Please cite this article in press as: Kosan et al., Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor Signaling at Early Commitment Stages of B Cell Differentiation, Immunity (2010), doi:10.1016/j.immuni.2010.11.028

Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor Signaling at Early Commitment Stages of B Cell Differentiation

Christian Kosan,[1] Ingrid Saba,[1,2] Maren Godmann,[3] Stefanie Herold,[4] Barbara Herkert,[4] Martin Eilers,[4] and Tarik Möröy[1,2,5,*]
[1]Institut de recherches cliniques de Montréal (IRCM), Montréal, Quebec H2W 1R7, Canada
[2]Département de Microbiologie et Immunologie, Université de Montréal, Montréal, Quebec H3C 3J7, Canada
[3]Department of Animal Science, McGill University, Ste-Anne-de-Bellevue, Quebec H9X 3V9, Canada
[4]Biozentrum Universität Würzburg, Würzburg D-97074, Germany
[5]Zentrum für Medizinische Biotechnologie (ZMB), Universität Duisburg-Essen, Essen D-45117, Germany
*Correspondence: tarik.moroy@ircm.qc.ca
DOI 10.1016/j.immuni.2010.11.028

SUMMARY

B cell development requires the coordinated action of transcription factors and cytokines, in particular interleukin-7 (IL-7). We report that mice lacking the POZ (Poxvirus and zinc finger) domain of the transcription factor Miz-1 (*Zbtb17*$^{\Delta POZ/\Delta POZ}$) almost entirely lacked follicular B cells, as shown by the fact that their progenitors failed to activate the Jak-Stat5 pathway and to upregulate the antiapoptotic gene *Bcl2* upon IL-7 stimulation. We show that Miz-1 exerted a dual role in the interleukin-7 receptor (IL-7R) pathway by directly repressing the Janus kinase (Jak) inhibitor suppressor of cytokine signaling 1 (*Socs1*) and by activating *Bcl2* expression. *Zbtb17*$^{\Delta POZ/\Delta POZ}$ (Miz-1-deficient) B cell progenitors had low expression of early B cell genes as transcription factor 3 (*Tcf3*) and early B cell factor 1 (*Ebf1*) and showed a propensity for apoptosis. Only the combined re-expression of *Bcl2* and *Ebf1* could reconstitute the ability of Miz-1-deficient precursors to develop into CD19^{+} B cells.

INTRODUCTION

The development of mature functional B cells capable of producing specific antibodies is a highly regulated, multistep process that is initiated in the bone marrow, where early B cell differentiation takes place to yield immature immunoglobulin-M (IgM)$^{+}$ B cells. These immature cells can leave the bone marrow and migrate to the peripheral lymphoid organs, where they complete their maturation. The earliest step on the way to become a B cell is the generation of multipotent progenitors (MPPs) from hematopoietic stem cells (HSCs), which have the potential to develop into multiple hematopoietic lineages (Christensen and Weissman, 2001; Morrison and Weissman, 1994). A subset of MPPs, which expresses the tyrosine kinase receptor Flt3, loses the ability to differentiate into the erythro-megakaryocytic lineage but retains myeloid and lymphoid potential. When these lymphoid-primed multipotent progenitors (LMPPs) upregulate Flt3 (FMS-like tyrosine kinase 3) expression, they lose their myeloid potential and become restricted to the lymphoid lineage (Adolfsson et al., 2005; Månsson et al., 2007). The LMPP population contains the early lymphoid progenitors (ELPs) (Igarashi et al., 2002), which give rise to the common lymphoid progenitors (CLPs) and, most probably, to the early T lineage progenitors (ETPs). CLPs can give rise to T cells, natural killer cells (NK cells), and dendritic cells, but in vivo are considered the sole progenitors of NK and B cells (Allman et al., 2003; Kondo et al., 1997b). CLPs retain expression of Flt3 and express the receptor for IL-7 (IL-7R), which are both critical for B cell development. Mice deficient for both receptors do not develop any B cells throughout fetal and adult life (Sitnicka et al., 2003).

Cytokine signaling and in particular IL-7 is indispensable for the adult B cell development in the mouse bone marrow (Miller et al., 2002; Namen et al., 1988). In CLPs, IL-7 signaling induces the expression of the early B cell factor 1 (*Ebf1*) (Kikuchi et al., 2005), which regulates the expression of the transcription factor *Pax5* (paired box gene 5) (O'Riordan and Grosschedl, 1999). In contrast, Malin and colleagues recently showed that Ebf1 regulation is independent of IL-7 signaling and suggest a role of IL-7 signaling solely in preventing apoptosis, as it has been shown for T cells (Grosschedl, 1995; Malin et al., 2010; Nutt et al., 1997; Urbánek et al., 1994). Consistent with this, it has been shown that the expression of Ebf1 is independent of IL-7 signaling during fetal B cell development (Kikuchi and Kondo, 2006) and that *Il7*$^{-/-}$ mice still have a few B cells, which may however originate from a fetal or neonatal precursor (Carvalho et al., 2001). Also, overexpression of Ebf1 in *Il7r*$^{-/-}$ mice only partially rescues early B cell development (Kikuchi et al., 2005), suggesting that other factors, induced by IL-7 signaling, must exist to support B cell development such as E2A (*Tcf3*), EBF, Pax5 that are required for expression recombination-activating genes 1 and 2 (*Rag1*, *Rag2*) and for a proper rearrangement of the immunoglobulin heavy chain locus (IgH) (Lin and Grosschedl, 1995; Nutt et al., 1997; Urbánek et al., 1994).

The Miz-1 (*Zbtb17*) gene encodes a 87 kDa protein with 13 C_2H_2 zinc finger domains at the carboxy terminal half and a POZ domain at its N terminus (Peukert et al., 1997). Miz-1 belongs to the group of POZ domain zinc finger transcription

Please cite this article in press as: Kosan et al., Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor Signaling at Early Commitment Stages of B Cell Differentiation, Immunity (2010), doi:10.1016/j.immunol.2010.11.028

factors that can act as both transcriptional activators and repressors. The POZ domain is required for multimerization and for the interaction with other cofactors that mediate their function (Staller et al., 2001). Miz-1 binds to core promoters of RNA polymerase II-dependent target genes and recruits the histone acetyltransferase EP300 (E1A binding protein 300) and nucleophosmin (Herold et al., 2002; Staller et al., 2001; Wanzel et al., 2008), which enables transcriptional activation of its targets such as the genes encoding the negative cell cycle regulators p15 (*Cdkn2b*) (Seoane et al., 2001; Staller et al., 2001) or p21 (*Cdkn1a*) (Phan et al., 2005; Seoane et al., 2002; Wu et al., 2003). Miz-1 can also form a complex with the bHLH (basic helix-loop-helix) leucine zipper transcription factor Myc, which displaces positive cofactors such as EP300 and nucleophosmin that leads to a repression of Miz-1 target genes (Herold et al., 2002; Seoane et al., 2001, 2002; Staller et al., 2001; Wanzel et al., 2008).

In this study we wished to elucidate the role of Miz-1 during hematopoiesis and have generated a loss-of-function mutant mouse by gene targeting introducing an allele that allowed the conditional deletion of the Miz-1 POZ domain. This strategy specifically disrupted the activity of Miz-1 as a transcriptional transregulator. Our studies demonstrated that Miz-1 is required for the very early steps of adult B cell development in the bone marrow. Importantly, we found that the deletion of the Miz-1 POZ domain severely disrupted signaling events triggered by IL-7 that substantially impairs B cell survival and differentiation through the regulation of early B cell genes and the antiapoptotic gene *Bcl2*.

RESULTS

Generation of Mice Carrying a Conditionally Deficient Allele of *Zbtb17*

The complete deletion of the *Zbtb17* gene leads to early embryonic lethality at E7.5 (Adhikary et al., 2003). To overcome this limitation, we generated mice carrying loxP (fl) sites flanking the region encoding the POZ domain to render it amenable to cre-mediated deletion. Analysis of protein extracts derived from $Zbtb17^{+/+}$, $Zbtb17^{+/\Delta POZ}$, or $Zbtb17^{\Delta POZ/\Delta POZ}$ embryonic fibroblasts (E12.5) confirmed that this targeting strategy yielded a truncated Miz-1 protein lacking the POZ domain (Figures S1A and S1B available online). Previous studies have shown that this truncated form is nonfunctional as a transcription factor (Staller et al., 2001; Wanzel et al., 2008) and we could show that this form is unable to stably bind chromatin (Figure S1C). Adult $Zbtb17^{fl/fl}$ or $Zbtb17^{+/\Delta POZ}$ mice were indistinguishable from wild-type (WT) littermates and were used as control animals throughout this study, whereas animals with two deleted *Zbtb17* alleles ($Zbtb17^{\Delta POZ/\Delta POZ}$) arrested development at around E14 (Figure S1D). Because $Zbtb17^{+/\Delta POZ}$ mice are phenotypically indistinguishable from $Zbtb17^{fl/fl}$ or wild-type mice, a dominant-negative effect of the truncated Miz-1 protein could be excluded.

Miz-1 Is Required for Early B Cell Development in Adult Mice

Zbtb17 is expressed in lymphoid cells notably in thymus, bone marrow, and spleen and in purified B and T cell populations (Figure S1E; data not shown). When $Zbtb17^{\Delta POZ/\Delta POZ}$ ES cells were used to generate chimeras, they did contribute to the formation of organs and hematopoietic cells with the exception of T and B cells (Figure S1F), strongly suggesting an important role of Miz-1 in lymphoid development. Consistent with this, we observed a severe defect in B and T cell development when a *Vav-cre* transgene was present in the conditional $Zbtb17^{fl/fl}$ mice, which enabled the deletion of the POZ domain encoding exons in all hematopoietic cells as previously described (Figure S1G; de Boer et al., 2003).

In the spleen, *Vav-cre* $Zbtb17^{fl/fl}$ mice showed a reduction of the B cell population ($B220^+$) to 10% of control mice (Figure 1A). Within the residual $B220^+$ cells of *Vav-cre* $Zbtb17^{fl/fl}$ mice, frequencies of mature B cells (IgM^-IgD^+) were reduced to half the values seen in wild-type mice (Figure 1B). In addition, follicular B cells (FO) were reduced in frequency (Figure 1B) and absolute numbers in *Vav-cre* $Zbtb17^{fl/fl}$ mice to about 4% of controls (Figure 1A), whereas marginal zone B cells (MZ) remained unaffected (Figures 1A and 1B). Similarly, in the bone marrow of *Vav-cre* $Zbtb17^{fl/fl}$ mice, cellular frequencies were strongly reduced and absolute numbers of $B220^+CD19^+$ cells dropped to about 3% of controls (Figures 1C and 1D), indicating that Miz-1 deficiency leads to a severe reduction of the B cell compartment, particularly affecting follicular B cells.

B Cell Development Is Arrested at the Pre-Pro-B to Pro-B Cell Transition in Miz-1-Deficient Animals

Hematopoietic progenitors including HSCs, MPPs, and LMPPs were present in *Vav-Zbtb17*$^{fl/fl}$ mice (Figure 2A). The frequencies and absolute numbers of CLPs (lin^- $IL\text{-}7R^+$ $AA4.1^+sca1^{med}$ $c\text{-}kit^{med}$) that are the precursors for the B cell lineage (Izon et al., 2001; Kondo et al., 1997b) as well as pre-pro-B cells ($lin^-B220^+CD43^+AA4.1^+CD19^-BP\text{-}1^-HSA^-$) that are contained within Fraction A ($B220^+CD43^+CD19^-BP\text{-}1^-HSA^-$) were not changed in *Vav-cre* $Zbtb17^{fl/fl}$ mice (Figures 2B–2F). However, the expression of AA4.1 on CLPs and pre-pro-B cells of *Vav-cre* $Zbtb17^{fl/fl}$ mice was severely reduced compared to control mice (Figures 2B and 2E), whereas IL-7R, c-kit, and Flt3 expression remained largely unchanged on pre-pro-B cells (Figure 2E). In addition, Fraction B ($B220^+CD43^+HSA^+BP\text{-}1^-CD19^+$), C, and C′ cells were almost absent in *Vav-cre* $Zbtb17^{fl/fl}$ mice (Figures 2C and 2F) and the proportion of $CD19^+$ cells in Fraction B was strongly reduced from 92.8% to 31.1% (Figure 2C). This suggested that Miz-1 is required for the generation of committed $CD19^+$ B cells; a phenotype that could be confirmed by adoptive transfer experiments of Miz-1-deficient bone marrow cells into syngeneic recipients (Figures 3A and 3B; Figures S2A–S2C). These experiments also showed that hematopoietic stem cells from *Vav-cre* $Zbtb17^{fl/fl}$ mice are able to home to the bone marrow and still have T lymphoid and myeloid potential (Figures S2A–S2C). However, although T cell development was severely perturbed, myeloid development was not affected in the absence of functional Miz-1 (data not shown; Figures S2A and S2B). Conversely, transfer of control bone marrow cells into *Vav-cre* $Zbtb17^{fl/fl}$ mice resulted in a normal lymphoid and myeloid reconstitution, indicating that the observed disruption of B cell development is a cell-autonomous phenotype (Figures S2D–S2F). Taken together, Miz-1 is required for

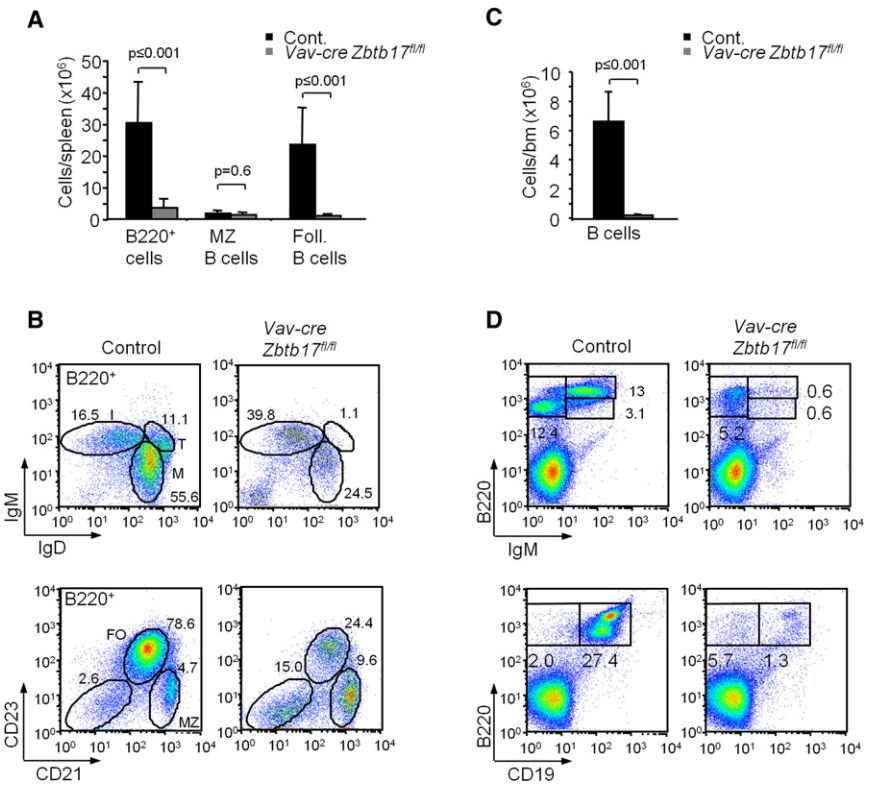

Figure 1. POZ Domain Transcription Factor Miz-1 Is Essential for B Cell Development

(A) Absolute numbers of splenic B cells (B220$^+$), marginal zone B cells (MZ; B220$^+$CD21$^+$CD23$^-$), and follicular B cells (Foll.; B220$^+$CD21$^+$CD23$^+$) of control mice (black) and *Vav-cre Zbtb17$^{fl/fl}$* (gray). A minimum of five mice was analyzed for each group. Error bars indicate standard deviation (±SD); p values are indicated in the figure.

(B) Flow cytometric analysis of splenic B cells from control mice and *Vav-cre Zbtb17$^{fl/fl}$* mice. B220$^+$ cells were analyzed for surface expression of IgM and IgD or CD21 and CD23. Immature (I), transitional (T), mature (M), follicular (FO), and marginal zone (MZ) B cells. Numbers in plots indicate percentages relative to gated B220$^+$ cells.

(C) Absolute numbers of bone marrow B cells (B220$^+$CD19$^+$; femur and tibia) were corrected to living cells (based on forward scatter [FSC] and side scatter [SSC] gating) of control mice (black) and *Vav-cre Zbtb17$^{fl/fl}$* (gray). A minimum of five mice was analyzed for each group. Error bars indicate standard deviation (±SD); a p value is given in the figure.

(D) Flow cytometric analysis of bone marrow B cells from control mice and *Vav-cre Zbtb17$^{fl/fl}$* mice. Cells were analyzed via antibodies for B220, IgM, and CD19; numbers in dot plots indicate percentages of each gate.

(B and D) Data represent at least three independent experiments. Numbers in plots indicate percentages calculated on live cells (based on FSC and SSC gating). Controls were either WT, *Vav-cre Zbtb17$^{fl/+}$*, or Zbtb17$^{fl/fl}$ mice.

See also Figure S1.

the development of CD19$^+$ B cells and T cells but does not affect progenitor function and myeloid development.

Adult Miz-1-Deficient B Cell Progenitors Fail to Develop In Vitro in Response to IL-7

LSKs and CLPs from adult *Vav-cre Zbtb17$^{fl/fl}$* were purified and cultured with IL-7 and Flt3L on OP9 cells. Under these conditions, Miz-1-deficient cells did not develop into B220$^+$CD19$^+$ B cells and were lost from the cultures reproducibly, whereas progenitors from control animals developed efficiently into B cells (Figures 3C and 3E). Under myeloid conditions and in the absence of IL-7, both wild-type and Miz-1-deficient LSK cells developed into Mac-1$^+$ cells (Figure 3D). To further analyze the response to IL-7, we cultured CLPs under stroma-free conditions, which have been shown to provide a survival and differentiation signal, but no proliferative stimulus (Miller et al., 2002). CLPs from control animals differentiated in the presence of IL-7 into B220$^+$CD19$^+$ B cells, whereas Miz-1-deficient CLPs did not develop and were again lost from the culture (Figure 3F). Because DNA content analysis and BrdU (bromodeoxyuridine)-labeling experiments demonstrated that progression through the cell division cycle is not disturbed in Miz-1-deficient CLPs or pre-pro-B cells in vivo (Figure S3), these findings suggested that loss of Miz-1 function affects those elements of IL-7 signaling that control differentiation and survival but not proliferation.

A key event in IL-7 signaling is the phosphorylation of Stat5, which is mediated by Janus kinases (Jaks) upon IL-7 stimulation (Lin et al., 1995). We found that in Miz-1-deficient lin$^-$CD19$^-$B220$^+$ or IL-7R$^+$ bone marrow cells, Stat5 phosphorylation was reduced upon IL-7 stimulation compared to similarly treated control cells (Figures 3G and 3H). To confirm the defect in IL-7 responsiveness, we stimulated CLPs with IL-7 and found that several IL-7-responsive genes such as *Socs1*, *Socs3*, and *Bcl2* were no longer IL-7- inducible in Miz-1-deficient cells, whereas a normal upregulation was seen in WT control cells (Figure 4A). We also observed that the expression of *Ebf1*, *Mcl1*, or *Bcl2l1* was not dependent on IL-7 in CLP cells and their expression level in the presence of IL-7 were not affected by Miz-1 deficiency (Figure 4A). In freshly isolated CLPs from *Vav-cre Zbtb17$^{fl/fl}$* mice, *Socs1* was (35-fold) increased in comparison to CLPs from control mice (Figure 4B). In contrast, the Miz-1-overexpressing 70Z/3 pre-B cell line showed a strong reduction in *Socs1* expression (Figure 4B). To identify potential binding sites of Miz-1 in the *Socs1* promoter, we performed chromatin immunoprecipitation (ChIP) with extracts from 70Z/3 pre-B cells transduced with a Miz-1-expressing retrovirus, 70Z/3 pre-B cells, or primary B cells (Figures 4C and 4D; Figures S4A and S4B). Of note, Miz-1 overexpression in 70Z/3 cells did not influence their cell cycle progression (Figure S4C). We analyzed a genomic region of 10 kb and found that Miz-1 bound to promoter sequences of the *Socs1* gene that are close to the transcription initiator site (Figures 4C and 4D; data not shown).

Please cite this article in press as: Kosan et al., Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor Signaling at Early Commitment Stages of B Cell Differentiation, Immunity (2010), doi:10.1016/j.immunol.2010.11.028

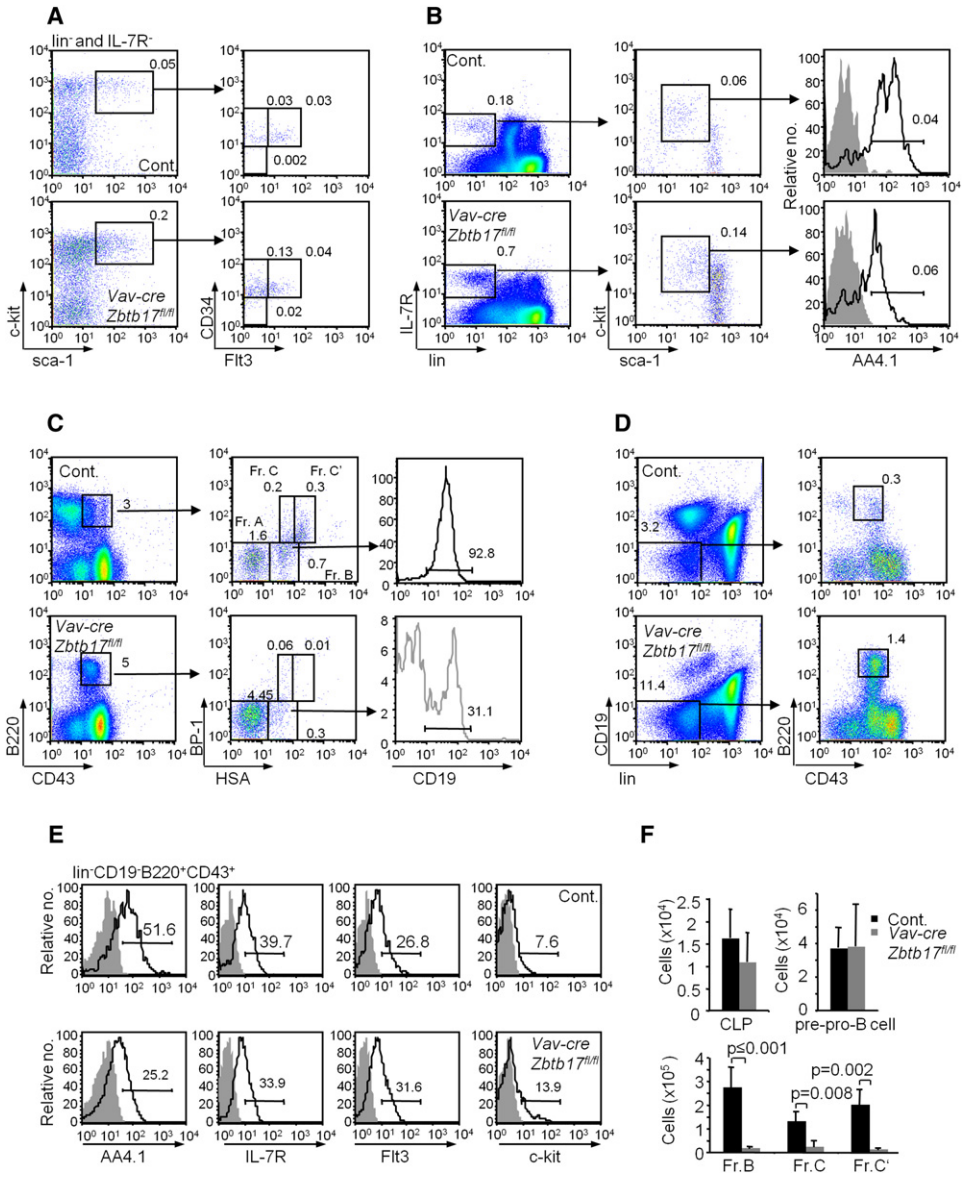

Figure 2. Miz-1 Function Is Required to Generate Pro-B Cells

Bone marrow cells from control and *Vav-cre Zbtb17*$^{fl/fl}$ mice were analyzed via flow cytometry.

(A) Lineage-negative and IL-7R-negative cells (lin$^-$ and IL-7R$^-$) were analyzed for c-kit, sca-1, CD34, and Flt3 expression.

(B) Surface expression of lineage marker (lin), IL-7R, sca-1, c-kit, and AA4.1 on bone marrow cells.

(C) Flow cytometric analysis of B220, CD43, HSA, BP-1, and CD19 surface expression on bone marrow cells. B220$^+$CD43$^+$ cells were electronically gated and analyzed for HSA and BP-1 expression. Fraction B (Fr. B) (B220$^+$CD43$^+$HSA$^+$BP1$^-$) cells were analyzed for CD19 expression. Numbers on gates represent percentages of living cells. Numbers in histograms represent percentages of cells in indicated gates. Fraction (Fr.) A–C′ are indicated on the gates.

(D) Expression of lin, CD19, B220, and CD43 on bone marrow cells. Lineage panel for pre-pro-B cells contains CD11b, Gr-1, Ter119, IgM, TCR-β, TCR-γδ, CD8, CD4, CD3, NK1.1, Ly-6c, and CD5. Lin$^-$CD19$^-$ cells were analyzed for the expression of B220 and CD43.

(E) The expression of AA4.1, IL-7R, Flt3, and c-kit on pre-pro-B cells (lin$^-$CD19$^-$B220$^+$CD43$^+$). Open histograms represent the expression of each surface marker and filled histograms represent isotype-matched irrelevant antibodies, respectively. Numbers in histograms represent percentages of (lin$^-$CD19$^-$B220$^+$CD43$^+$).

(F) Absolute numbers of CLPs (lin$^-$IL-7R$^+$AA4.1$^+$sca-1^{med}c-kit^{med}), pre-pro-B cells (lin$^-$CD19$^-$B220$^+$CD43$^+$AA4.1$^+$), Fr. B cells (B220$^+$CD43$^+$HSA$^-$BP1$^-$CD19$^+$), Fr. C cells (B220$^+$CD43$^+$HSA$^+$BP1$^+$CD19$^+$), and Fr. C′ cells (B220$^+$CD43$^+$HSA^{hi}BP1$^+$ CD19$^+$). Absolute cells numbers (femur and tibia) were corrected to living cells (based on FSC and SSC gating) from control mice (black) and *Vav-cre Zbtb17*$^{fl/fl}$ (gray).

A minimum of five mice was analyzed for each group. Error bars indicate the standard deviation (±SD); a p value is given in the figure. See also Figure S2.

Please cite this article in press as: Kosan et al., Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor Signaling at Early Commitment Stages of B Cell Differentiation, Immunity (2010), doi:10.1016/j.immuni.2010.11.028

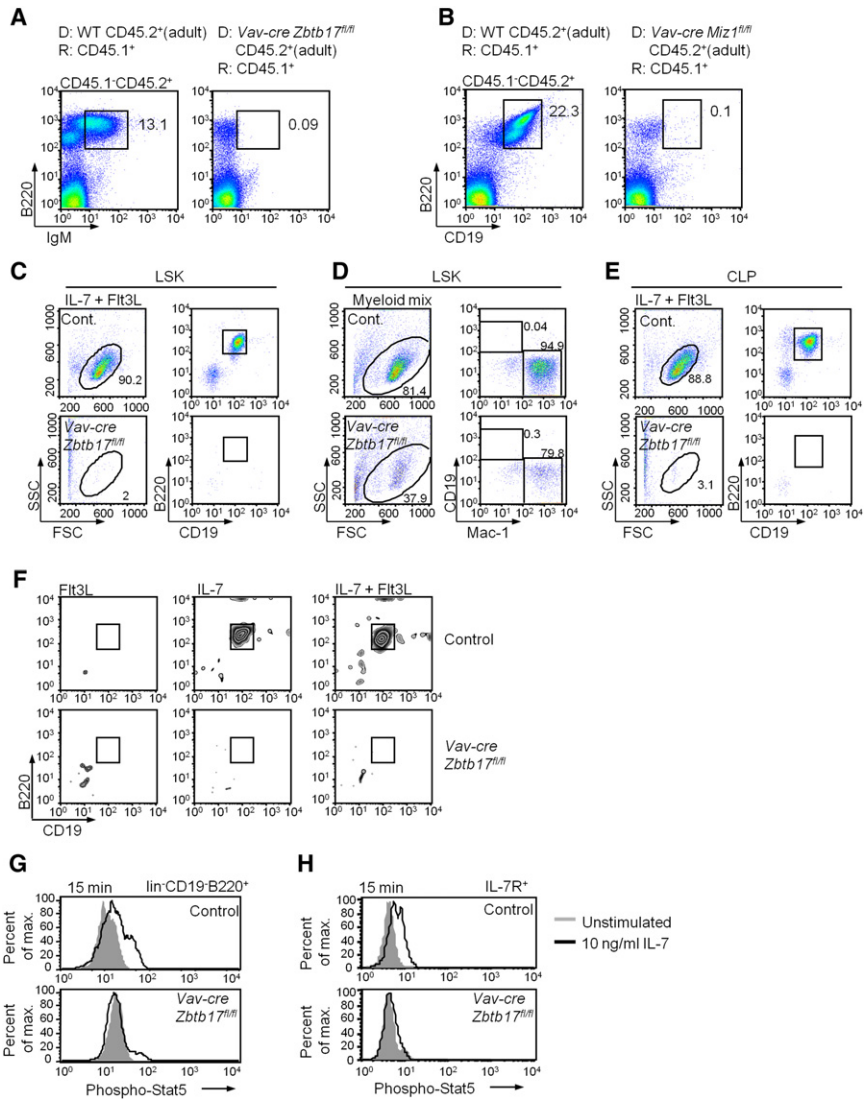

Figure 3. Miz-1 Is Indispensable for Progenitor Differentiation and Survival upon Cytokine Stimulation

(A and B) Lethally irradiated recipients (CD45.1) were reconstituted with bone marrow cells (Terr119$^-$B220$^-$CD19$^-$) from WT control mice or *Vav-cre Zbtb17*$^{fl/fl}$ mice (both CD45.2$^+$). Bone marrow cells were analyzed 3–4 months after transplantation by flow cytometry with antibodies for CD45.1, CD45.2, B220, and IgM (A) and with antibodies for CD45.1, CD45.2, B220, and CD19 (B). Data represent three independent experiments. Numbers on gates indicate percentages. (D, donor; R, recipient.)

(C) LSK (lin$^-$sca-1$^+$c-kit$^+$) cells from control mice or *Vav-cre Zbtb17*$^{fl/fl}$ mice were flow cytometrically sorted and cocultured on OP9 cells in the presence of Flt3L and IL-7 for 10–12 days. Cells were analyzed for forward (FSC) and side (SSC) scatter and the surface expression of B220 and CD19 by flow cytometry.

(D) LSK (lin$^-$sca-1$^+$c-kit$^+$) cells from control mice or *Vav-cre Zbtb17*$^{fl/fl}$ mice were flow cytometrically sorted and cocultured on OP9 cells in the presence of a myeloid cytokine cocktail for 6–8 days. Cells were analyzed for FSC and SSC and the surface expression of Mac1 and CD19 by flow cytometry.

(E) CLP (lin$^-$IL-7R$^+$sca-1^{med}c-kit^{med}) cells from control mice or *Vav-cre Zbtb17*$^{fl/fl}$ mice were flow cytometrically sorted and cocultured on OP9 cells in the presence of Flt3L and IL-7 for 10–12 days. Cells were analyzed for FSC and SSC as well as the surface expression of B220 and CD19 by flow cytometry.

(F) CLP were sorted and cultured under stroma-free conditions in the presence of Flt3L, IL-7 or Flt3L, and IL-7 for 6 days. Cells were analyzed for the surface expression of B220 and CD19 by flow cytometry.

Data represent at least four (C, E, F) or two (D) independent experiments.

(G and H) Flow cytometry of Stat5 phosphorylation in lin$^-$CD19$^-$B220$^+$ (lin: Mac1, Terr119) (G) or in IL-7R$^+$ bone marrow cells (H) from control mice or *Vav-cre Zbtb17*$^{fl/fl}$ mice. Open histograms represent the expression of phospho-Stat5 after 15 min IL-7 stimulation and filled histograms represent phospho-Stat5 in unstimulated cells, respectively. Data represent three (G) or two (H) independent experiments.

See also Figure S3.

We could not find a similar binding site in the *Socs3* promoter region (data not shown). This suggested that Miz-1 functions as a direct transcriptional repressor of the *Socs1* gene and that *Socs3* regulation by Miz-1 must be indirect.

Apoptosis in *Zbtb17*-Deficient B Cells Can Be Partially Rescued by *Bcl2* or Inhibition of *Socs1*

CLPs or Fraction A cells from *Vav-cre Zbtb17*$^{fl/fl}$ mice did not indicate a deregulation of survival genes such as *Bcl2* and *Mcl1* or proapoptotic genes like *Bad* and *Bax* (Figure 5A), which is also consistent with findings reported for the *IL7r*$^{-/-}$ mice (Kikuchi et al., 2005). One exception is *Bcl2l1*, which we found slightly downregulated in CLPs from *Vav-cre Zbtb17*$^{fl/fl}$ mice (Figure 5A). ChIP on the promoter regions of *Bcl2l1* or *Ebf1* did not show Miz-1 binding (data not shown); however, scanning over a 10 kb region of the *Bcl2* promoter revealed a Miz-1 binding site about 1.4 kb upstream of the initiator site (Figure 5B; Figure S5A). Together with the fact that *Bcl2* can no longer be induced by IL-7, these findings suggest that Miz-1 may be required for the upregulation of *Bcl2* upon IL-7 and acts by directly binding to the *Bcl2* promoter at a site different from the initiator.

To test whether absence of a functional Miz-1 affected the survival of B lineage cells in vivo, we stained CLPs and pre-pro-B cells (lin$^-$B220$^+$CD43$^+$CD19$^-$) of Miz-1-deficient mice or controls with AnnexinV, but we did not find different rates of cell death (Figure 5C). In contrast, 5- to 10-fold increased frequencies of apoptotic cells (AnnexinV$^+$) were observed in lin$^-$B220$^+$CD43$^+$CD19$^+$ cells (Fraction B–C′) and in B220$^+$CD43$^-$ cells, respectively, in the absence of a functional Miz-1 (Figure 5C), which may explain the loss of committed CD19$^+$ B cells (described in Figure 2).

Please cite this article in press as: Kosan et al., Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor Signaling at Early Commitment Stages of B Cell Differentiation, Immunity (2010), doi:10.1016/j.immuni.2010.11.028

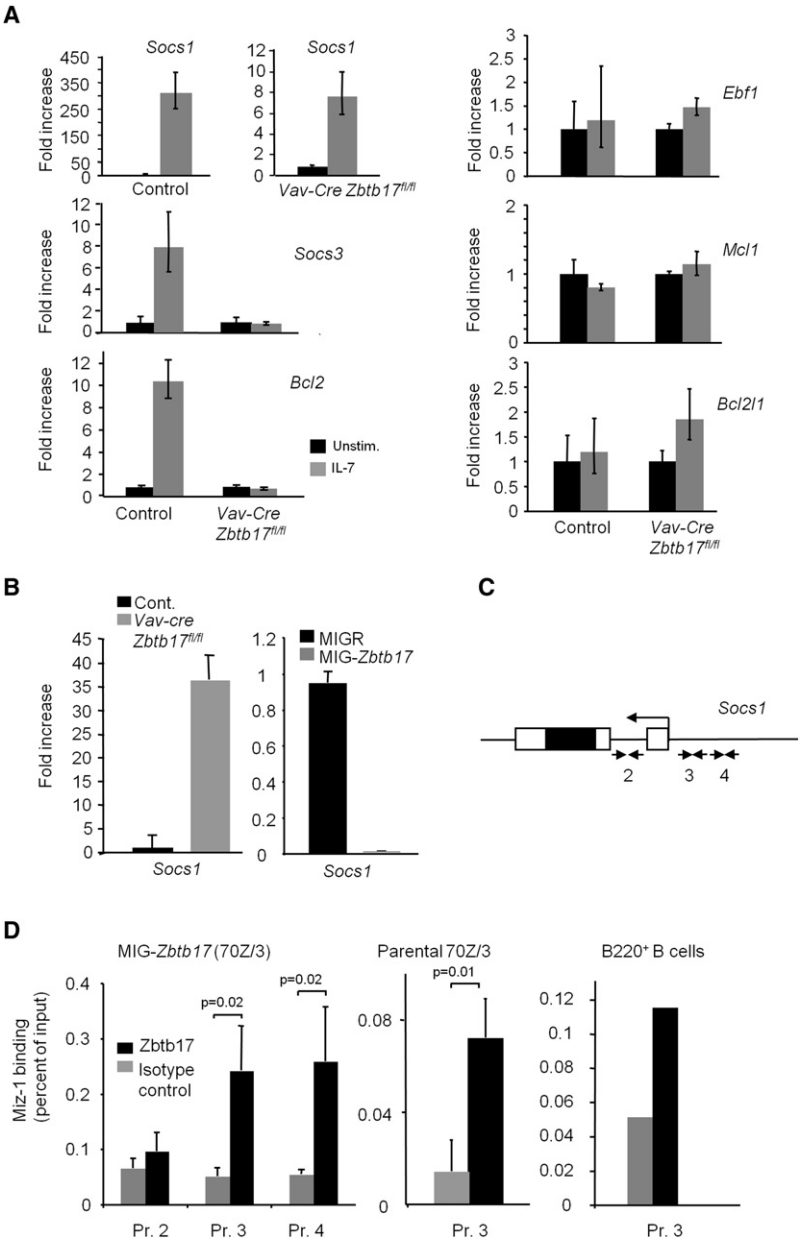

Figure 4. Miz-1 Is Required to Regulate the Expression of IL-7-Responsive Genes in CLPs

(A) CLPs from the bone marrow of control and *Vav-cre Zbtb17$^{fl/fl}$* mice were separated by flow cytometric cell sorting and cultured for 120 min in the absence and presence of IL-7. RNA was extracted and reverse-transcribed and used for quantitative PCR. Expression of the indicated genes was measured and normalized to the expression of the *Gapdh* gene and is presented as the fold increase relative to cDNA from control mice or unstimulated cells (set as 1-fold). Data represent three independent experiments each done in triplicate. Error bars indicate the standard deviation (±SD).

(B) Expression of *Socs1* determined by qRT-PCR in CLPs from control and and *Vav-cre Zbtb17$^{fl/fl}$* mice (left), and in 70Z/3 cells transduced with empty vector (MIGR1) or with a MIG-*Zbtb17*-GFP virus (right). Error bars indicate the standard deviation (±SD).

(C) *Socs1* locus, showing coding regions (black boxes) and noncoding regions (open boxes) and location of primers (black triangles).

(D) Chromatin immunoprecipitation (ChIP) showing binding of Miz-1 to the promoter region of *Socs1*. Used were α-Miz-1 antibodies (H190) and α-isotype control antibodies on extracts of MIG-*Zbtb17*-GFP transduced 70Z/3 cells (left), 70Z/3 cells (middle), or primary B cells (B220$^+$ cells) (right), followed by a quantitative RT-PCR via primer pairs of indicated regions. Data represent two independent experiments each done in triplicate (PCR on primary B cells was done in duplicates). Error bars indicate the standard deviation (±SD).

See also Figure S4 and Tables S1 and S2.

In an attempt to counteract this enhanced rate of cell death, we crossed an *H2-K1-Bcl2* transgene, which directs a high constitutive expression of Bcl2 throughout hematopoiesis, including HSCs and early lymphoid progenitors (Figure S5B; Domen et al., 1998; Kondo et al., 1997a) into *Vav-cre Zbtb17$^{fl/fl}$* mice. In the resulting combinatorial mutant animals, B220$^+$ CD19$^+$ B cells were now detected in the bone marrow, reaching about 40% of control frequencies (Figure 6A). Importantly, lin$^-$CD19$^+$B220$^+$CD43$^+$ pro-B cells, which were almost undetectable in Miz-1-deficient mice, now reappeared in the presence of the *H2-K1-Bcl2* transgene (Figure 6A). We also found that in contrast to CLPs from *Vav-cre Zbtb17$^{fl/fl}$*, CLPs from *Vav-cre Zbtb17$^{fl/fl}$ H2-K1-Bcl2* mice now survived and expanded in vitro on OP9 cells (Figure 6B), but gave rise to only a very small number of CD19$^+$ cells, possibly reflecting the partial rescue seen in vivo (Figure 6A). This indicated that *Bcl2* alone is not sufficient to overcome the developmental block imposed by Miz-1 deficiency.

Socs1 is a negative regulator of the IL-7 signaling pathway and we did not only observe high *Socs1* expression in Miz-1-deficient CLPs, but our ChIP experiments also suggested that Miz-1 directly binds to *Socs1* promoter, possibly modulating its expression. Hence, we reasoned that inhibition of *Socs1* could restore IL-7 responsiveness in Miz-1-deficient cells and allow the development of CD19-positive cells. To test this, we performed a silencing experiment with gene-specific "Morpholino" oligonucleotides that are able to interfere with Socs1 protein expression (Figure 6C). LSKs from *Vav-cre Zbtb17$^{fl/fl}$* transfected with *Socs1*-specific Morpholinos showed a higher percentage of survival compared to *Vav-cre Zbtb17$^{fl/fl}$* transfected with a control Morpholino. These cells were able to expand on OP9 cells in the presence of IL-7 but did not upregulate CD19 (Figure 6D). This suggested that high *Socs1* expression was at least partially responsible for the lack of survival of Miz-1-deficient B lineage precursors on OP9 cells in vitro.

Ebf1 and Bcl2 Restore the Ability of Miz-1-Deficient Precursors to Generate CD19$^+$ B Cells

The inability to fully rescue B cell differentiation in Miz-1-deficient progenitors by restoration of the IL-7-Stat5-Bcl2 pathway either

Please cite this article in press as: Kosan et al., Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor Signaling at Early Commitment Stages of B Cell Differentiation, Immunity (2010), doi:10.1016/j.immuni.2010.11.028

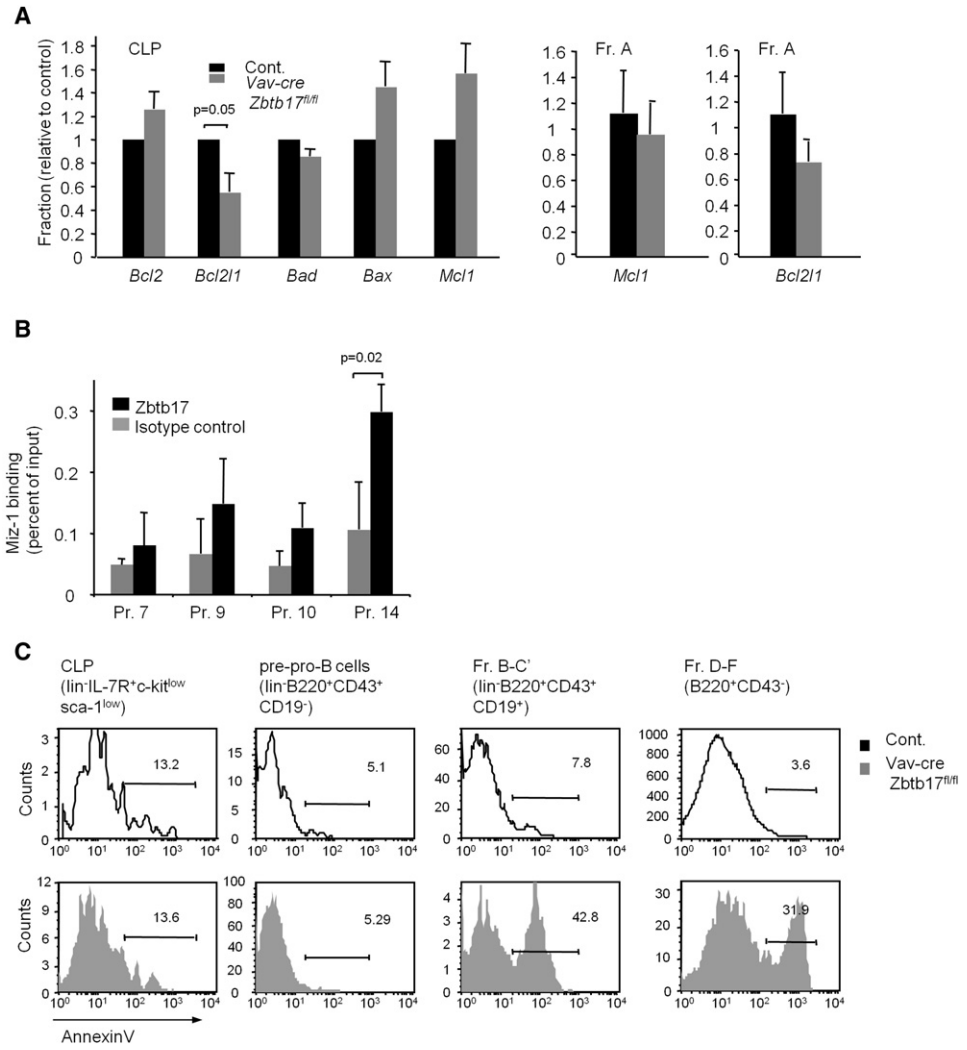

Figure 5. Apoptosis during B Cell Development in Miz-1-Deficient Mice

(A) CLPs or Fraction A ($CD19^-B220^+CD43^+$) cells from control and *Vav-cre Zbtb17$^{fl/fl}$* mice were separated by flow cytometric cell sorting. RNA was extracted and reverse transcribed and used for quantitative PCR with indicated primer. All values were normalized to the expression of the *Gapdh* gene and are presented as the fraction relative to cDNA from control mice (set as 1-fold). Data show three independent experiments, each done in triplicates. Error bars indicate the standard error of the mean (±SEM).

(B) Chromatin immunoprecipitation (ChIP) of the binding of Miz-1 to the promoter region of *Bcl2*. Used were a Miz-1 antibody (H190) and an isotype control antibody and extracts of MIG-*Zbtb17*-GFP transduced 70Z/3 cells, followed by a qRT-PCR of the indicated genomic regions. Data represent two independent experiments, each done in triplicate. Error bars indicate the standard deviation (±SD). A p value is indicated.

(C) CLPs (lin^-IL-7Rα^+c-$kit^{med}$$sca\text{-}1^{med}$), pre-pro-B ($lin^-B220^+CD43^+CD19^-$), pro-B ($lin^-B220^+CD43^+CD19^+$), and Fr. D–F B ($B220^+CD43^-$) cells were electronically gated and analyzed for apoptotic cells via AnnexinV staining. Numbers in histograms indicate percentages of each indicated gate. Data represent three independent experiments.

See also Figure S5 and Tables S1 and S2.

by providing excess Bcl2 or by inhibiting *Socs1* suggested that an additional defect exists (Domen et al., 1998; Kondo et al., 1997a). Comparative expression analysis showed that lymphoid-specific genes such as *Tcf3*, *Ebf1*, *Dntt*, *Pax5*, *Rag1*, and *Rag2* were decreased in Miz-1-deficient CLPs versus controls (Figure 7A). Ebf1 is essential for B cell development and responsible for the expression of Pax5 and eventually also Rag1 and Rag2. Therefore, we expected to be able to rescue B cell differentiation in Miz-1-deficient cells by overexpressing Ebf1. We restored Ebf1 expression in Miz-1-deficient progenitors via a murine stem cell virus (MSCV) containing the *Ebf1* gene and GFP as a marker (Figure S6A). However, LSK cells from *Vav-cre Zbtb17$^{fl/fl}$* mice transduced with the *Ebf1*-expressing virus did not develop into $CD19^+$ cells and almost all cells were lost when cultured on OP9-stroma cells, whereas control LSK cells showed an accelerated differentiation into $CD19^+$ B cells (Figure 7B). Similar to this, forced expression of a rearranged transgenic V(D)J segment at the IgH locus (Cascalho et al., 1996) did not rescue adult B cell development in Miz-1-deficient mice (Figure S6B).

Please cite this article in press as: Kosan et al., Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor Signaling at Early Commitment Stages of B Cell Differentiation, Immunity (2010), doi:10.1016/j.immunol.2010.11.028

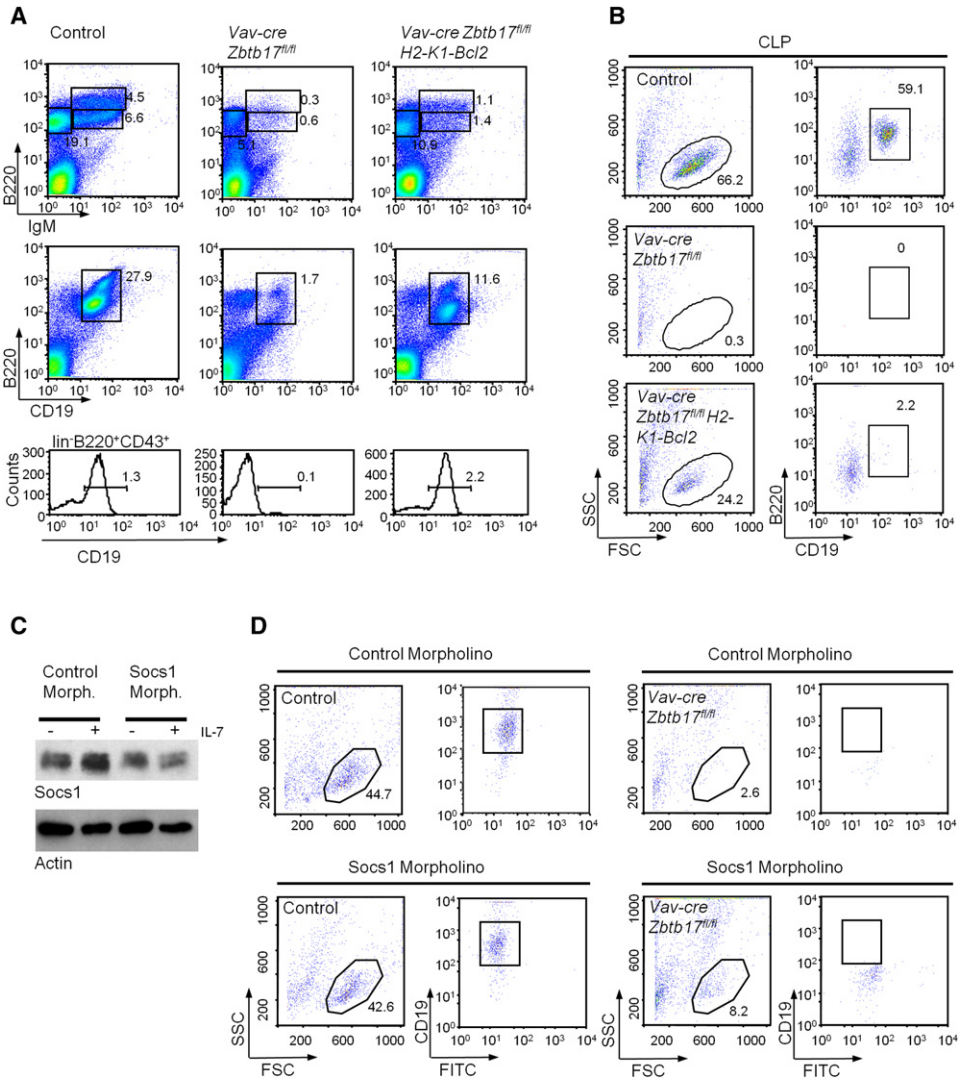

Figure 6. *Bcl2* Expression or *Socs1* Inhibition Enables Miz-1-Deficient Precursors to Survive with IL-7

(A) Flow cytometry analysis of bone marrow from control, *Vav-cre Zbtb17*$^{fl/fl}$, and *Vav-cre Zbtb17*$^{fl/fl}$ *H2-K1-Bcl2* mice with the indicated surface markers. Numbers in dot plots indicate percentages of each gate. Bone marrow cells were analyzed with the pre-pro-B lineage markers (CD11b, Gr-1, Ter119, IgM, TCR-β, TCR-γδ, CD8, CD4, CD3, NK1.1, Ly-6c, and CD5) and B220, CD43 CD19, and IgM to define the pro-B cell population. Numbers in histograms indicate percentages of pro-B cells for the indicated mice. Data are representative of three independent experiments.

(B) CLP (lin$^-$IL-7R$^+$sca-1^{med}c-kit^{med}) cells from control, *Vav-cre Zbtb17*$^{fl/fl}$, and *Vav-cre Zbtb17*$^{fl/fl}$ *H2-K1-Bcl2* were flow cytometrically sorted and cocultured on OP9 stroma cells in the presence of Flt3L and IL-7 for 7–8 days. Cells were analyzed for FSC and SSC as well as the surface expression of B220 and CD19 by flow cytometry. Data are representative of two independent experiments.

(C) Expression of Socs1 protein in 70Z/3 cells stimulated or not with IL-7 in the presence or absence of an anti-Socs1 Morpholino demonstrates its effect to knockdown Socs1 protein expression in the presence of IL-7.

(D) Sorted LSKs from control or *Vav-cre Zbtb17*$^{fl/fl}$ mice were incubated with Morpholino against *Socs1* mRNA or a control Morpholino. Shown is the fluorescein isothyocyanate fluorescence after incubation, indicative of an almost complete and efficient transfer of morpholino. Cells from control or *Vav-cre Zbtb17*$^{fl/fl}$ treated either with control or anti-Socs1 Morpholinos were analyzed after 8 days of OP9 coculture (representative of at least three independent experiments).

We hypothesized that because both the IL-7-Stat5-Bcl2 signaling pathway that regulates cell survival and the Ebf1-Pax5-Rag1-Rag2 axis that regulates differentiation are both affected by Miz-1 deficiency, a full rescue of B lineage differentiation might be achieved only if both pathways are restored. To test this, we sorted LSK cells from *Vav-cre Zbtb17*$^{fl/fl}$ *H2-K1-Bcl2* mice and transduced them with a retroviral vector expressing *Ebf1* or a virus made with an empty vector control. When GFP$^+$ cells obtained after infection were cultured on OP9 cells, those that had received the *Ebf1*-expressing retrovirus were indeed able to fully differentiate into CD19$^+$ cells, but LSKs from *Vav-cre Zbtb17*$^{fl/fl}$ *H2-K1-Bcl2* infected with the control retrovirus alone did not (Figure 7C). This indicated that re-expression of both Bcl2 and Ebf1 is needed to restore B cell differentiation from Miz-1-deficient progenitors and confirms that the transcription factor Miz-1 is required to

Please cite this article in press as: Kosan et al., Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor Signaling at Early Commitment Stages of B Cell Differentiation, Immunity (2010), doi:10.1016/j.immuni.2010.11.028

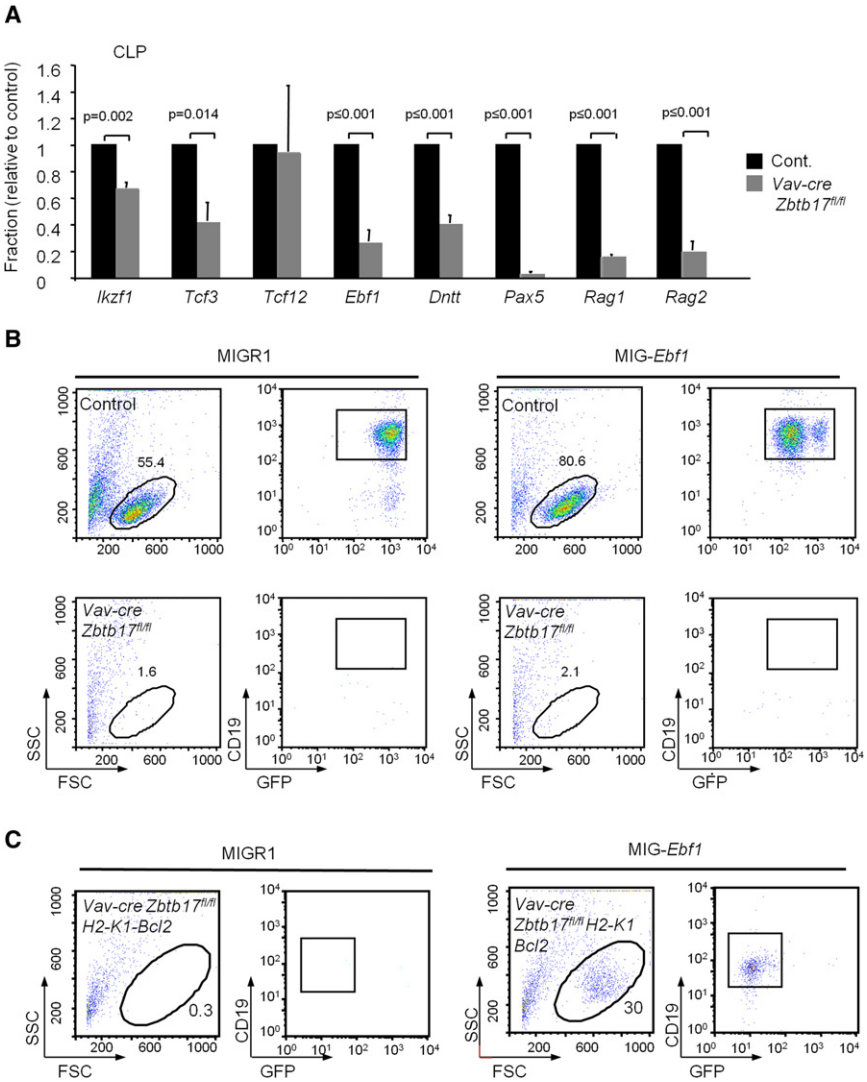

Figure 7. Reconstitution of B Cell Development in Miz-1-Deficient Precursors by Ebf1 and Bcl2

(A) CLPs (lin$^-$IL-7R$^+$c-kit^{med}sca-1^{med}) were isolated by flow cytometric cell sorting from the bone marrow of control mice and *Vav-cre Zbtb17$^{fl/fl}$* mice. RNA was extracted and reverse-transcribed and used for quantitative PCR. All values are normalized to the expression of the *Gapdh* gene and are presented as the fraction relative to cDNA from control mice (set as 1-fold). Data show three independent experiments each done in triplicates. Error bars indicate the standard error of the mean (±SEM).

(B) LSK cells sorted from bone marrow were infected with MIGR1-GFP or MIG-*Ebf1*-GFP retroviruses. Transduced cells (GFP$^+$) were FACS sorted (Figure S6) and equal cell numbers were directly cultured on OP9 cells in the presence of SCF, Flt3L, and IL-7. Cells were analyzed for CD19 and GFP expression by flow cytometry 10–14 days after infection. Numbers on gates indicate percentages. Data are representative of three independent experiments.

(C) LSK cells were sorted from *Vav-cre Zbtb17$^{fl/fl}$ H2-K1-Bcl2* mice and were infected with MIGR1-GFP or MIG-*Ebf1*-GFP retroviruses. After infection, LSK cells were cultured in the presence of SCF, Flt3L, and IL-7 on OP9 cells and the GFP$^+$ fraction was isolated by sorting and further cultured under the same conditions. Cells were analyzed for CD19 and GFP expression by flow cytometry 16 days after infection, Numbers on gates indicate percentages.

Data are representative of two independent experiments. See also Figure S6 and Table S2.

regulate survival and developmental networks during early B cell development.

DISCUSSION

The development of B cells in the adult bone marrow is a well-defined process, in which cytokine signaling, V(D)J recombination, and the regulation of gene expression by transcription factors play a central role (Medina et al., 2004; Singh et al., 2007). The signal transduction process initiated by IL-7 and its direct and indirect downstream effectors and also the transcription factors Ebf1 and Pax5 are essential for the early commitment and differentiation stages of B cell development. In this study, we present evidence that the POZ-domain protein Miz-1 (*Zbtb17*) is essential for B cell development. Our data suggest that Miz-1 regulates IL-7 signaling by monitoring on one hand the expression of *Socs1*, a negative regulator, and on the other hand *Bcl2*, a positive effector of IL-7 signaling. In addition, Miz-1 is required for the proper expression of *Tcf3* and *Ebf1* and thus assures the functioning of the Ebf1-Pax5-Rag1-Rag2 axis.

Cre-mediated *Zbtb17* deletion initiated in hematopoietic stem cells by the *Vav* promoter confirmed this and suggested a role of Miz-1 at a stage of early uncommitted B cell progenitors. Flow cytometry data further supported the view that Miz-1 deficiency affects mainly follicular B cells and their differentiation, but not the formation of marginal zone B cells. This is similar to the phenotype reported in *Il7*- or *Il7r*-deficient mice (Carvalho et al., 2001; Hesslein et al., 2006), where most residual peripheral B cells are marginal zone (MZ) B cells. MZ B cells are long-lived cells and are less affected by the absence of IL-7 signaling (Hesslein et al., 2006; Lu and Cyster, 2002), which might explain their accumulation in Miz-1-deficient mice. Hence, the fact that we see a loss of follicular B cells but not MZ B cells could point to a possible role of Miz-1 in IL-7 signaling. Along this line, the severe drop in absolute CD19$^+$ pro-B cell numbers and the fact that CLP or pre-pro-B cell numbers were unaffected in Miz-1-deficient mice suggested a block precisely at the pre-pro-B to pro-B cell transition, which is very similar to the reported observations in mice deficient for *IL7* or *IL7r* (Dias et al., 2005; Kikuchi et al., 2005).

Our data clearly show that activation of Stat5 by Jak-mediated phosphorylation in response to IL-7 is almost undetectable in

Please cite this article in press as: Kosan et al., Transcription Factor Miz-1 Is Required to Regulate Interleukin-7 Receptor Signaling at Early Commitment Stages of B Cell Differentiation, Immunity (2010), doi:10.1016/j.immuni.2010.11.028

Miz-1-deficient cells. This is probably due to the fact that *Vav-cre Zbtb17$^{fl/fl}$* CLPs highly express the Jak inhibitor *Socs1* and are thus unable to upregulate IL-7-responsive target genes. Moreover, our ChIP and mRNA expression data very strongly suggest that the normal function of Miz-1 during IL-7-dependent early B cell development is to dampen the expression of *Socs1* through direct binding to its transcription initiator site and to allow the activation of Stat5 and the subsequent upregulation of IL-7 target genes. Because the inhibition of *Socs1* allows Miz-1-deficient cells to survive on OP9 and in the presence of IL-7, it is conceivable that Miz-1 plays an important role in regulating IL-7-mediated survival signals.

In a close perspective to our findings, it has been shown that during early B cell development, *Bcl2* expression is highly upregulated in uncommitted "Fraction A" B cells that contain pre-pro-B cells to provide a survival signal (Li et al., 1993). It has also been reported that under stroma-free conditions, IL-7 alone enables CLPs to both survive and differentiate into B220^{+}CD19^{+} pro-B cells (Kikuchi et al., 2005; Miller et al., 2002). Our expression analysis showed that in the absence of Miz-1, *Bcl2* expression cannot be upregulated upon IL-7 stimulation in CLPs, and ChIP experiments demonstrated a direct binding of Miz-1 to the *Bcl2* promoter. This suggests that Miz-1 exerts a second function in the IL-7R pathway, which is different from the repression of *Socs1* and acts as a direct transcriptional activator of *Bcl2*, probably in response to IL-7. Such a role would be consistent with two different reports that have previously demonstrated that *Bcl2* is a direct effector gene of Miz-1 (Patel and McMahon, 2007; Saito et al., 2009).

Miz-1 has been discovered as a Myc binding protein and its function as a repressor has been described in a complex with Myc on the promoters of cell cycle regulator genes such as *Cdkn1a* and *Cdkn2b* (Phan et al., 2005; Seoane et al., 2001, 2002; Staller et al., 2001; Wu et al., 2003). None of these Miz-1 targets that regulate cell cycle progression were altered in Miz-1-deficient cells (data not shown). Consistent with this, we could not find evidence for disturbed cell cycle progression in Miz-1-deficient B cell progenitors, suggesting that the regulatory function of Miz-1 that we describe here is independent of Myc. In addition, the few remaining peripheral follicular B cells did not show a proliferation defect (data not shown) in spite of the fact that they emerged from a disturbed early B cell development. To obtain further insights in the role of Miz-1 in peripheral follicular B cells, other Cre deleter strains will have to be used.

The knockout of either *Ebf1* or *Tcf3* leads to a complete block at the pre-pro-B to pro-B transition (Bain et al., 1994; Lin and Grosschedl, 1995), which resembles the phenotype seen in *Miz-1*-deficient mice. However, *Tcf3* is necessary for CLP formation and E2A-deficient mice have a marked reduction in their CLP numbers (Bain et al., 1997; Borghesi et al., 2005), which is different from our observations in the Miz-1-deficient mice. In addition, E2A is necessary to upregulate *Ebf1* for allowing progenitors to develop into B cells, and ectopic expression of Ebf1 in *Tcf3*-deficient progenitors rescued B lymphocyte differentiation (Bain et al., 1994; Seet et al., 2004). In Miz-1-deficient CLPs, *Tcf3* expression is reduced to about 50% and this residual expression might be sufficient to maintain CLP numbers in Miz-1-deficient mice but may be the cause of the reduced [illegible] amounts. It is unlikely that Miz-1 regulates *Ebf1* expression directly because ChIP experiments did not provide evidence for Miz-1 binding on the *Ebf1* promoter. It is, however, possible that Miz-1 acts further upstream and interferes with the expression of *Tcf3* via a mechanism that remains to be elucidated.

If regulation of the E2A-Ebf1-Pax5-Rag1-Rag2 axis would fully describe the biochemical function of Miz-1, the retrovirally re-expression of *Ebf1* or a rearranged variable heavy chain gene should have rescued B cell differentiation in Miz-1-deficient progenitors. However, this is not the case, suggesting that another defect outside the Ebf1-Pax5-Rag1-Rag2 signaling pathway must exist in cells lacking Miz-1. A very likely solution to this is the role of Miz-1 in the part of IL-7 signaling that provides a survival signal to B cell progenitors described above. Ectopic expression of Ebf1 in *IL7r*$^{-/-}$ progenitor cells leads to an initial development of B220^{+}CD19^{+}IgM^{+} B cells but cannot overcome a defect in cell survival (Kikuchi et al., 2005). Our data are consistent with this and corroborate recent reports indicating that regulation of Ebf1 is independent of IL-7 (Malin et al., 2010). Our own findings that *Ebf1* cannot be induced by IL-7 and that re-expression of *Ebf1* alone was not sufficient to rescue B cell development in Miz-1-deficient mice confirm this notion further.

When we crossed Miz-1-deficient mice with transgenic mice overexpressing *Bcl2*, a substantial but still partial rescue of B cell differentiation was seen in vivo, but differentiation of Miz-1-deficient B cell precursors expressing *Bcl2* did not occur when cultured on OP9 stroma in vitro. The introduction of both Bcl2 and Ebf1, which has been shown to promote B cell development by upregulating B cell genes like *Pax5*, *Rag1* and *Rag2*, and *Cd19* (Medina et al., 2004; O'Riordan and Grosschedl, 1999; Pongubala et al., 2008) finally enabled us to fully reconstitute B cell commitment in Miz-1-deficient precursors when cultured in vitro. This was evident because only with *Bcl2* and *Ebf1*, Miz-1-deficient progenitors were able to significantly upregulate CD19 expression, which indicates that B cell lineage commitment has taken place. We found that *Cd19-Cre Zbtb17$^{fl/fl}$* mice, which excise later than *Vav-Cre Zbtb17$^{fl/fl}$* at pro-B and pre-B stages (Rickert et al., 1997), have normal B cell development (data not shown), so we conclude that Miz-1 acts precisely at the pre-pro-B to pro-B transition, where Ebf1 is critical for commitment. This experiment supports a comprehensive model, in which Miz-1 exerts two functions in early B lineage progenitors: one in the regulation of the IL-7-independent E2A-Ebf1-Pax5-Rag1-Rag2 axis responsible for B cell differentiation and another role in the IL-7-dependent upregulation of *Bcl2* that ensures survival of B cell progenitors. In summary, the evidence that we present here establishes the POZ transcription factor Miz-1 as a regulatory element required at a critical point where signals from the IL-7 receptor have to be relayed to effector genes that mediate survival (such as *Bcl2*) and to be coordinated with signals that enable differentiation (such as *Ebf1*) to allow full lineage commitment and differentiation along the B cell lineage.

EXPERIMENTAL PROCEDURES

Mice

All experiments performed on mice were approved by the IRCM animal care committee and done in accordance with the regulation of the Canadian Council of Animal Care.

For additional information see Supplemental Experimental Procedures.

Flow Cytometry, Cell Surface Staining, Stimulation, and Cell Sorting

Single-cell suspensions were prepared at the time of autopsy from thymus, bone marrow, or spleen in PBS supplemented with 1% FCS (staining solution). Antibody incubation was performed at 4°C for 15 min in 1% FCS in PBS (for more detail see Supplemental Information). For the intracellular flow cytometry analysis of pStat-5, cells were incubated for 1.5 hr at 37°C to shut off endogenous signaling prior to stimulation. Cells were harvested, washed with PBS, and incubated with or without 10 ng/ml IL-7 (Peprotech 217-17) for 15 min at 37°C. After stimulation, cells were fixed with formaldehyde (BD cytofix) and additionally permeabilized with methanol (BD Phosflow Perm III). Samples were then stained with anti-phospho-Stat5(Y694)-Alexa488 (BD Phosflow 612598) or Alexa488 Mouse IgG1κ isotype control (BD Phosflow). AnnexinV staining was performed with the AnnexinV-FITC Detection Kit I (Becton Dickinson) and by following the manufacturer's instructions.

Chromatin Immunoprecipitation Assay

ChIP assays were performed with ChIP-IT Express (Active Motif) according to the manufacturer's instructions. Cells were fixed with 1% paraformaldehyde (PFA) neutralized with glycine, washed, and lysed with IGEPAL lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% IGEPAL, 1 mM PMSF). The lysate was sonicated (Branson Digital Sonifier). One percent of the soluble fraction (input) was kept and the rest was precleared with salmon sperm DNA-Protein G-agarose (Upstate). This was then divided into two tubes and incubated with 15 μg/ml of rabbit anti-Miz-1 polyclonal IgG (H190, Santa Cruz) or 15 μg/ml rabbit control IgG-ChIP grade antibodies (Abcam). The immune complexes were then precipitated with Protein G-agarose and eluted according to the manufacturer's instruction. DNA was purified with a polymerase chain reaction (PCR) purification kit (QIAGEN). Quantitative PCR was performed with the SYBR Green system on the Invitrogen Mx3005. Primers used for experiments are listed in Table S1.

Retroviral Transduction

Retroviruses were generated with 293-GPG cells. GP+E cells were infected with retroviruses from the 293-GPG cells for coculture and virus production. Hematopoietic cells were transduced spin-infection. For spin infection, sorted LSK cells were resuspended in viral supernatant in the presence of polybrene (12.5 μg/ml) and were centrifuged by 1400 × g for 2 hr. LSK cells were washed with Opti-MEM and cocultured on OP9 cells (Opti-MEM [10% (vol/vol) FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM β-mercaptoethanol] containing 10 ng/ml SCF, 10 ng/ml Flt3L, and 1 ng/ml IL-7). Four to five days after infection, transduced cells were cell sorted by flow cytometry on the basis of GFP expression. Coculture transduced cells were plated on OP9 cells. After 4 days of coculture, cells were transferred onto new stroma cells supplied with new cytokine containing media and cultured for the indicated time periods.

Morpholino Silencing

LSK cells sorted by flow cytometry were cultured in the presence of SCF, Flt3L, and IL-7 for 1 hr under stroma-free conditions. Morpholinos were added to the culture in the presence of EndoPorter followed the manufacturer's instructions (Gene Tools, USA). After 4 hr progenitor cells were transferred on OP9 cells. Every 4 days, cells were transferred on new OP9 cells and new cytokines were added (see Supplemental Information).

Statistics

Two-tailed Student's t tests were used to calculate p values where indicated. A p value ≤ 0.05 was indicated as statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at doi:10.1016/j.immuni.2010.11.028.

ACKNOWLEDGMENTS

We thank H. Singh (University of Chicago) for the EBF retroviral construct and H.M. Jaeck (Erlangen, Germany) for the VHT-KI mice. This work was supported by the IRCM, the Canadian Foundation for Innovation (CFI), the CIHR operating grant 84526, and a Canada Research Chair (Tier1) to T.M.

Received: November 11, 2009
Revised: August 10, 2010
Accepted: October 1, 2010
Published online: December 16, 2010

REFERENCES

Adhikary, S., Peukert, K., Karsunky, H., Beuger, V., Lutz, W., Elsässer, H.P., Möröy, T., and Eilers, M. (2003). Miz1 is required for early embryonic development during gastrulation. Mol. Cell. Biol. *23*, 7648–7657.

Adolfsson, J., Månsson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A., et al. (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell *121*, 295–306.

Allman, D., Sambandam, A., Kim, S., Miller, J.P., Pagan, A., Well, D., Meraz, A., and Bhandoola, A. (2003). Thymopoiesis independent of common lymphoid progenitors. Nat. Immunol. *4*, 168–174.

Bain, G., Maandag, E.C., Izon, D.J., Amsen, D., Kruisbeek, A.M., Weintraub, B.C., Krop, I., Schlissel, M.S., Feeney, A.J., van Roon, M., et al. (1994). E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. Cell *79*, 885–892.

Bain, G., Robanus Maandag, E.C., te Riele, H.P., Feeney, A.J., Sheehy, A., Schlissel, M., Shinton, S.A., Hardy, R.R., and Murre, C. (1997). Both E12 and E47 allow commitment to the B cell lineage. Immunity *6*, 145–154.

Borghesi, L., Aites, J., Nelson, S., Lefterov, P., James, P., and Gerstein, R. (2005). E47 is required for V(D)J recombinase activity in common lymphoid progenitors. J. Exp. Med. *202*, 1669–1677.

Carvalho, T.L., Mota-Santos, T., Cumano, A., Demengeot, J., and Vieira, P. (2001). Arrested B lymphopoiesis and persistence of activated B cells in adult interleukin 7(-/-)- mice. J. Exp. Med. *194*, 1141–1150.

Cascalho, M., Ma, A., Lee, S., Masat, L., and Wabl, M. (1996). A quasi-monoclonal mouse. Science *272*, 1649–1652.

Christensen, J.L., and Weissman, I.L. (2001). Flk-2 is a marker in hematopoietic stem cell differentiation: A simple method to isolate long-term stem cells. Proc. Natl. Acad. Sci. USA *98*, 14541–14546.

de Boer, J., Williams, A., Skavdis, G., Harker, N., Coles, M., Tolaini, M., Norton, T., Williams, K., Roderick, K., Potocnik, A.J., and Kioussis, D. (2003). Transgenic mice with hematopoietic and lymphoid specific expression of Cre. Eur. J. Immunol. *33*, 314–325.

Dias, S., Silva, H., Jr., Cumano, A., and Vieira, P. (2005). Interleukin-7 is necessary to maintain the B cell potential in common lymphoid progenitors. J. Exp. Med. *201*, 971–979.

Domen, J., Gandy, K.L., and Weissman, I.L. (1998). Systemic overexpression of BCL-2 in the hematopoietic system protects transgenic mice from the consequences of lethal irradiation. Blood *91*, 2272–2282.

Herold, S., Wanzel, M., Beuger, V., Frohme, C., Beul, D., Hillukkala, T., Syvaoja, J., Saluz, H.P., Haenel, F., and Eilers, M. (2002). Negative regulation of the mammalian UV response by Myc through association with Miz-1. Mol. Cell *10*, 509–521.

Hesslein, D.G., Yang, S.Y., and Schatz, D.G. (2006). Origins of peripheral B cells in IL-7 receptor-deficient mice. Mol. Immunol. *43*, 326–334.

Igarashi, H., Gregory, S.C., Yokota, T., Sakaguchi, N., and Kincade, P.W. (2002). Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. Immunity *17*, 117–130.

Izon, D., Rudd, K., DeMuth, W., Pear, W.S., Clendenin, C., Lindsley, R.C., and Allman, D. (2001). A common pathway for dendritic cell and early B cell development. J. Immunol. *167*, 1387–1392.

Kikuchi, K., and Kondo, M. (2006). Developmental switch of mouse hematopoietic stem cells from fetal to adult type occurs in bone marrow after birth. Proc. Natl. Acad. Sci. USA *103*, 17852–17857.

Kikuchi, K., Lai, A.Y., Hsu, C.L., and Kondo, M. (2005). IL-7 receptor signaling is necessary for stage transition in adult B cell development through up-regulation of EBF. J. Exp. Med. *201*, 1197–1203.

Kondo, M., Akashi, K., Domen, J., Sugamura, K., and Weissman, I.L. (1997a). Bcl-2 rescues T lymphopoiesis, but not B or NK cell development, in common gamma chain-deficient mice. Immunity *7*, 155–162.

Kondo, M., Weissman, I.L., and Akashi, K. (1997b). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661–672.

Li, Y.S., Hayakawa, K., and Hardy, R.R. (1993). The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. J. Exp. Med. *178*, 951–960.

Lin, H., and Grosschedl, R. (1995). Failure of B-cell differentiation in mice lacking the transcription factor EBF. Nature *376*, 263–267.

Lin, J.X., Migone, T.S., Tsang, M., Friedmann, M., Weatherbee, J.A., Zhou, L., Yamauchi, A., Bloom, E.T., Mietz, J., John, S., et al. (1995). The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. Immunity *2*, 331–339.

Lu, T.T., and Cyster, J.G. (2002). Integrin-mediated long-term B cell retention in the splenic marginal zone. Science *297*, 409–412.

Malin, S., McManus, S., Cobaleda, C., Novatchkova, M., Delogu, A., Bouillet, P., Strasser, A., and Busslinger, M. (2010). Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development. Nat. Immunol. *11*, 171–179.

Månsson, R., Hultquist, A., Luc, S., Yang, L., Anderson, K., Kharazi, S., Al-Hashmi, S., Liuba, K., Thorén, L., Adolfsson, J., et al. (2007). Molecular evidence for hierarchical transcriptional lineage priming in fetal and adult stem cells and multipotent progenitors. Immunity *26*, 407–419.

Medina, K.L., Pongubala, J.M., Reddy, K.L., Lancki, D.W., Dekoter, R., Kieslinger, M., Grosschedl, R., and Singh, H. (2004). Assembling a gene regulatory network for specification of the B cell fate. Dev. Cell *7*, 607–617.

Miller, J.P., Izon, D., DeMuth, W., Gerstein, R., Bhandoola, A., and Allman, D. (2002). The earliest step in B lineage differentiation from common lymphoid progenitors is critically dependent upon interleukin 7. J. Exp. Med. *196*, 705–711.

Morrison, S.J., and Weissman, I.L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity *1*, 661–673.

Namen, A.E., Lupton, S., Hjerrild, K., Wignall, J., Mochizuki, D.Y., Schmierer, A., Mosley, B., March, C.J., Urdal, D., and Gillis, S. (1988). Stimulation of B-cell progenitors by cloned murine interleukin-7. Nature *333*, 571–573.

Nutt, S.L., Urbánek, P., Rolink, A., and Busslinger, M. (1997). Essential functions of Pax5 (BSAP) in pro-B cell development: difference between fetal and adult B lymphopoiesis and reduced V-to-DJ recombination at the IgH locus. Genes Dev. *11*, 476–491.

O'Riordan, M., and Grosschedl, R. (1999). Coordinate regulation of B cell differentiation by the transcription factors EBF and E2A. Immunity *11*, 21–31.

Patel, J.H., and McMahon, S.B. (2007). BCL2 is a downstream effector of MIZ-1 essential for blocking c-MYC-induced apoptosis. J. Biol. Chem. *282*, 5–13.

Peukert, K., Staller, P., Schneider, A., Carmichael, G., Hänel, F., and Eilers, M. (1997). An alternative pathway for gene regulation by Myc. EMBO J. *16*, 5672–5686.

Phan, R.T., Saito, M., Basso, K., Niu, H., and Dalla-Favera, R. (2005). BCL6 interacts with the transcription factor Miz-1 to suppress the cyclin-dependent kinase inhibitor p21 and cell cycle arrest in germinal center B cells. Nat. Immunol. *6*, 1054–1060.

Pongubala, J.M., Northrup, D.L., Lancki, D.W., Medina, K.L., Treiber, T., Bertolino, E., Thomas, M., Grosschedl, R., Allman, D., and Singh, H. (2008). Transcription factor EBF restricts alternative lineage options and promotes B cell fate commitment independently of Pax5. Nat. Immunol. *9*, 203–215.

Rickert, R.C., Roes, J., and Rajewsky, K. (1997). B lymphocyte-specific, Cre-mediated mutagenesis in mice. Nucleic Acids Res. *25*, 1317–1318.

Saito, M., Novak, U., Piovan, E., Basso, K., Sumazin, P., Schneider, C., Crespo, M., Shen, Q., Bhagat, G., Califano, A., et al. (2009). BCL6 suppression of BCL2 via Miz1 and its disruption in diffuse large B cell lymphoma. Proc. Natl. Acad. Sci. USA *106*, 11294–11299.

Seet, C.S., Brumbaugh, R.L., and Kee, B.L. (2004). Early B cell factor promotes B lymphopoiesis with reduced interleukin 7 responsiveness in the absence of E2A. J. Exp. Med. *199*, 1689–1700.

Seoane, J., Pouponnot, C., Staller, P., Schader, M., Eilers, M., and Massagué, J. (2001). TGFbeta influences Myc, Miz-1 and Smad to control the CDK inhibitor p15INK4b. Nat. Cell Biol. *3*, 400–408.

Seoane, J., Le, H.V., and Massagué, J. (2002). Myc suppression of the p21 (Cip1) Cdk inhibitor influences the outcome of the p53 response to DNA damage. Nature *419*, 729–734.

Singh, H., Pongubala, J.M., and Medina, K.L. (2007). Gene regulatory networks that orchestrate the development of B lymphocyte precursors. Adv. Exp. Med. Biol. *596*, 57–62.

Sitnicka, E., Brakebusch, C., Martensson, I.L., Svensson, M., Agace, W.W., Sigvardsson, M., Buza-Vidas, N., Bryder, D., Cilio, C.M., Ahlenius, H., et al. (2003). Complementary signaling through flt3 and interleukin-7 receptor alpha is indispensable for fetal and adult B cell genesis. J. Exp. Med. *198*, 1495–1506.

Staller, P., Peukert, K., Kiermaier, A., Seoane, J., Lukas, J., Karsunky, H., Möröy, T., Bartek, J., Massagué, J., Hänel, F., and Eilers, M. (2001). Repression of p15INK4b expression by Myc through association with Miz-1. Nat. Cell Biol. *3*, 392–399.

Urbánek, P., Wang, Z.Q., Fetka, I., Wagner, E.F., and Busslinger, M. (1994). Complete block of early B cell differentiation and altered patterning of the posterior midbrain in mice lacking Pax5/BSAP. Cell *79*, 901–912.

Wanzel, M., Russ, A.C., Kleine-Kohlbrecher, D., Colombo, E., Pelicci, P.G., and Eilers, M. (2008). A ribosomal protein L23-nucleophosmin circuit coordinates Miz1 function with cell growth. Nat. Cell Biol. *10*, 1051–1061.

Wu, S., Cetinkaya, C., Munoz-Alonso, M.J., von der Lehr, N., Bahram, F., Beuger, V., Eilers, M., Leon, J., and Larsson, L.G. (2003). Myc represses differentiation-induced p21CIP1 expression via Miz-1-dependent interaction with the p21 core promoter. Oncogene *22*, 351–360.