Cutting Edge: IRF8 Regulates Bax Transcription In Vivo in Primary Myeloid Cells

Jine Yang, Xiaolin Hu, Mary Zimmerman, Christina M. Torres, Dafeng Yang, Sylvia B. Smith and Kebin Liu

*J Immunol* published online 26 September 2011
http://www.jimmunol.org/content/early/2011/09/25/jimmunol.1101034

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/09/27/jimmunol.1101034.DC1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: IRF8 Regulates Bax Transcription In Vivo in Primary Myeloid Cells

Jine Yang,*† Xiaolin Hu,‡ Mary Zimmerman,† Christina M. Torres,† Dafeng Yang,† Sylvia B. Smith,‡ and Kebin Liu†‡§

A prominent phenotype of IRF8 knockout (KO) mice is the uncontrolled expansion of immature myeloid cells. The molecular mechanism underlying this myeloproliferative syndrome is still elusive. In this study, we observed that Bax expression level is low in bone marrow progenitor cells and increases dramatically in primary myeloid cells in wt mice. In contrast, Bax expression level remained at a low level in primary myeloid cells in IRF8 KO mice. However, in vitro IRF8 KO bone marrow-differentiated myeloid cells expressed Bax at a level as high as that in wild type myeloid cells. Furthermore, we demonstrated that IRF8 specifically binds to the Bax promoter region in primary myeloid cells. Functional analysis indicated that IRF8 deficiency results in increased resistance of the primary myeloid cells to Fas-mediated apoptosis. Our findings show that IRF8 directly regulates Bax transcription in vivo, but not in vitro during myeloid cell lineage differentiation. The Journal of Immunology, 2011, 187: 000–000.

All types of immune cells are derived from the hematopoietic stem cells in the bone marrow (BM) through a hierarchical differentiation process. This differentiation process is tightly regulated by lineage-specific transcription factors (1). IFN regulatory factor 8 (IRF8/ICSBP) is a key transcription factor that regulates myeloid cell and B cell differentiation under physiologic conditions (2–4). IRF8 KO mice and mice with a mutation in the IRF8/ICSBP gene develop a myeloproliferative syndrome of IRF8-deficient mice is still elusive. Previous studies have identified multiple apoptosis-related genes, including FAP-1, FLIP, Bcl-xL, Bcl-2, that are regulated by IRF8 in myeloid cell lines in vitro (6–12). These prominent studies suggest that the myeloproliferative syndrome of IRF8-deficient mice might be the result of altered sensitivity of myeloid cells to apoptosis in vitro; however, these studies also suggest that regulation of apoptosis-related genes by IRF8 is dependent on cell type. Furthermore, it has been shown that regulation of Bcl-2 by IRF8 observed in vitro might not be observed in vivo (10).

In this study, we compared the genome-wide gene expression profiles of CD11b+ primary myeloid cells purified from wild type (wt) and IRF8 KO mice and identified that Bax is an IRF8 target gene in vivo but not in vitro. Our data suggest that IRF8 directly binds to the Bax promoter region to activate Bax transcription and that the loss of IRF8 expression significantly decreases primary myeloid cell sensitivity to Fas-mediated apoptosis. Thus, our results suggest that IRF8 mediates myeloid cell differentiation partially through regulating Bax expression and the Fas-mediated apoptosis pathway in vivo.

Materials and Methods

Mice

IRF8 KO mice (provided by Dr. Keiko Ozato, National Institutes of Health, Bethesda, MD) were maintained as described (5). All IRF8 KO mice used for the study were between the ages of 43 and 60 d. To generate chimeric mice, wt C57BL/6 recipient mice were irradiated with a dose of 950 rad. BM cells from IRF8 KO and age-matched littermate wt mice (5 × 10^6 cells per mouse) were injected 4 h later to the irradiated mice. Mice were used 30 d after BM transplantation. All mice were studied in accordance with approved Georgia Health Sciences University protocols.

DNA microarray

CD11b+ cells were isolated from spleen cells using anti-CD11b mAb (Biolegend) and magnetic beads. DNA microarray analysis of genome-wide gene expression with mouse OneArray (Phalanx Biotech, Belmont, CA) was performed as previously (13).

RT-PCR analysis

RT-PCR analysis was performed as previously (14). The PCR primer sequences are as follows: mouse Bax, forward: 5′-GGATGCGTCCAC-CAAGAAGC-3′; reverse: 5′-GGAGGAAGTCCAGTGTCCAGCC-3′; mouse PARP, poly(ADP-ribose) polymerase; wt, wild type.

Copyright © 2011 by The American Association of Immunologists, Inc. 0022-1767/11/$16.00
CD11b⁺, CD4⁺, CD8⁺, and NK⁺ cells were isolated from minced spleens with the respective mAb (Biolegend, San Diego, CA) and magnetic beads. Western blot analysis was performed as previously (15). The blots were probed with the following Abs: anti-Cytochrome C (BD Biosciences, San Diego, CA) at 1:500, anti-Bax (Santa Cruz Biotech, Santa Cruz, CA) at 1:2000, anti-cleaved polyADP-ribose) polymerase (PARP) (Cell Signal, Danvers, MA) at 1:500, and anti-β-actin (Sigma, St Louis, MO) at 1:8000. Blots were detected using the ECL Plus (Amersham Pharmacia Biotech, Piscataway, NJ) Western detection kit.

**Cell surface marker analysis**

Spleens were minced to make single-cell suspension through a cell strainer (BD Biosciences). The cell suspension was stained with FITC-conjugated anti-mouse CD11b mAb (BD Biosciences). The stained cells were analyzed by flow cytometry as previously (16).

**Cytosol and mitochondria fractionation**

Cytosol and mitochondrion-enriched fractions were prepared as described previously (17).

**Chromatin immunoprecipitation**

CD11b⁺ cells were purified by depleting other subsets of cells with respective mAbs and magnetic beads. Chromatin immunoprecipitation (ChIP) assays were performed according to protocols from Upstate Biotech (Lake Placid, NY) as previously (13). Immunoprecipitation was performed using anti-IRF8 Ab (C-19; Santa Cruz) and agarose-protein A beads. The PCR primers used to amplify the promoter region of the Bax gene as depicted in Fig. 3A are as follows. ChIP 1, forward: 5'-GGGAAGGGCAGTTTGAGC-3', reverse: 5'-TGCCCTAGGGAATGGAGTCAC-3'; ChIP 2, forward: 5'-TGCGTTCTTCCGAAGGT-3', reverse: 5'-TGCAGCTTCAGCCTTTT-3'; ChIP 3, forward: 5'-ATGGACTTGAGCAGCTTTT-3', reverse: 5'-TTTATCCAGGCCTCCAGAAA-3'; ChIP 2, forward: 5'-TGCAGCTTCAGCCTTTT-3', reverse: 5'-TTTATCCAGGCCTCCAGAAA-3'; ChIP 3, forward: 5'-ATGGACTTGAGCAGCTTTT-3', reverse: 5'-TTTATCCAGGCCTCCAGAAA-3'; ChIP 4, forward: 5'-TGCAGCTTCAGCCTTTT-3', reverse: 5'-TTTATCCAGGCCTCCAGAAA-3'.

**Protein–DNA interaction assay**

EMSA was performed as previously (18). The DNA probes are as follows: GAS wt probe, forward: 5'-ACCTTCGGAAGAACAG-3', reverse: 5'-CTGTGTCCTCGAAGAAGTGGTGTT-3'; ChIP4, forward: 5'-TGCAGCTTCAGCCTTTT-3', reverse: 5'-TTTATCCAGGCCTCCAGAAA-3'; ChIP 2, forward: 5'- TGCGTTCTTCCGAAGGT-3', reverse: 5'-TGCAGCTTCAGCCTTTT-3'; ChIP 3, forward: 5'-ATGGACTTGAGCAGCTTTT-3', reverse: 5'-TTTATCCAGGCCTCCAGAAA-3'; ChIP 4, forward: 5'-TGCAGCTTCAGCCTTTT-3', reverse: 5'-TTTATCCAGGCCTCCAGAAA-3'. Oligonucleotides were annealed and end-labeled with γ[32P]-ATP and used for EMSA as previously (18).

**Measurement of apoptotic cell death**

Total spleen cells were cultured in the absence or presence of Fas ligand (FasL) (10 ng/ml) overnight. The cells were then collected and stained with anti-CD11b mAb and Alex Fluor 647-conjugated annexin V. The CD11b⁺ cells were gated out to determine the percentage of annexin V⁺ CD11b⁺ cells. The percentage of cell death was calculated by the following formula: % cell death = % annexin V⁺ cells with FasL treatment − % annexin V⁺ cells without FasL treatment.

**Results**

**Identification of Bax as a target of IRF8**

As noted before, the size of the spleen from IRF8 KO mice was significantly larger than that from wt mice (Fig. 1A). The number of Gr1⁺ and CD11b⁺ cells that likely include macrophages and granulocytes are dramatically increased in the spleen of the IRF8 KO mice and accounts for 50% of the total spleen cells (Fig. 1B), thus validating the function of IRF8 in apoptosis.

Western blotting analysis revealed that Bax protein level is also dramatically lower in IRF8 KO CD11b⁺ primary cells than in wt CD11b⁺ primary cells (Fig. 2A). However, Bax protein level is not significantly different in CD4⁺CD8⁺ and NK cells between the IRF8 KO and wt control mice (Fig. 2A). Quantitative RT-PCR analysis indicates that the Bax mRNA level is significantly lower in IRF8 KO CD11b⁺ primary cells than in the CD11b⁺ primary cells of age-matched wt littermate control mice (Fig. 2A). The Bax protein level is low in BM cells of both wt and IRF8 KO mice (Fig. 2A).

Lethally irradiated wt mice that received BM from IRF8 KO mice also exhibited splenomegaly (Fig. 2B). These IRF8 KO chimera mice showed immune cell profiles (Fig. 2C) similar to the conventional IRF8 KO mice (Fig. 1B). The Bax protein level of these IRF8 KO chimera mice is also dramatically lower than that in wt chimera mice (Fig. 2C). In vitro differentiation of BM cells from both wt and IRF8 KO mice with GM-CSF and M-CSF generated CD11b⁺ cells (Fig. 2C). However, Bax protein level of these in vitro-differentiated IRF8 KO CD11b⁺ cells is as high as that in wt CD11b⁺ cells (Fig. 2C, 2D). Our data suggest that IRF8 regulates Bax expression in vivo but not in vitro during myeloid cell differentiation (Fig. 2D).

**IRF8 protein binds to the Bax promoter**

ChIP assays were performed with purified wt CD11b⁺ primary cells to determine whether IRF8 protein is associated with the Bax promoter. As illustrated in Fig. 3A, the Bax promoter region contains several IRF8 consensus binding elements.

**FIGURE 1.** Identification of Bax as an IRF8 target gene in primary myeloid cells. A, Spleens of wt (left) and IRF8 KO (right) mice. B, A single-cell suspension was prepared from the spleens of IRF8 KO and age-matched wt littermate control mice and was analyzed for subsets of immune cells as indicated. The number in the box indicates the percentage of that subset of cells. Shown are representative data from one of three pairs of mice. C, Differential Bax gene expression between wt and IRF8 KO primary CD11b⁺ myeloid cells. Data were derived from DNA microarray analysis. The expression of Bax in wt CD11b⁺ myeloid cells was set as 1.
Specific IRF8 binding was detected in one region of the Bax promoter (Fig. 3B). EMSA with nuclear extracts from purified wt and IRF8 KO CD11b+ cells indicated that IRF8 directly interacts with one of the GAS elements in the Bax promoter region (GAS1; Fig. 3C). Initial attempts did not detect IRF8 binding to a DNA probe containing GAS2-4 (data not shown). Our data suggest that IRF8 directly binds to the Bax promoter in primary CD11b+ cells.

IRF8-deficient myeloid cells exhibit decreased sensitivity to Fas-mediated apoptosis

The above observation that IRF8 KO myeloid cells exhibit decreased Bax expression leads us to speculate that the IRF8 KO cells might acquire resistance to apoptosis. To test this hypothesis, we first measured cytochrome C (CytC) release, a biochemical marker for the intrinsic apoptosis pathway, in purified primary CD11b+ cells. Spontaneous CytC release is higher in purified wt CD11b+ cells ex vivo as compared with IRF8 KO cells (Fig. 4A). Treatment with FasL induced a rapid increase in CytC release in wt CD11b+ cells, but not in IRF8 KO CD11b+ cells (Fig. 4A). Consistent with the CytC release pattern, cleaved PARP, a biochemical marker of both extrinsic and intrinsic apoptosis, was also observed in wt but not in IRF8 KO CD11b+ primary cells (Fig. 4A). At the functional level, the wt CD11b+ primary cells exhibited significantly more spontaneous apoptosis ex vivo with FasL further increasing the apoptosis rate. However, the IRF8 KO CD11b+ cells exhibited significantly less spontaneous apoptosis and became less sensitive to FasL-induced apoptosis ex vivo (Fig. 4B). Our data suggest that the loss of IRF8 function decreases CD11b+ primary cell sensitivity to Fas-mediated apoptosis.

Discussion

IRF8 is essential for myeloid cell differentiation, and loss of IRF8 expression leads to uncontrolled clonal expansion of CD11b+ myeloid cells (5). It has been proposed that acquisition of apoptosis resistance is responsible for the impaired...
myeloid cell differentiation (6–12). Several apoptosis regulators, including Bcl-xl, Bcl-2, FAP-1, and acid ceramidase, have been shown to have a role in regulating apoptosis in myeloid leukemia cell lines in vitro (6–8, 16). IRF8 has also been shown to regulate FLIPL expression in primary myeloid cells in vivo (17). The function of IRF8 in apoptosis has also been demonstrated in other nonhematopoietic cell types (17, 22–24). In addition, it has been shown that the correlation between IRF8 and the Bcl-2 family members observed in vitro may not be observed in vivo (10). Therefore, the function of IRF8 in the regulation of apoptosis-related genes might be cell type-dependent and different between in vitro and in vivo conditions. Our data indicate that IRF8 directly binds to the Bax promoter to regulate Bax transcription in primary myeloid cells in vivo but not in vitro.

The Fas-mediated apoptosis pathway plays an essential role in the elimination of unwanted cells by the host immune system during lineage differentiation and homeostasis (25, 26). Bax is a key mediator of the mitochondrion-dependent intrinsic apoptosis pathway (27). Our data indicate that IRF8 is a transcription activator of Bax. Therefore, IRF8 might mediate myeloid homeostasis and differentiation through regulating Bax expression to maintain the myeloid cell sensitivity to Fas-mediated apoptosis. However, IRF8 also regulates the expression of other apoptosis-related genes in primary myeloid cells in vivo (Supplemental Figs. 1, 2). Therefore, the relative role of Bax and other IRF8-regulated and apoptosis-related genes in IRF8-mediated myeloid cell differentiation in vivo requires further study.

Acknowledgments

We thank Dr. Jeanene Pihkala for assistance in flow cytometry analysis.

Disclosures

The authors have no financial conflicts of interest.

References


