} mice in vivo might reflect the essential requirements for the two signaling proteins for Fc ϵ RI-mediated cytokine production.

Bcl10 and Malt1 are dispensable for IgE-induced mast cell survival

In lymphocytes, Bcl10 and Malt1 are not only vital for cell activation but also involved in the regulation of cell survival (15). Because recent studies demonstrated a role for Fc ϵ RI-derived signals in the control of mast cell survival (4, 5), we also studied the contributions of Bcl10 and Malt1 to this

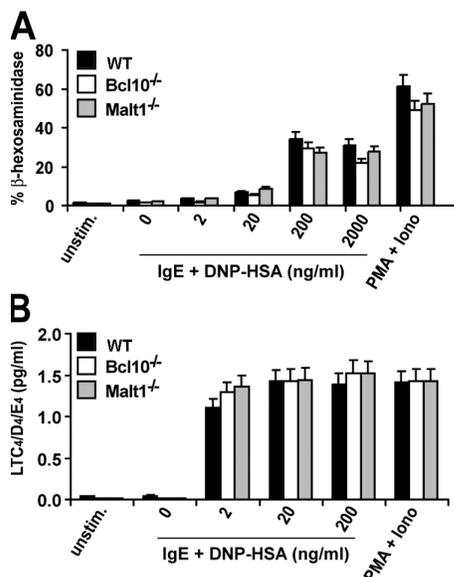


Figure 3. Normal degranulation and leukotriene synthesis in Bcl10^{-/-} and Malt1^{-/-} BMMCs. (A) Regular degranulation of Bcl10^{-/-} and Malt1^{-/-} mast cells. BMMCs from WT, Bcl10^{-/-}, and Malt1^{-/-} mice were loaded with anti-DNP IgE and subsequently stimulated for 30 min with the indicated concentrations of DNP-HSA. Alternatively, BMMCs were left unsensitized and stimulated with PMA/Iono (100 nM each) for 30 min. The extent of degranulation was determined by measuring the activity of the granular enzyme β -hexosaminidase in supernatants and cell lysates and calculated as described in Material and methods. Data are means \pm SEM from triplicate samples and are representative of four independent experiments. (B) Regular leukotriene synthesis in Bcl10^{-/-} and Malt1^{-/-} mast cells. Anti-DNP IgE-sensitized BMMCs from all three genotypes were stimulated with DNP-HSA at the indicated concentrations, or BMMCs were left unsensitized and stimulated with PMA/Iono (100 nM each). 60 min later, the supernatants of the stimulated cells were collected and assayed for the concentrations of LTC₄, LTD₄, and LTE₄. Data are means \pm SEM from triplicate samples and representative of four independent experiments.

pathway (Fig. 5). BMMCs of WT, Bcl10^{-/-}, and Malt1^{-/-} mice were incubated for 4 d in media with low concentrations of FCS without IL-3 and SCF. This growth factor withdrawal induced apoptotic death of WT, Bcl10^{-/-}, and Malt1^{-/-} BMMCs. The frequency of apoptotic cells was independent of the presence of Bcl10 or Malt1 as determined by flow cytometry after annexin V/propidium iodide staining. (Fig. 5 A). Stimulation of cells with IgE alone or in combination with antigen (unpublished data) rescued cell death and up-regulated the expression of the prosurvival protein Bcl-X_L in mast cells of all three genotypes similarly (Fig. 5 B). We thus conclude that Bcl10 and Malt1 are not essential for the Fc ϵ RI-controlled survival pathway.

Bcl10 and Malt1 are essential regulators of Fc ϵ RI-controlled NF- κ B activation

To define the molecular mechanisms responsible for the defective cytokine gene expression in Bcl10^{-/-} and Malt1^{-/-}

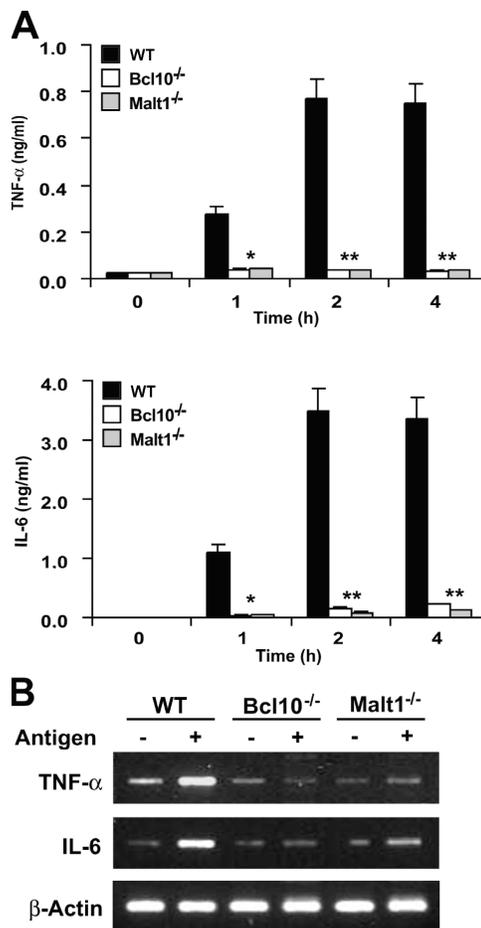


Figure 4. Defective cytokine production in Bcl10^{-/-} and Malt1^{-/-} BMMCs. (A) Impaired cytokine secretion in Bcl10^{-/-} and Malt1^{-/-} mast cells. WT, Bcl10^{-/-}, and Malt1^{-/-} BMMCs were loaded with anti-DNP IgE and stimulated with DNP-HSA for the indicated intervals. Supernatants were collected, and TNF- α and IL-6 protein concentrations were determined by ELISA. Data are means \pm SEM from triplicate samples and representative of at least five independent experiments. * and **, $P \leq 0.05$ and $P \leq 0.01$ between WT and Bcl10^{-/-} or WT and Malt1^{-/-} BMMCs, respectively. (B) Defective expression of cytokine mRNAs in Bcl10^{-/-} and Malt1^{-/-} mast cells. BMMCs from WT, Bcl10^{-/-}, and Malt1^{-/-} mice were stimulated for 30 min as in A. RNA was purified, and TNF- α , IL-6, and β -actin mRNAs were detected by semiquantitative RT-PCR using specific primers. Data are representative of three independent experiments.

deficient mast cells, we analyzed the signaling pathways downstream of Fc ϵ RI stimulation (Fig. 6 and see Fig. 7).

Ligation of IgE-occupied Fc ϵ RI molecules with antigen results in the rapid activation of receptor-proximal tyrosine kinases, which is a prerequisite for all mast cell effector functions (2). Consistent with the normal degranulation and lipid mediator synthesis in Bcl10^{-/-} and Malt1^{-/-} mast cells, receptor-proximal signaling does not involve Bcl10 or Malt1 because the tyrosine phosphorylation patterns induced by Fc ϵ RI stimulation did not differ in kinetics or intensity among BMMCs of the three genotypes (Fig. 6 A).

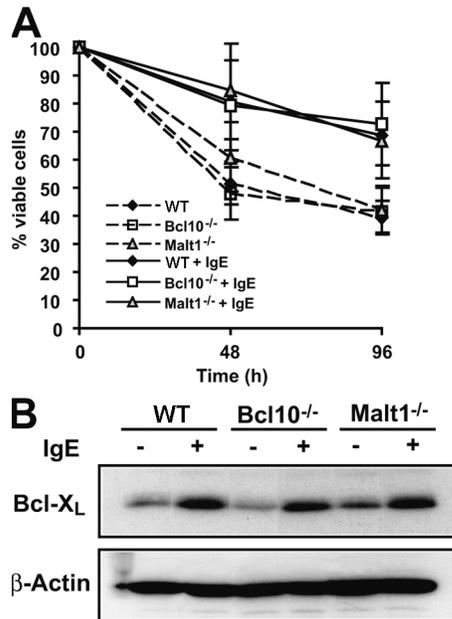


Figure 5. Regular IgE-induced survival signaling in the absence of Bcl10 or Malt1. (A) BMMCs from all three genotypes were deprived of the mast cell survival cytokines IL-3 and SCF and cultured in media with low concentrations of FCS (1%) with or without 5 μ g/ml anti-DNP IgE mAb. 48 and 96 h later, apoptotic cell death was determined by flow cytometry after annexin V/propidium iodide staining. The percentages of viable mast cells are shown. Data are means \pm SEM from triplicate samples and are representative of four independent experiments. No important differences were detected among the three genotypes. (B) BMMCs were cultured as in A and left unstimulated or stimulated for 24 h with 5 μ g/ml anti-DNP IgE. Total cell lysates were resolved by SDS-PAGE and immunoblotted with anti-Bcl-X_L antibody and, after stripping, reprobed with anti- β -actin antibody to control for equal loading.

Fc ϵ RI-proximal signaling activates the Erk, Jnk, and p38 pathways, as well as the phosphatidylinositol 3-kinase pathway that results in the activation of the serine kinase Akt. These cascades regulate both cytokine production and generation of arachidonic acid metabolites upon Fc ϵ RI ligation (1, 2). Bcl10 and Malt1 have been implicated in the regulation of Jnk and p38 signaling in lymphocytes (18, 19). However, using immunoblotting with activation state-specific phosphoantibodies against Jnk, p38, the p42 and p44 MAP kinases Erk1 and Erk2, or Akt, we could not detect differences in the Fc ϵ RI-induced activation of any of these pathways among WT, Bcl10^{-/-}, and Malt1^{-/-} mast cells (Fig. 6 B). We additionally activated the cells with PMA/Iono and again detected similar activation for Jnk, p38, Erk1, Erk2, and Akt in the presence or absence of either Bcl10 or Malt1 (Fig. 6 C).

As earlier mentioned, the expression of TNF- α and IL-6 critically depends on the activation of NF- κ B. Based on known functions of Bcl10 and Malt1 in lymphocytes and their newly discovered requirement in mast cells, we reasoned that the two proteins might be involved in Fc ϵ RI-

mediated NF- κ B activation. To test this hypothesis, we analyzed I κ B- α phosphorylation and its subsequent degradation in Fc ϵ RI-stimulated BMMCs of the three genotypes. Fc ϵ RI signaling in WT cells induced rapid I κ B- α phosphorylation and, consequently, its degradation (Fig. 7 A). In contrast, signaling to I κ B- α was completely abolished in Bcl10^{-/-} or in Malt1^{-/-} mast cells. Direct PKC activation via PMA/Iono stimulation also resulted in I κ B- α phosphorylation and degradation only in the WT cells but not in BMMCs deficient for either Bcl10 or Malt1, indicating a requirement for Bcl10 and Malt1 downstream of PKC activation.

To directly investigate NF- κ B nuclear translocation and DNA binding, we performed gel shift assays with nuclear extracts from stimulated mast cells. Consistent with the marked impairment of I κ B- α phosphorylation and degradation in Bcl10^{-/-} and Malt1^{-/-} BMMCs, NF- κ B was only activated in WT mast cells but not in those deficient for Bcl10 or Malt1 (Fig. 7 B). Thus, Bcl10 and Malt1 are both essential to specifically couple Fc ϵ RI and PKC signaling to the activation of the NF- κ B pathway in mast cells.

DISCUSSION

In this paper, we have demonstrated on a genetic basis that Bcl10 and Malt1 are essential and nonredundant positive regulators of mast cell activation and effector function. Although mast cells seem to differentiate and survive normally in the absence of either Bcl10 or Malt1, both proteins are critically required for NF- κ B activation induced by the high-affinity IgE receptor Fc ϵ RI. Bcl10- or Malt1-deficient mast cells do not produce the NF- κ B-regulated proinflammatory cytokines TNF- α and IL-6 and, consistently, late phase PCA reactions are severely impaired in Bcl10- or Malt1-deficient mice in vivo.

Activation of mast cells through the Fc ϵ RI initiates a cascade of events that lead to degranulation, production of lipid mediators such as leukotrienes, and transcriptional up-regulation and release of proinflammatory cytokines such as TNF- α or IL-6. These all represent normal mast cell functions that, depending on the context, may be beneficial or harmful in the setting of innate or allergic immune responses. Bcl10 and Malt1 are not involved in Fc ϵ RI-proximal signaling events that are known to lead to the activation of PKC enzymes (2). The PKC family consists of at least 10 serine/threonine kinases, and previous studies have indicated that PKC activation is involved in all aspects of mast cell effector functions (26). Although comprehensive studies of the individual roles of all PKC isoforms in mast cell biology are missing, chemical or genetic inhibition of the PMA- and calcium-sensitive conventional isoforms PKC α and PKC β identified these kinases as essential inducers of degranulation and cytokine and leukotriene production (27–29). The PMA-sensitive but calcium-insensitive isoform PKC δ is involved in the activation of leukotriene synthesis (30) and can simultaneously function as a negative regulator of antigen-induced degranulation (31).

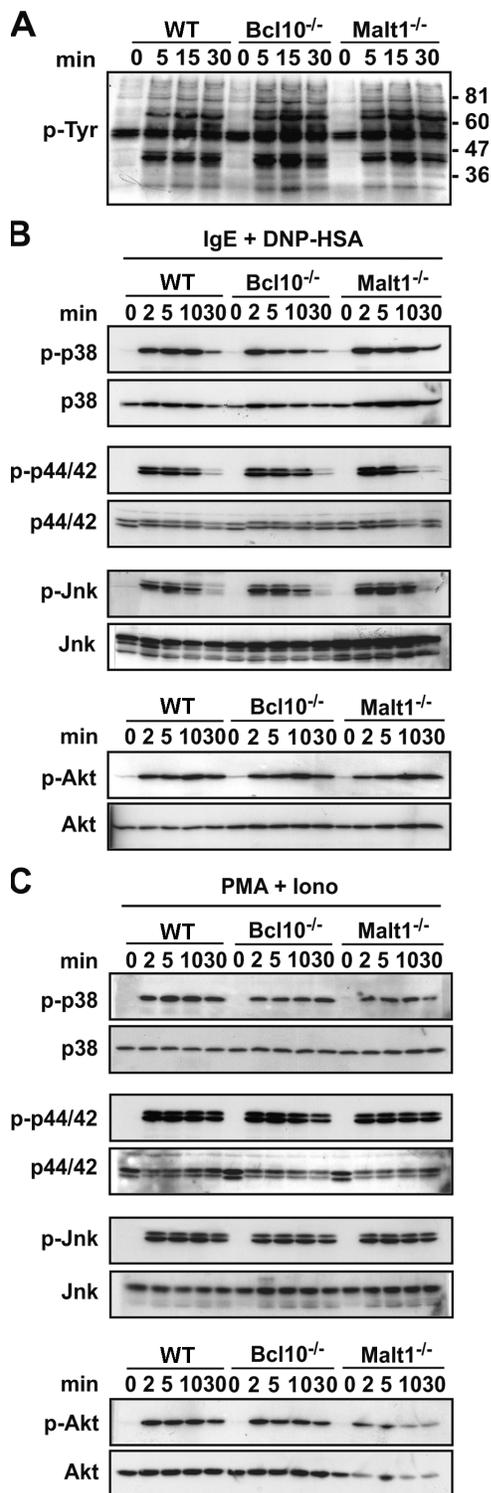


Figure 6. Normal Fc ϵ RI proximal signaling and regular p38, Erk, Jnk, and Akt activation in Bcl10 $^{-/-}$ and Malt1 $^{-/-}$ mast cells. (A and B) WT, Bcl10 $^{-/-}$, and Malt1 $^{-/-}$ BMMCs sensitized with anti-DNP IgE were stimulated with DNP-HSA for the indicated time intervals (in minutes) as described in Material and methods. Total cell lysates were separated by SDS-PAGE (2×10^5 cell equivalents). (A) Immunoblot with anti-phosphotyrosine antibodies. Molecular mass (kD) markers

In this paper, we show that Fc ϵ RI- and PMA/Iono-induced I κ B- α phosphorylation, degradation, and NF- κ B activation are completely abolished in Bcl10- or Malt1-deficient mast cells. These results reveal an essential requirement for Bcl10-Malt1 signaling downstream of the Fc ϵ RI and downstream of all PMA-responsive PKCs in mast cells. Importantly, however, Fc ϵ RI- or PMA/Iono-induced activation of the Jnk, p38, Erk, and Akt pathways are intact in Bcl10 $^{-/-}$ and Malt1 $^{-/-}$ mast cells, indicating that the Bcl10-Malt1 complex does not affect all PKC downstream pathways. Consistent with the regular activation of selected Fc ϵ RI or PKC effector signals, we observed normal mast cell survival, degranulation, and leukotriene production in the absence of Bcl10 or Malt1. The observation that TNF- α and IL-6 transcription and protein production are severely impaired in Bcl10- or Malt1-deficient mast cells is in agreement with our findings that Bcl10-Malt1 specifically controls Fc ϵ RI-induced NF- κ B activation and the known role of NF- κ B for cytokine gene expression. Considering all in vitro results collectively, we propose a model for the molecular functions of Bcl10 and Malt1 in mast cell signaling that is depicted in Fig. 7 C. This signaling model is consistent with the in vivo observations in Bcl10- and Malt1-deficient mice, as immediate phase PCA reactions that depend on mast cell degranulation (21) are normal, whereas late phase reactions that are at least partially dependent on transcriptional induction and the release of mast cell-derived cytokines (1, 24) are severely impaired in the absence of either Bcl10 or Malt1. However, because in the mutant animals all cells lack Bcl10 or Malt1, signaling deficiencies in other cells besides mast cells could additionally contribute to the defective late phase PCA response.

Experiments with genetically altered mice have recently elucidated many aspects of positive and negative regulation of Fc ϵ RI-induced mast cell activation (1). Collectively, these findings demonstrate that antigen receptor-specific molecules required for Fc ϵ RI signaling generally affect several aspects of mast cell function simultaneously. In contrast, we identified the Bcl10-Malt1 signaling complex as a molecular structure that selectively regulates one downstream effector arm of Fc ϵ RI, namely NF- κ B-mediated cytokine production, but does not affect mast cell survival, degranulation, or the synthesis of leukotrienes.

The conclusion that the Bcl10-Malt1 complex operates selectively downstream of PKCs in Fc ϵ RI signaling to NF- κ B

are shown on the left. Data are representative of three independent experiments. (B) Immunoblot with anti-phospho-p38, anti-phospho-p44/42, anti-phospho-Jnk, and anti-phospho-Akt. After stripping, membranes were reprobed with anti-p38, anti-p44/p42, anti-Jnk, and anti-Akt antibody, respectively. Data are representative of at least three independent experiments. (C) Unsensitized WT, Bcl10 $^{-/-}$, and Malt1 $^{-/-}$ BMMCs were stimulated with PMA/Iono for the indicated time intervals (in minutes) as described in Material and methods and immunoblotted as in B. Results are representative of three independent experiments.

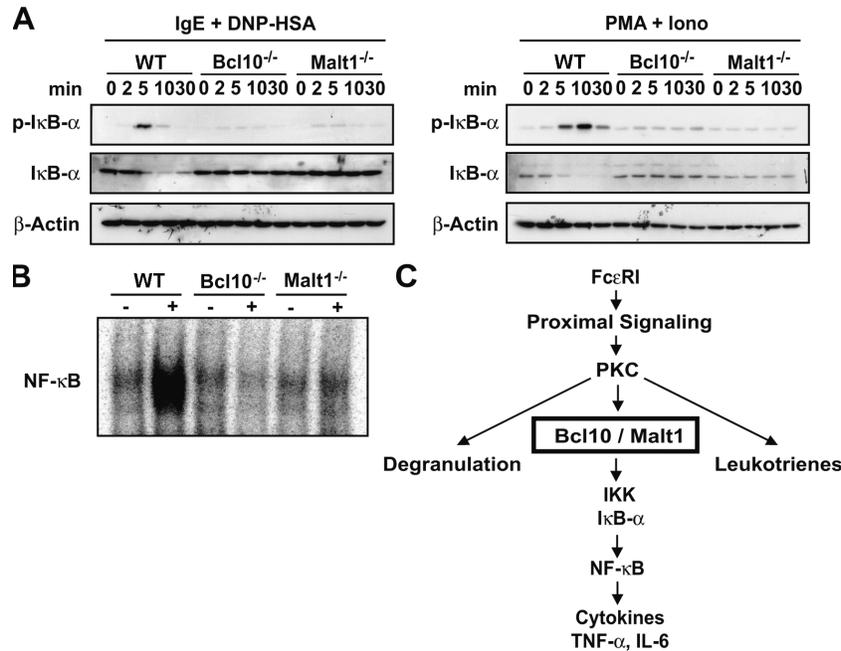


Figure 7. Bcl10 and Malt1 are required for NF-κB signaling in mast cells. (A) Defective IκB-α phosphorylation and degradation after FcεRI ligation or PMA/Iono treatment in Bcl10^{-/-} or Malt1^{-/-} mast cells. BMMCs from all genotypes were sensitized with anti-DNP IgE and stimulated with DNP-HSA for the indicated time intervals (in minutes). Alternatively, cells were left unsensitized and stimulated with PMA/Iono (100 nM each). IκB-α phosphorylation and degradation were determined by Western blotting. Membranes were reprobed with anti-β-actin antibody to control for equal loading. Data are representative of at least four independent experiments. (B) Defective NF-κB activation in Bcl10^{-/-} and Malt1^{-/-} mast cells. BMMCs were left unstimulated or stimulated with

PMA/Iono (100 nM each) for 60 min. Subsequently, nuclear extracts were prepared and subjected to gel mobility shift assays using a radiolabeled probe containing NF-κB binding site sequences. Results are representative of three independent experiments. (C) A model for the role of Bcl10 and Malt1 in FcεRI-mediated signal transduction after FcεRI receptor stimulation, Bcl10 and Malt1 operate downstream of PKCs and serve as selective signal transducers that activate the IκB-dependent NF-κB pathway. The Bcl10-Malt1 complex selectively controls proinflammatory cytokine production but does not regulate mast cell degranulation or leukotriene synthesis. For details, see Discussion.

provides novel insights into context-specific immune regulation. The FcεRI on mast cells belongs to the family of multisubunit immunoreceptors that also includes the TCR and the B cell receptor (BCR), but numerous genetic studies have demonstrated that each of these receptors uses unique sets of downstream molecules to differentially mediate cell activation. For example, the FcεRI requires the tyrosine kinases Syk and Btk and the adaptor molecule linker for activation of T cells to mediate mast cell activation (32–34). Syk and Btk are additionally required for BCR signaling but are completely dispensable for T cell activation, whereas the linker for activation of T cells is essential for TCR signaling but has no role in B cells (35–37). The Bcl10-Malt1 complex is also known to be differentially used by immunoreceptors. Bcl10 can bind to Carma molecules and recruit Malt1 and various TNF receptor-associated factor molecules and kinases such as TAK1 into signaling complexes to mediate downstream activation of NF-κB, Jnk, and p38 (15, 19, 38–40). TCR signaling to NF-κB critically depends on Bcl10, Malt1, and the kinase TAK1 (39). However, Malt1 is partially expendable for BCR signaling to NF-κB (18), and TAK1 is completely dispensable for BCR-

induced NF-κB activation but selectively controls p38 MAP kinase signaling in response to BCR ligation (40). Our findings that FcεRI signaling requires both Bcl10 and Malt1 for NF-κB control but not for Jnk or p38 activation extend this idea of receptor-specific utilization of the signaling module and reinforces the necessity to investigate the precise function of each signal transducer in distinct cell lineages. So far there are no other reports available that explore roles of Bcl10, Malt1, or any of their binding partners in FcεRI signaling. It will thus be additionally important to study mast cells in mice deficient in Carma proteins, TNF receptor-associated factor molecules, and MAP kinase-activating kinases, including TAK1, to understand precisely how the FcεRI differentially couples to NF-κB and Jnk/p38 downstream signaling. These studies could also reveal the Bcl10-Malt1-independent mechanisms of Jnk and p38 activation and give further insights into the selective control of distinct mast cell effector functions.

Recent biochemical studies have shown that Bcl10-Malt1 signaling to IKK and NF-κB involves the oligomerization of Bcl10 and Malt1 and subsequent Malt1-dependent regulatory lysine-63-linked ubiquitinylation of IKKγ/NEMO,

which induces IKK activity (39, 41). These findings open the possibility that Bcl10–Malt1 signaling could potentially be inhibited by targeted drugs because numerous kinase inhibitors and many compounds that target the ubiquitin/proteasome system are in preclinical or clinical development. Mast cell–derived TNF- α recruits and activates neutrophils and lymphocytes at sites of inflammation and has been implicated in tissue remodeling processes, angiogenesis, and fibrosis that can be so prominent in IgE-associated chronic allergic diseases (1, 23, 42, 43). Consequently, there is a tremendous interest in inhibiting TNF- α production in allergic inflammation. In this paper, we demonstrate that a complete genetic blockage of Bcl10–Malt1 signaling thoroughly abolishes Fc ϵ RI-specific NF- κ B activation and TNF- α and IL-6 production in mast cells. Thus, we suggest that specific therapeutic targeting of Bcl10–Malt1 signaling could potentially contribute to the therapy of mast cell–mediated inflammatory diseases without the toxic side effects that can be expected from general NF- κ B inhibition.

MATERIALS AND METHODS

Mice. The generation of Bcl10- and Malt1-deficient mice was reported previously (17, 18). Mice were housed under specific pathogen-free conditions. The study was conducted under federal guidelines for the use and care of laboratory animals and was approved by the Government of the District of Upper Bavaria. Mice were of a C57BL/6 and 129/J mixed background. Littermates were used in each experiment.

Skin histology. Naive animals at 10–12 wk of age were killed, and skin samples from the ear, neck, and groin regions were removed and fixed in 4% formalin. 4- μ m tissue sections were stained with toluidine blue, and mast cells were counted in the dermis of each sample at 40 \times . 10 fields at 10 \times (per mouse and location) were used to delineate and integrate the dermal area (KS 300 software; Carl Zeiss MicroImaging, Inc.) that was analyzed.

BMMC culture and FACS analysis. Bone marrow cells were cultured in suspension in RPMI 1640 with 20% FCS, 30 ng/ml murine IL-3 (R&D Systems), 50 ng/ml murine SCF (R&D Systems), and 150 μ M monothio-glycerol (Sigma-Aldrich). To quantify BMMC frequencies, cells were stained with FITC-labeled anti-Fc ϵ RI and PE-labeled anti-c-kit antibody (eBioscience) and analyzed by flow cytometry (FACScan; Becton Dickinson).

PCA. For immediate phase PCA reactions, mice were passively sensitized by intradermal injection of 250 ng anti-DNP IgE mAb (clone H1- ϵ -26; provided by F.-T. Liu [University of California, Davis, Sacramento, CA] and D.H. Katz [Avanir Pharmaceuticals, San Diego, CA]) into one ear and PBS injection into the contralateral ear. 24 h later, mice were challenged by i.v. injection of 150 μ g of DNP–HSA (30–40 moles DNP/mol HSA; Sigma-Aldrich) in 100 μ l Evans blue dye (0.5%; Sigma-Aldrich). To determine the amount of extravasated dye, mice were killed, ears were removed, and Evans blue dye was extracted with potassium hydroxide and photometrically quantified as described previously (22).

For late phase PCA reactions, mice were passively sensitized by i.v. injection of 2 μ g anti-DNP IgE mAb (clone H1- ϵ -26) or left unsensitized. 24 h later, sensitized and nonsensitized mice were challenged by epicutaneous application of 10 μ l of DNFB (0.2% wt/vol) in acetone/olive oil (4:1) to both sides of both ears. The ear swelling response was assessed by measuring the ear thickness using an engineer's micrometer dial thickness gauge. The increment of ear thickness (postchallenge value – prechallenge baseline value) was expressed as the percentage of the baseline value obtained before antigen challenge.

Mast cell degranulation. To induce degranulation, 2×10^6 BMDCs/ml were loaded with 5 μ g/ml anti-DNP IgE mAb (SPE-7; Sigma-Aldrich) for 1 h on ice in Tyrode's buffer (10 mM Hepes, pH 7.4, 130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, and 0.1% BSA). After washing, sensitized cells were stimulated with the concentrations of DNP–HSA indicated in the figures. Alternatively, cells were left unsensitized and stimulated with 100 nM PMA (Sigma-Aldrich) and 100 nM Iono (Sigma-Aldrich) in Tyrode's buffer. The enzymatic activities of β -hexosaminidase in supernatants and cell pellets solubilized with 0.5% Triton X-100 in Tyrode's Buffer were measured with *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide (Sigma-Aldrich), and the percentage of degranulation was calculated as previously described (25).

Measurements of leukotrienes and cytokines. 2×10^6 BMDCs/ml were loaded with 0.5 μ g/ml anti-DNP IgE mAb (SPE-7) overnight, washed twice, and stimulated with DNP–HSA or left unsensitized and stimulated with PMA and Iono (100 nM each) as indicated in the figures. Cell supernatants were harvested, and leukotrienes LTC₄, LTD₄, and LTE₄ were determined by enzyme-linked immunoassay (GE Healthcare), whereas IL-6 and TNF- α were determined by ELISA DuoSets (R&D Systems), as recommended by the manufacturer.

RT-PCR. 2×10^6 BMDCs/ml were loaded with 0.5 μ g/ml anti-DNP IgE mAb (SPE-7) overnight, washed twice, and stimulated with 20 ng/ml DNP–HSA for 30 min. Total RNA was isolated using TRIzol reagent (Invitrogen), according to the manufacturer's instructions, and reverse transcribed using the Superscript first-strand synthesis system (Invitrogen). Primers and PCR conditions were described elsewhere (25).

Survival assay. BMDCs were cultured as indicated in the figures. Cell viability was quantified by flow cytometry after annexin V/propidium iodide staining as recommended by the manufacturer (BD Biosciences).

Signal transduction. For Fc ϵ RI signaling, 2×10^6 BMDCs/ml were preloaded with 0.5 μ g/ml anti-DNP IgE mAb (SPE-7) and subsequently activated by adding 20 ng/ml DNP–HSA, as indicated in the figures. Alternatively, BMDCs were left unsensitized and stimulated with PMA and Iono (100 nM each) or with 5 μ g/ml anti-DNP IgE mAb for Bcl-X_L induction. Cells were lysed, and denatured proteins were separated on 10% polyacrylamide gels and subjected to immunoblotting using antibodies against phosphotyrosine (PY99; Santa Cruz Biotechnology, Inc.), phospho-p38, p38, phospho-p44/42, p44/42, phospho-Jnk, Jnk, phospho-Akt, Akt, phospho-I κ B- α , I κ B- α , Bcl-X_L (all from Cell Signaling), or β -actin (Sigma-Aldrich). For electromobility shift assays, 10^7 BMDCs/sample were stimulated with PMA/Iono, and gel shifts were performed as described previously (17).

Statistical analysis. Results were analyzed for statistical significance with the unpaired two-tailed Student's *t* test (Excel; Microsoft). Data from Bcl10^{-/-} and Malt1^{-/-} mice or BMDCs were separately compared with the WT. Differences between groups were considered significant at $P \leq 0.05$.

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