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Bcl10/Malt1 Signaling Is Essential for TCR-Induced NF-κB Activation in Thymocytes but Dispensable for Positive or Negative Selection[1]

Philipp J. Jost,* Stephanie Weiss,* Uta Ferch,* Olaf Gross,* Tak W. Mak,† Christian Peschel,* and Jürgen Ruland[2]*

During T cell development in the thymus, high-affinity/avidity TCR engagement induces negative selection by apoptosis, while lower affinity/avidity TCR interactions lead to positive selection and survival of thymocytes. Yet, the mechanisms that discriminate between positive and negative selection are not fully understood. One major regulator of survival and apoptosis in lymphoid cells is the transcription factor NF-κB. Several reports have indicated key roles for NF-κB in positive and negative selection. In peripheral T cells, TCR ligation activates NF-κB through a selective pathway that involves protein kinase Cθ, Bcl10, and Malt1. While protein kinase Cθ is dispensable for thymic TCR signaling, the molecular roles of Bcl10 and Malt1 in thymocytes have not been investigated. In the present study, we show that both Bcl10 and Malt1 are essential for TCR signaling in thymocytes as a genetic disruption of either molecule blocks TCR-induced NF-κB activation in these cells. To investigate the function of this pathway in thymic selection, we introduced the Bcl10 or Malt1 mutations into three well-established TCR transgenic mouse models. Surprisingly, using several in vivo or in vitro assays, we were unable to demonstrate a role for TCR-induced NF-κB activation in either positive or negative selection. Thus, while TCR signaling to NF-κB controls the activation of mature T cells, we suggest that this pathway is not involved in the positive or negative selection of thymocytes. ***The Journal of Immunology,*** **2007, 178: 953–960.**

The development of T cells in the thymus is characterized by a series of cellular differentiation stages that are distinguishable by the surface expression of CD4 and CD8 coreceptor molecules (1). After a successful rearrangement of TCR α- and β-chain genes, immature double-negative (DN)[3] $CD4^-CD8^-$ thymocytes differentiate into αβ TCR expressing double-positive (DP) $CD4^+CD8^+$ cells, which represent the major adult thymocyte population. Before these DP cells further differentiate into single-positive (SP) $CD4^+$ or $CD8^+$ T cells, they undergo two critical selection steps, which ensure the generation of a self-restricted and self-tolerant peripheral TCR repertoire. DP cells that recognize self-peptides in the context of MHC molecules with high affinity are deleted by apoptosis in a process termed "negative selection" (2). DP cells that recognize self-peptides plus MHC with lower affinity receive a survival signal and differentiate into SP cells in a process termed "positive selection." Paradoxically these opposing thymocyte fates are both triggered by the same CD3/TCR complex. It remains poorly understood which selected CD3/TCR induced downstream pathways discriminate between thymocyte survival/differentiation vs apoptosis/clonal deletion during T cell selection, although these signals are frequently controlled by the same molecules, which later in the life of a T lymphocyte mediate activation and clonal expansion.

One key transcription factor that controls survival vs death decisions in many cell lineages including developing T cells is NF-κB (3). NF-κB is a small family of dimerizing proteins, known as REL family, that consists of Rel-A, Rel-B, cRel, NF-κB1/p50 and NF-κB2/p52. The family members Rel-A, cRel, and p50 are all expressed in DP and in SP thymocytes (4, 5). In nonstimulated cells, NF-κB dimers are retained in an inactive form in the cytoplasm by binding to inhibitory IκB proteins. A wide variety of signals can readily activate NF-κB through distinct upstream pathways (6). Most pathways for NF-κB activation converge on the IκB kinase (IKK), a multiprotein complex that is composed of a regulatory subunit, IKKγ (also known as NEMO), and two catalytic subunits, IKKα and IKKβ. Upon stimulation, IKK phosphorylates IκB proteins on two conserved serine residues to target them for polyubiquitinylation and subsequent proteolytic degradation. These events release NF-κB dimers from IκB molecules allowing them to enter the nucleus and to bind to cognate DNA binding-sites, where gene transcription is initiated.

TCR and pre-TCR signaling in thymocytes induce NF-κB activation (7, 8) and various in vitro and in vivo studies have indicated essential roles for NF-κB activation in either positive or negative thymic selection (7–12). In one in vitro selection model NF-κB activation was blocked with a mutated degradation-resistant IκB-α superinhibitor protein, which thoroughly inhibits TCR-induced differentiation of a $CD4^+CD8^+$ DP thymocyte cell line into $CD4^+$

*Third Medical Department, Technical University of Munich, Klinikum rechts der Isar, Munich, Germany; and †Campbell Family Institute for Breast Cancer Research and Ontario Cancer Institute, University Health Network, University of Toronto, Toronto, Ontario, Canada

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[2] Address correspondence and reprint requests to Dr. Jürgen Ruland, Third Medical Department of Hematology and Oncology, Klinikum rechts der Isar, Technical University of Munich, Ismaninger Strasse 22, 81675 Munich, Germany. E-mail address: jruland@lrz.tum.de

[3] Abbreviations used in this paper: DN, double negative; DP, double positive; SP, single positive; IKK, IκB kinase; PKC, protein kinase C; Iono, ionophore; 7-AAD, 7-aminoactinomycin D.

SP cells (13). This report suggested a function for NF-κB in positive selection at least for the CD4 lineage. Additional support for a role of NF-κB in positive selection came from in vivo studies with transgenic mice that express an IκB superinhibitor selectively in the T cell lineage (9). However, in these settings NF-κB inhibition selectively blocked the positive selection of $CD8^+$ thymocytes but did not affect the development of $CD4^+$ cells (9). Other reports in contrast indicated essential roles for NF-κB activation in negative selection. Hettmann et al. (10) demonstrated that blocking NF-κB induction makes DP thymocytes resistant to anti-CD3-mediated apoptosis both in vivo and in fetal thymic organ cultures in vitro, suggesting that NF-κB activation is critical for clonal deletion during negative selection. Independent in vivo findings support this hypothesis because transgenic inhibition of NF-κB activation also interferes with negative selection induced by endogenous MHC ligands in TCR transgenic systems, which were either MHC class I and class II restricted (11). Finally, transgenic expression of DN IKKβ inhibits anti-CD3-mediated apoptosis of DN thymocytes in vivo whereas DN IKKα makes these cells more sensitive to TCR-induced cell death (8). This particular study suggested a differential requirement of IKKα and IKKβ in the regulation of thymocyte survival and apoptosis. Together all these reports indicate important roles for NF-κB signaling in thymic selection. However, all conclusions are based on the use of artificial DN signaling molecules that do not specifically block NF-κB activation in response to TCR ligation. Since the use of various DN molecules resulted in differential effects on either positive or negative selection, the specific contribution of TCR-induced NF-κB signaling to selection is still unclear.

Lately, much has been learned about the signal transduction pathway that connects TCR ligation in peripheral T cells to IKK mediated NF-κB activation (14). After TCR proximal signaling, protein kinase C (PKC)θ phosphorylates the scaffold molecule Carma1 (15, 16), which then recruits Bcl10 and Malt1 to mediate activation of IKK. Genetic disruptions of either PKCθ, Carma1, Bcl10, or Malt1 result in identical defects in peripheral T cells with a complete blockage of TCR-mediated NF-κB induction and severe defects in TCR-induced cell proliferation, cytokine production and effector function (17–23). However, this pathway is not conserved between peripheral T cells and thymocytes as the deletion of PKCθ selectively affects TCR-induced NF-κB activation only in the periphery but not in the thymus (22). The roles of Bcl10 and Malt1 in thymic TCR signaling and thymocyte selection are still undefined, although both proteins are required for normal thymocyte development (19, 20).

In the present study, we investigate the functions of Bcl10 and Malt1 in TCR signaling in the thymus. We report that both molecules are essential for TCR-mediated NF-κB activation in DP and in SP thymocytes. To study the role of this pathway in thymic selection, we introduced three transgenic TCR mouse models into the Bcl10- or Malt1-deficient backgrounds and performed in vivo and in vitro assays for thymic selection. Surprisingly, we were unable to demonstrate a critical role for Bcl10/Malt1 signaling in either positive or negative selection. Thus, we conclude that while TCR signaling to NF-κB is essential for the activation of peripheral T cells, this pathway is not critically involved in the selection of thymocytes.

Materials and Methods

Mice

Bcl10-deficient ($Bcl10^{-/-}$) and Malt1-deficient ($Malt1^{-/-}$) mice were reported previously (20, 21). These mice were crossed with mice bearing a transgenic MHC class I restricted TCR specific for the male H-Y Ag peptide (H-Y mice) (24) or with mice expressing the transgenic P14 TCR, which binds to the immunodominate peptide of lymphocytic choriomeningitis virus glycoprotein p33 in context with MHC class I molecules (25) or with mice expressing the transgenic OT-2 TCR, that recognizes the peptide 323–339 of OVA in the context of MHC class II molecules (26). Mice were of a B6/129J mixed genetic background and were kept under specific pathogen-free conditions according to animal experimental ethics committee guidelines and used for experiments 6–8 wk after birth. Littermates were used as controls in all experiments.

Gel shift assays

For EMSAs, cells were stimulated with mAbs against CD3 (BD Pharmingen) with or without anti-CD28 (BD Pharmingen), PMA (Sigma-Aldrich) plus calcium ionophore (Iono) (Sigma-Aldrich), TNF-α (R&D Systems), or medium alone (Invitrogen Life Technologies). Nuclear protein extracts from unstimulated and stimulated cells (10^6 cells/lane) were prepared as described (20). Extracts (4 μg of protein) were incubated in 20 μl of binding buffer with radiolabeled, dsDNA probes containing NF-κB binding sites (5′-ATCAGG GACTTTCCGCTGGGGACTTTCCG-3′) and fractionated on a 5% polyacrylamide gel. Binding buffer contains 5 mM HEPES (pH 7.8), 50 mM KCL, 0.5 mM DTT, 2 μg of poly(deoxyinosinic-deoxycytidylic acid), and 10% glycerol.

Western blots

Protein lysates from thymocytes that were stimulated with PMA (Sigma-Aldrich) plus calcium Iono (Sigma-Aldrich) or TNF-α (R&D Systems) were subjected to Western blotting using Abs against IκB-α and β-actin as described previously (20).

Immunofluorescence staining and flow cytometry analysis

Thymocytes, splenocytes, and lymph node cells were stained with surface marker-specific mAb, including anti-CD3, anti-Vα5.1/5.2, anti-CD4, anti-CD8, anti-Vα2, T3.70, anti-Vβ8, or with 7-aminoactinomycin D (7-AAD) to determine cell viability (all BD Biosciences). For peptide induced IκB-α degradation OT-2 TCR transgenic thymocytes were cocultured with 0,3 mM OVA-peptide pulsed EL-4 cells as APCs for 4 h in the presence of 5 μg/ml cycloheximide to prevent novel protein synthesis. After cell surface staining and permeabilization with Cytofix/Cytoperm[a] Solution Kit (BD Biosciences) intracellular IκBα was stained using an anti-IκB-α Ab (Cell Signaling Technology) and a secondary FITC-labeled Ab (BD Biosciences). Analysis was performed on a FACSCalibur (BD Biosciences). Data were analyzed using FlowJo Software, Tree Star. H-Y TCR transgenic mice were genotyped using the specific mAb T3.70 against the α-chain of the H-Y TCR. P14 TCR transgenic mice were genotyped using mAbs directed against the P14 TCR subunits Vα2 and Vβ8. OT-2 TCR transgenic mice were genotyped using mAbs against the OT-2 TCR subunits Vα2 and Vβ5.1/5.2. To specifically isolate DP and SP thymocyte populations, single cell suspensions were stained with anti-CD4 and anti-CD8 and cells were sorted by FACS using a CyAn FACS sorter (DakoCytomation) and standard protocols.

Peptide-specific clonal deletion

For peptide-specific clonal deletion in vitro the thymoma cell line EL-4 was used as APC seeded on a 96-well culture dish plate at a concentration of 5×10^5 cells/ml. EL-4 cells were loaded with either P14 peptide (KAVYNFATC) (27) for P14-TCR transgenic thymocytes or with OVA-peptide (OVA323–339: ISQAVHAAHAEINEAGR) for OT-2 TCR transgenic thymocytes at a concentration ranging from 10^{-1} to 10^{-3} M. Freshly isolated thymocytes from transgenic mice were added to peptide-loaded APCs at 1×10^7 cells/ml in a 96-well culture dish plate. Cells were incubated for 24 h before immunofluorescence staining and flow cytometry analysis. Percentage survival was determined from the number of viable DP thymocytes remaining after culture in a given concentration of each peptide compared with the number of viable thymocytes remaining after culture without peptide.

Proliferation assay

Peripheral $CD4^+$ T lymphocytes were isolated from OT-2 TCR transgenic mice to >95% purity using immunomagnetic beads (Dynabeads). T cells were stained with CFSE as previously described (28) and plated at on 96-well plates at a concentration of 2×10^6 cells/ml together with agonistic Abs against CD3 (10 μg/ml) and CD28 (2 μg/ml). After 72 h, cells were analyzed by FACS for Vα2 and CD4 surface expression and CFSE fluorescence.

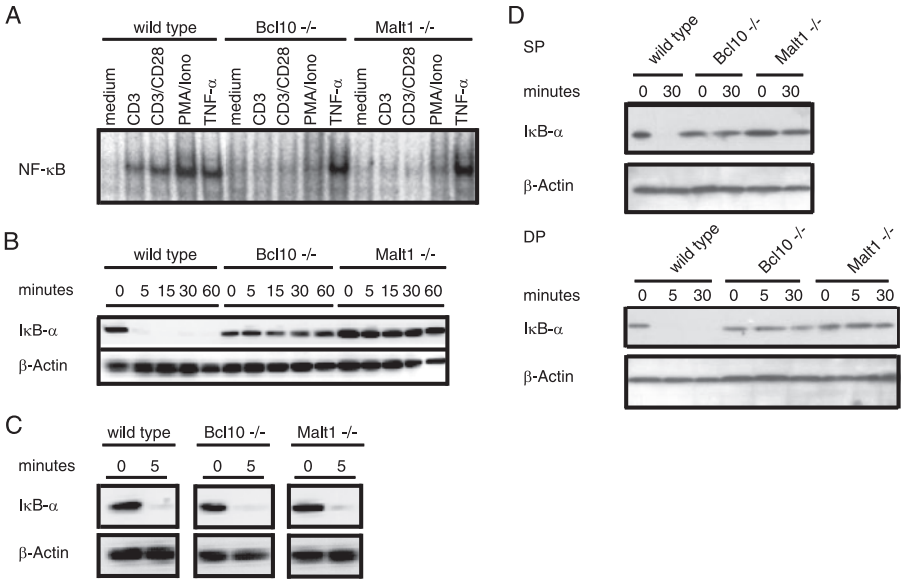

FIGURE 1. Bcl10 and Malt1 control TCR-induced NF-κB activation in thymocytes. *A*, NF-κB DNA binding activity. Purified wild-type, Bcl10$^{-/-}$, or Malt1$^{-/-}$ thymocytes were stimulated with medium alone, anti-CD3 (1 μg/ml) with or without anti-CD28 (1 μg/ml), PMA (50 μg/ml) plus Iono (10 ng/ml), or TNF-α (10 ng/ml) for the indicated time points. Eight hours later, nuclear extracts were prepared and subjected to gel mobility shift assays for detection of NF-κB DNA binding activity. *B*, Defective IκB-α degradation in response to PMA/Iono. Wild-type, Bcl10$^{-/-}$, and Malt1$^{-/-}$ thymocytes were stimulated with PMA (50 μg/ml) plus Iono (10 ng/ml) for the indicated time points in minutes. Degradation of IκB-α was investigated by Western blotting using Abs against IκB-α and β-actin (loading control). *C*, Regular degradation of IκB-α after stimulation with TNF-α. Wild-type, Bcl10$^{-/-}$, and Malt1$^{-/-}$ thymocytes were stimulated with TNF-α (10 ng/ml) for 5 min. Degradation of IκB-α was investigated by Western blotting using Abs against IκB-α and β-actin (loading control). *D*, Defective IκB-α degradation in both SP and DP thymocytes. SP and DP thymocytes from wild-type, Bcl10$^{-/-}$, and Malt1$^{-/-}$ mice were sorted by FACS and stimulated for the indicated time points shown in *B*. Degradation of IκB-α was investigated by Western blotting using Abs against IκB-α and β-actin (loading control).

Results

Bcl10 and Malt1 control TCR-induced NF-κB activation in thymocytes

To investigate the roles of Bcl10 and Malt1 in TCR signaling in the thymus, we isolated thymocytes from Bcl10- or Malt1-deficient mice and stimulated these cells in vitro by TCR cross-linking with Abs against CD3 either alone or in combination with anti-CD28 or with PMA and calcium Iono (Fig. 1*A*). As a control for TCR independent NF-κB activation we treated the cells with TNF-α. Subsequently, we isolated nuclear extracts and performed EMSAs with NF-κB binding site containing oligonucleotides. All these stimuli robustly activated NF-κB in wild-type thymocytes in comparison to stimulation with medium alone. However, neither TCR signaling nor pharmacological PKC stimulation with PMA plus Iono induced NF-κB DNA binding activity in the absence of Bcl10 or Malt1. In contrast, TNF-α stimulation induced regular NF-κB activation in Bcl10- and in Malt1-deficient thymocytes. To further characterize the roles of Bcl10 and Malt1 in thymic NF-κB signaling, we studied PMA plus Iono or TNF-α-induced IκB-α degradation in Bcl10- and Malt1-deficient thymocytes by Western blotting (Fig. 1, *B* and *C*). Consistent with a requirement of Bcl10 and Malt1 in TCR and PMA plus Iono-mediated NF-κB activation, IκB-α was only degraded in PMA plus Iono-stimulated wild-type thymocytes, but not in those deficient for Bcl10 or Malt1 (Fig. 1*B*). In contrast, TNF-α stimulation induced regular IκB-α degradation in the absence of Bcl10 or Malt1 (Fig. 1*C*).

To selectively study the roles of Bcl10 and Malt1 in DP and SP thymocyte subsets, we isolated these specific populations using FACS. Subsequently, we stimulated these cell types individually with PMA plus Iono and measured IκBα degradation by Western blotting. Whereas a strong IκBα degradation was observed in both DP and SP cells of wild-type mice, we did not detect IκBα degradation in either DP or SP cells from Bcl10-deficient or from Malt1-deficient animals (Fig. 1*D*). Together these results demonstrate a specific requirement for Bcl10 and Malt1 in TCR signaling to NF-κB in thymocytes. In addition, these experiments indicate that Bcl10/Malt1 signaling controls NF-κB activation downstream of all PMA- and/or Iono-responsive PKC isoenzymes in both DP and SP subpopulation.

Regular positive and negative selection of H-Y TCR transgenic T cells in the absence of Bcl10 or Malt1

To define the specific roles of TCR-mediated NF-κB activation to positive and negative thymocyte selection, we introduced several rearranged αβ TCR transgenes into Bcl10$^{-/-}$ and Malt1$^{-/-}$ backgrounds. We first crossed Bcl10 or Malt1-deficient animals to transgenic mice that express a TCR specific for the male H-Y Ag (24, 29). In female mice, H-Y TCR expressing (T3.70^{+}) thymocytes are positively selected and develop mainly into CD8^{+} T cells. Flow cytometric analysis of thymocytes from H-Y TCR transgenic female mice revealed normal positive selection of H-Y TCR^{+} cells into the CD8^{+} lineage in the absence of either Bcl10 or Malt1 (Fig. 2*A*). We also detected H-Y TCR^{+} CD8^{+} cells in normal frequencies (Fig. 2*B*) and numbers (data not shown) in peripheral lymphoid organs of female Bcl10- and Malt1-deficient mice.

We then studied the contributions of Bcl10 and Malt1 to negative selection in the H-Y TCR model. In male mice, the H-Y TCR is self-reactive and induces deletion of H-Y TCR^{+} cells during thymocyte development resulting in markedly reduced thymocyte numbers (24, 29). As shown in Fig. 3 the total thymocyte numbers and the CD4/CD8 profiles are equivalent in H-Y TCR^{+} wild-type, Bcl10$^{-/-}$, and Malt1$^{-/-}$ mice indicating regular negative selection of H-Y TCR^{+} thymocytes in the absence of Bcl10 or Malt1. These in vivo studies indicate that neither positive nor negative

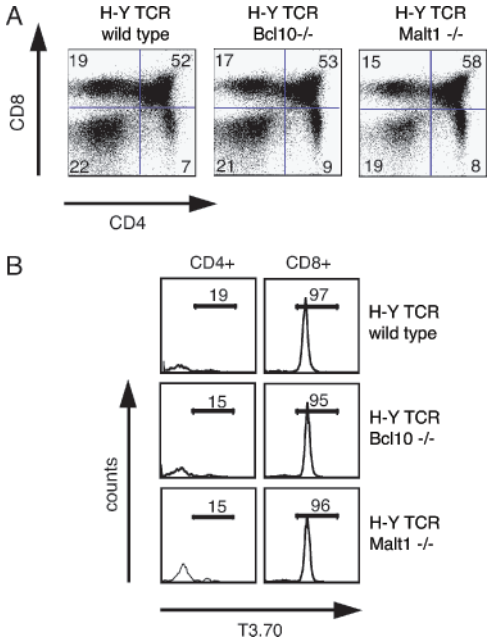

FIGURE 2. Analysis of Bcl10$^{-/-}$ and Malt1$^{-/-}$ H-Y TCR transgenic thymocytes and T cells in female mice. *A*, Regular positive selection of thymocytes from female H-Y TCR transgenic mice. Thymocytes were isolated from female H-Y TCR transgenic mice and stained with anti-CD4 and anti-CD8 Abs. Percentages of positive cells within each quadrant are indicated. One result representative of four independent experiments is shown. Total thymocyte numbers are as follows (mean values ± SD; n = 12): wild-type female: 8.0 ± 2.1 × 10^7; Bcl10$^{-/-}$ female: 6.3 ± 2.4 × 10^7; Malt1$^{-/-}$ female: 6.1 ± 1.9 × 10^7. *B*, Regular frequencies of CD8$^+$ H-Y TCR transgenic T cells in lymph nodes. CD4$^+$ or CD8$^+$ wild-type, Bcl10$^{-/-}$ and Malt1$^{-/-}$ T lymphocytes were isolated from lymph nodes of female H-Y TCR transgenic mice and stained with anti-CD4, anti-CD8 and T3.70 Abs. Frequencies of T3.70$^+$ cells in the CD4$^+$ and CD8$^+$ compartment are indicated. One result representative of five experiments is shown.

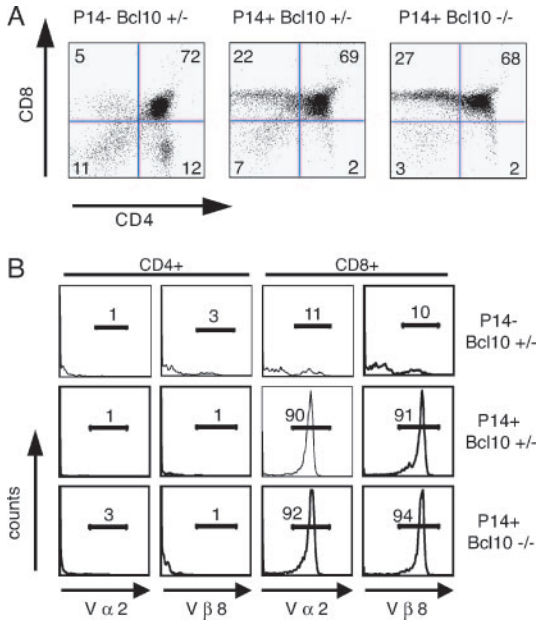

FIGURE 4. P14 TCR transgenic thymocytes and T cells in Bcl10-deficient mice. *A*, Regular positive selection. Thymocytes from Bcl10$^{+/-}$ background compared with P14 TCR$^+$ Bcl10$^{+/-}$ and P14 TCR$^+$ Bcl10$^{-/-}$ mice were isolated and stained with anti-CD4 and anti-CD8 Abs. Percentages of positive cells within each quadrant are indicated. One result representative of three individual experiments is shown. Total thymocyte numbers are as follows (mean values ± SD; n = 9): Bcl10$^{+/-}$: 8.4 ± 2.1 × 10^7; P14-TCR$^+$Bcl10$^{+/-}$: 7.7 ± 2.8 × 10^7; P14-TCR$^+$ Bcl10$^{-/-}$: 5.7 ± 2.8 × 10^7. *B*, Regular frequencies of CD8$^+$ P14 TCR transgenic T cells in lymph nodes of Bcl10$^{-/-}$ mice. Lymph node cell suspensions from Bcl10$^{+/-}$, P14 TCR$^+$ Bcl10$^{+/-}$ or P14 TCR$^+$ Bcl10$^{-/-}$ mice were stained with anti-Vα2 and anti-Vβ8 Abs and analyzed by FACS. Frequencies of the indicated surface markers are shown. One result representative of four experiments is shown.

selection of this particular MHC class I-restricted TCR depends on Bcl10/Malt1 signal transduction.

Bcl10 is dispensable for positive selection and peptide specific apoptosis of P14 TCR transgenic thymocytes

To further elucidate the role of TCR-induced NF-κB signaling in thymic selection in an independent system, we used P14 TCR transgenic mice. All subsequent studies were solely performed in Bcl10$^{-/-}$ animals. The P14 TCR is specific for a peptide epitope of the lymphocytic choriomeningitis virus glycoprotein p33 in the context of the MHC class I (25). In wild-type animals, P14 TCR-expressing DP thymocytes are positively selected into the CD8$^+$ lineage resulting in a skewing of the CD4/CD8 ratio toward CD8$^+$ cells (25). Similar frequencies and absolute numbers of P14 TCR$^+$ CD8$^+$ T cells were detected in the thymus of Bcl10$^{+/-}$ or Bcl10-deficient mice (Fig. 4*A*), whereas P14 TCR$^-$ Bcl10$^{+/-}$ thymocytes develop normally into CD4$^+$ and CD8$^+$ T cells (Fig. 4*A*). P14 TCR transgenic cells are exported into the periphery and comparable frequencies of P14 TCR$^+$CD8$^+$ T cells were detected in the lymph nodes of P14 TCR$^+$ Bcl10$^{+/-}$ and Bcl10$^{-/-}$ mice (Fig. 4*B*).

We then studied the role of Bcl10 in peptide-specific apoptosis with the P14 TCR transgenic system in vitro, which is considered as a model for negative selection (30). Thymocytes from P14 TCR transgenic wild-type or Bcl10$^{-/-}$ mice and nontransgenic littermates were isolated and incubated with APCs that have been preloaded with the agonistic p33 peptide. Twenty-four hours later, cells were stained with anti-CD4 and anti-CD8 mAbs and 7-AAD to quantify viability of DP cells by flow cytometry (Fig. 5). As expected, wild-type P14 TCR$^+$ DP cells readily underwent apoptosis in a dose-dependent manner in response to treatment with p33 peptide whereas nontransgenic T cells were not deleted. Bcl10-deficient P14 TCR transgenic DP cells also underwent dose-dependent deletion with the same kinetics and to a similar extend as wild-type P14 TCR transgenic cells. Taken together, the results obtained with the P14 TCR transgenic system show that Bcl10 signaling is also dispensable for the positive selection and for peptide-specific deletion of DP cells that express this receptor.

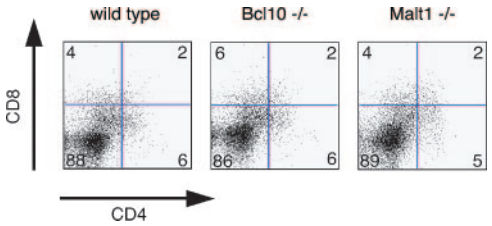

FIGURE 3. Regular negative selection of male H-Y TCR transgenic thymocytes in the absence of Bcl10 and Malt1. Thymocytes were isolated from male H-Y TCR transgenic mice and stained with anti-CD4 and anti-CD8 Abs. Percentages of positive cells within each quadrant are indicated. One result representative of three individual experiments is shown. Total thymocyte numbers are as follows (mean values ± SD; n = 12): wild-type male: 1.1 ± 0.7 × 10^7; Bcl10$^{-/-}$ male: 0.9 ± 0.5 × 10^7; Malt1$^{-/-}$ male: 0.9 ± 0.8 × 10^7.

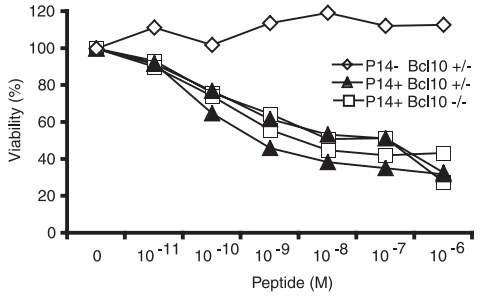

FIGURE 5. Regular peptide-specific clonal deletion of P14 TCR transgenic DP thymocytes in vitro. Thymocytes were isolated from P14 TCR transgenic Bcl10$^{+/-}$ (▲), P14 TCR transgenic Bcl10$^{-/-}$ mice (□) and wild-type Bcl10$^{+/-}$ mice (open rectangles) and cultured on EL-4 cells as APCs pulsed with the indicated concentrations of p33 peptide. Twenty-four hours later, viability of DP cells was determined by FACS after staining with anti-CD4, anti-CD8, and 7-AAD. One result representative of three independent experiments is shown. Results from two mice of each experimental group plus one control mouse are shown. ◇: P14 TCR^{-} Bcl10$^{+/-}$ thymocytes (control mouse); ▲: P14 TCR^{+} Bcl10$^{+/-}$ thymocytes; □: P14 TCR^{+} Bcl10$^{-/-}$ thymocytes.

Bcl10 is not required for positive selection and peptide specific apoptosis of OT-2 TCR transgenic thymocytes

Both the H-Y TCR and the P14 TCR are selected into the CD8^{+} T cell lineage due to MHC class I restriction. Since previous work has indicated that TCR-mediated activation of NF-κB could play differential roles in the selection of CD4^{+} and CD8^{+} cells (9, 13), we additionally investigated the role of Bcl10 signaling in the selection of a MHC class II-restricted TCR transgene: the OT-2 TCR that is selected toward the CD4^{+} lineage (Figs. 6 and 7). Flow cytometric analysis of the thymi of OT-2 TCR transgenic Bcl10$^{-/-}$ mice showed CD4/CD8 profiles that are comparable to those of OT-2 TCR transgenic wild-type mice with a strong skewing toward the CD4^{+} lineage (Fig. 6*A*). Analysis of the OT-2 TCR expression after staining with TCR Vα2 and TCR Vβ5.1/5.2 Abs revealed that OT-2 TCR^{+}Bcl10$^{-/-}$ T cells are regularly selected into the CD4^{+} lineage and they are found in normal frequencies in the periphery of OT-2 TCR^{+}Bcl10$^{-/-}$ animals (Fig. 6*B*).

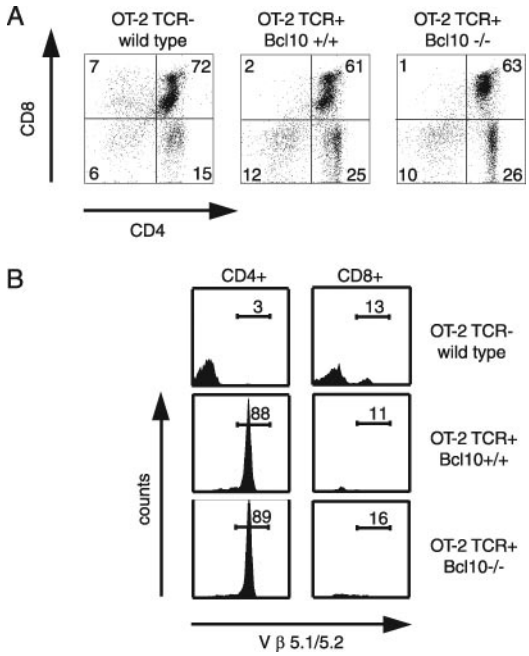

FIGURE 6. Regular positive selection of OT-2 TCR transgenic thymocytes. *A*, Regular positive selection. Thymocytes were isolated from wild-type and OT-2 TCR^{+} wild-type and OT-2 TCR^{+} Bcl10$^{-/-}$ mice and cells were stained with anti-CD4 and anti-CD8 Abs and analyzed by FACS. Percentages of positive cells within each quadrant are indicated. One result representative of three individual experiments is shown. Total thymocyte numbers are as follows (mean values ± SD; $n = 9$): wild-type: $8.6 \pm 2.9 \times 10^{7}$; OT-2 TCR^{+} Bcl10$^{+/+}$: $8.4 \pm 2.1 \times 10^{7}$; OT-2 TCR^{+} Bcl10$^{-/-}$: $5.9 \pm 2.7 \times 10^{7}$. *B*, Regular frequencies of CD4^{+} OT-2 TCR transgenic T cells in lymph nodes. Lymph node cells were isolated from wild-type and OT-2 TCR expressing wild-type or Bcl10$^{-/-}$ mice and stained with anti-Vβ5.1/5.2 Abs. Percentages of Vβ5.1/5.2^{+} cells are shown and histograms for the indicated surface marker are indicated. One result representative of three individual experiments is shown.

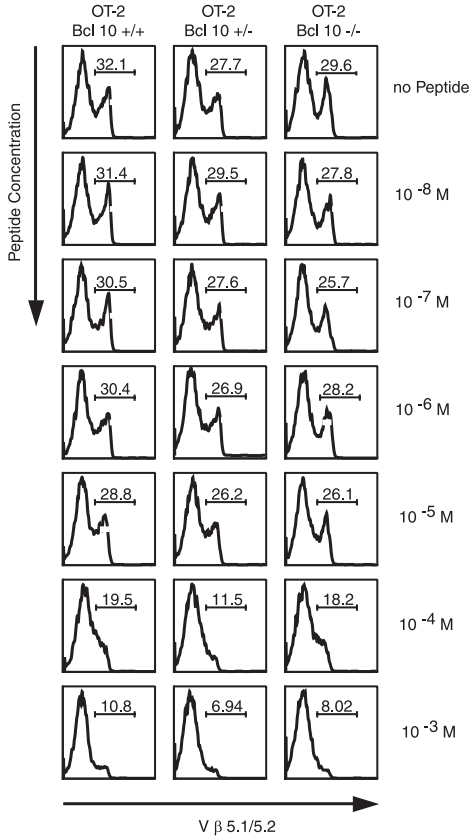

FIGURE 7. Peptide-specific apoptosis of OT-2 TCR transgenic thymocytes in vitro. Regular clonal deletion of OT-2 TCR^{+} thymocytes. Thymocytes were purified from OT-2 expressing wild-type (first column), Bcl10$^{+/-}$ (second column), and Bcl10$^{-/-}$ (third column) mice and cultured on EL-4 APCs pulsed with the indicated concentrations of OVA-peptide. Cells were harvested after 24 h of culture. Numbers of OT-2 expressing thymocytes were determined by FACS staining using anti-CD4, anti-CD8 and anti-Vβ5.1/5.2 Abs. Percentages of OT-2 TCR transgenic thymocytes remaining after incubation in a given concentration of OVA-peptide are indicated in the histograms. One result representative of three independent experiments is shown.

Next, we studied the role of Bcl10 in peptide-specific apoptosis in the OT-2 TCR transgenic system. Thymocytes of wild-type OT-2 TCR transgenic mice and Bcl10$^{-/-}$ OT-2 TCR transgenic mice were cocultured with APCs that have been pulsed with increasing concentrations of the OT-2 TCR agonistic OVA peptide. Twenty-four hours later, the cells were stained for viability with 7-AAD and costained with Abs against CD4, CD8, and Vβ5.1/5.2. Subsequently, the frequencies of viable Vβ5.1/5.2^{+} cells were determined in the DP population. We observed a comparable dose dependent deletion of OT-2 TCR^{+}Bcl10$^{+/+}$, Bcl10$^{+/-}$, or

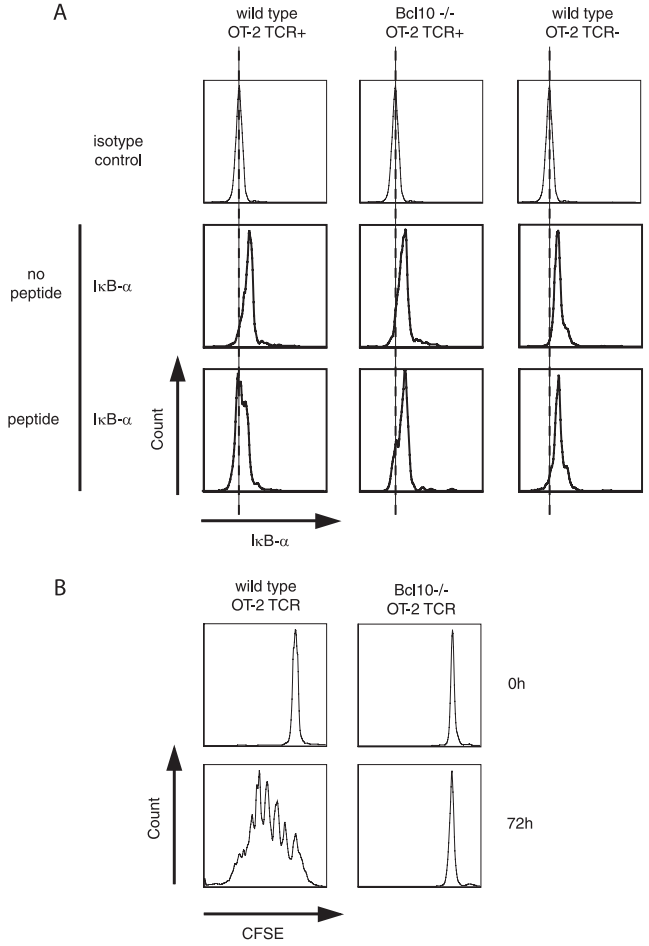

FIGURE 8. Peptide induced NF-κB activation in DP thymocytes and proliferation of positively selected mature OT-2 TCR transgenic T cells. *A*, Bcl10 signaling is essential for peptide induced NF-κB activation in DP OT-2 TCR transgenic thymocytes. OT-2 TCR transgenic thymocytes from wild-type or Bcl10$^{-/-}$ mice were incubated for 4 h with OVA peptide (0.3 mM)-pulsed EL-4 cells as APCs. Subsequently the cells were labeled with fluorescently conjugated anti-CD4, anti-CD8, and anti-Vα2 Abs, permeabilized, and then intracellularly stained with anti-IκBα Abs and fluorescently conjugated secondary Abs. IκBα degradation was analyzed by FACS in DP cells after gating for CD4 and CD8 coexpression. Histograms represent IκBα protein levels in OT-2 TCR^{+} and in OT-2 TCR^{-} DP thymocytes. *B*, Defective TCR-induced proliferation of mature Bcl10$^{-/-}$ OT-2 TCR transgenic cells. Mature OT-2 TCR transgenic T cells were isolated from wild-type and Bcl10$^{-/-}$ mice, labeled with CFSE, and cultured with Abs against CD3 (10 μg/ml) and CD28 (2.5 μg/ml) for 72 h. Subsequently, cells were harvested and stained with Abs against CD4, CD8, and Vα2. Cell proliferation was investigated by measuring CFSE dilution by FACS and gating for CD4 and TCR Vα2 expression. Histograms represent CFSE fluorescence specifically in OT-2 TCR transgenic Vα2^{+}CD4^{+} cells.

Bcl10$^{-/-}$ cells (Fig. 7). Together the results using the OT-2 TCR transgenic system indicate that Bcl10 signaling is dispensable for the positive or negative selection of an MHC class II-restricted TCR.

In a final set of experiments, we investigated the role of Bcl10 in NF-κB signaling directly in ex vivo thymocytes from TCR-transgenic mice exposed to their cognate peptide and additionally studied the proliferative response of mature peripheral Bcl10$^{-/-}$ TCR-transgenic T cells that had been positively selected (Fig. 8). We first coincubated OT-2 transgenic thymocytes from wild-type or Bcl10$^{-/-}$ mice with APCs that were loaded with the agonistic OVA peptide. We monitored NF-κB signaling specifically in TCR transgenic DP cells after surface staining for TCR Vα2, CD4, and CD8 and intracellular staining for IκBα using FACS (Fig. 8*A*). Four hours of APC/peptide stimulation resulted in a clear degradation of IκBα in DP wild-type cells that express the OT-2 TCR. However, IκBα was neither degraded in control wild-type cells that do not express the transgenic TCR, nor in DP OT-2 TCR transgenic cells that lack Bcl10. Thus, Bcl10 signaling is essential for NF-κB activation in TCR transgenic thymocytes exposed to their cognate peptide.

We then isolated mature T cells that had been positively selected from OT-2 TCR transgenic mice, labeled these with CFSE to track cell division and stimulated them through the TCR (Fig. 8*B*). Wild-type OT-2 TCR transgenic T cells proliferated readily and performed several cell division cycles in response to TCR ligation. However, mature OT-2 TCR transgenic T cells from Bcl10-deficient animals show severe proliferation defects in response to TCR ligation. Therefore, we conclude that, while Bcl10 signaling is dispensable for the selection of OT-2 TCR transgenic T cells, the pathway is required to mediate activation/proliferation of OT-2 TCR transgenic T cells in the periphery.

Discussion

Our data demonstrate that Bcl10 and Malt1 are key regulators of the TCR-induced NF-κB signaling pathway in DP and SP thymocytes. Various in vitro and in vivo studies have indicated essential roles for NF-κB activation in positive or negative thymic selection (3, 8–12). However, using three TCR transgenic models, we surprisingly find that the TCR-induced signaling pathway to NF-κB is unlikely to be involved in thymic selection, although it is essential for the activation of peripheral T cells.

The analysis of PKCθ-deficient mice has established that the signaling cascade from the TCR to NF-κB differs between developing and mature T cells. PKCθ controls TCR-induced NF-κB activation in peripheral T cells, but not in thymocytes (22). We show here together with previous work (19–21), that the Bcl10/Malt1 complex plays an analogous role in mature and immature T lymphocytes. These results indicate a conserved mechanism between thymic and mature TCR signaling. In addition, our finding that PMA plus Iono stimulation requires the Bcl10/Malt1 complex for NF-κB activation in thymocytes indicates that a PKC enzyme distinct from PKCθ controls thymic TCR signaling to NF-κB. Although that PKC enzyme remains to be identified this hypothesis is consistent with related roles for Carma1 in thymocytes and in mature T cells (17, 18) and the fact that various PKC isoforms are able to phosphorylate the linker region of Carma1 for the activation of downstream signaling via Bcl10 (15, 16). The Carma1 deficiency has previously been introduced into the H-Y TCR transgenic background without any effects on the selection of this particular TCR (17). The interpretation of these experiments is that other Carma family proteins like Carma2 or Carma3 might transduce the TCR signals for NF-κB-dependent thymocyte selection (17). Although the physiological functions of Carma2 and Carma3 in thymocytes are unknown, all Carma proteins use Bcl10 for downstream signaling to NF-κB (31, 32). Thus, the genetic disruption of Bcl10 is likely to block NF-κB activation downstream of all Carma proteins. Therefore, we feel that our current study can be considered as a comprehensive analysis of the specific contribution of TCR signaling to NF-κB in thymic selection.

The initial characterization of Bcl10- and Malt1-deficient mice reported high frequencies of T lymphocyte precursors that are undergoing apoptosis (19, 20). These dying thymocytes do not express CD4 or CD8 or they have lost coreceptor expression and are therefore detected in the DN FACS gate. These cells have passed the pre-TCR checkpoint as they express mature αβ TCRs at levels comparable to normal DP cells. We have therefore hypothesized

that Bcl10 could regulate an $\alpha\beta$ TCR controlled survival signal whose strengths might depend on the clonotypic TCR of the individual thymocyte (20). In our present study, we find a requirement for thymic Bcl10 signaling in peptide induced NF-κB activation but still regular positive selection of three distinct TCRs in the absence of Bcl10 or Malt1. These findings suggest that the Bcl10/Malt1 complex does not control the survival signal that is required for positive selection. Recent studies have shown that thymocytes that lack Calcineurin b1 have selective defects in positive but not negative selection (33). In addition, ERK signaling and serum response factor accessory protein 1 play key roles in this process (34–39). Although previous papers have indicated requirements for NF-κB activity in positive selection (9, 11, 12), our results demonstrate that the specific TCR signaling pathway to NF-κB is not required for this process. The DN IκB molecules that were used in previous studies to investigate NF-κB signaling in thymic selection are not specific for IκBα. Moreover, they do not selectively block TCR signaling but inhibit NF-κB induction in response to most upstream stimuli including pathways from CD30, Fas or other TNF receptor superfamily members (40). Although these pathways presumably signal independent of Bcl10 or Matl1, they could potentially operate as coregulators for selection. DN IκB superinhibitors would block such Bcl10 and Malt1 independent pathways that are controlled by either IκBα or IκBβ. The fact that the DN IκB molecules block positive selection in certain experimental systems could therefore indicate together with our results that a non-TCR signal for NF-κB activation might set the threshold of cell viability, which could indirectly influence the low-intensity TCR signals that mediate positive selection via Calcineurin b1 and ERK.

We also find Bcl10/Malt1 signaling dispensable for negative selection or peptide specific apoptosis of DP thymocytes, although it has previously been suggested that negative selection is at least in part controlled by TCR-induced cell intrinsic NF-κB activation (8, 10–12, 41). We have considered the hypothesis that the activation of contaminating mature T cells could indirectly influence the death of immature Bcl10/Malt1 thymocytes in our in vitro deletion experiments, e.g., via Fas/Fas ligand interaction. However, mature T cells from Bcl10- or Malt1-deficient mice have severe activation defects and TCR induce up-regulation of Fas ligand is specifically dependent on Bcl10 signaling (data not shown). Thus, we regard this possibility as unlikely. The final mediator of apoptosis for the negative selection of DP cells is the proapoptotic BH3 only protein Bim (42). Bim is both necessary and sufficient for the induction of cell death in this context. Several mechanisms of Bim activation for autoreactive lymphocyte deletion have been proposed including posttranslational modifications (43) and importantly transcriptional induction mediated by a PKC and Ca^{2+}-dependent mechanism (44). The transcription factors and the pathway that connects PKC and Ca^{2+} signaling to Bim induction remain unknown (44). We show that the Bcl10/Malt1 complex controls PKC downstream signaling to the transcription factor NF-κB. Yet, our results also argue against a role of the Bcl10/Malt1 pathway in the direct or indirect regulation of Bim activity, as the absence of Bcl10 signaling does not influence negative selection in three distinct models. As discussed above for positive selection, the functions for NF-κB in negative selection were also largely deduced from experiments with mice that ectopically express artificial IκB superrepressors (8, 10, 11, 41). Thus, it can again be speculated that non-TCR signals (e.g., signals from CD30, Fas, or other TNFR family members) might modulate negative selection via NF-κB and indirectly influence the apoptosis inducing TCR signal in certain settings.

In conclusion, while TCR signaling to NF-κB is indispensable for the activation of mature T cells we provide strong genetic evidence that this signaling cascade does not influence positive or negative thymocyte selection. However, various studies with transgenic mice that overexpress artificial DN signaling molecules have implicated NF-κB in the regulation of thymic selection. Whether non-TCR signals to NF-κB modulate the susceptibility of thymocytes to positive or negative selection in physiological settings requires further investigation.

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Disclosures

The authors have no financial conflict of interest to disclose.

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