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IFN Regulatory Factor 4 and 8 Promote Ig Light Chain \( \kappa \) Locus Activation in Pre-B Cell Development

Shibin Ma, Anna Turetsky, Long Trinh, and Runqing Lu

Previous studies have shown that B cell development is blocked at the pre-B cell stage in IFN regulatory factor (IRF)4 (pip) and IRF8 (IFN consensus sequence binding protein) double mutant mice (IRF4,8\(^{-/-}\)). In this study, the molecular mechanism by which IRF4,8 regulate pre-B cell development was further investigated. We show that IRF4,8 function in a B cell intrinsic manner to control pre-B cell development. IRF4,8\(^{-/-}\) mice expressing a Bcl-2 transgene fail to rescue pre-B cell development, suggesting that the defect in B cell development in IRF4,8\(^{-/-}\) mice is not due to a lack of survival signal. IRF4,8\(^{-/-}\) pre-B cells display a high proliferation index that may indirectly inhibit the L chain rearrangement. However, forced cell cycle exit induced by IL-7 withdrawal fails to rescue the development of IRF4,8\(^{-/-}\) pre-B cells, suggesting that cell cycle exit by itself is not sufficient to rescue the development of IRF4,8\(^{-/-}\) pre-B cells and that IRF4,8 may directly regulate the activation of L chain loci. Using retroviral mediated gene transduction, we show that IRF4 and IRF8 function redundantly to promote pre-B cell maturation and the generation of IgM\(^+\) B cells. Molecular analysis indicates that IRF4, when expressed in IRF4,8\(^{-/-}\) pre-B cells, induces \( \kappa \) germline transcription, enhances V(D)J rearrangement activity at the \( \kappa \) locus, and promotes L chain rearrangement and transcription. Chromatin immunoprecipitation assay further reveals that IRF4 expression leads to histone modifications and enhanced chromatin accessibility at the \( \kappa \) locus. Thus, IRF4,8 control pre-B cell development, at least in part, by promoting the activation of the \( \kappa \) locus. *The Journal of Immunology,* 2006, 177: 7898–7904.

Development of B cells can be characterized by the sequential rearrangement of Ig loci and the expression of distinct cell surface markers (1). During B cell development the H chain locus is rearranged at the pro-B stage while the L chain locus is rearranged at the pre-B stage. After productive H chain rearrangement, the H chain protein \( \mu \) will pair with surrogate L chain Vpre-B and A5 and form pre-BCR on the cell surface. The pre-BCR acts as an important developmental checkpoint and plays a critical role in pre-B cell proliferation and differentiation. This effect is evidenced by the fact that mutations in components of the pre-BCR or the signaling molecules presumably downstream of pre-BCR, block pre-B cell development (2–9). Pre-B cells undergo two phases of maturation, a clonal expansion phase, in which the cells are highly proliferative, and a resting phase, in which cells stop proliferating and rearrange the L chain, thereby giving rise to IgM\(^+\) B cells.

The rearrangement and transcription of the \( \kappa \) L chain is regulated by two redundant enhancer elements, one in the 3’ noncoding region (Ex3) and one in an intron (Exi). Deletion of either enhancer has a modest effect on \( \kappa \) rearrangement, but deletion of both enhancers completely blocks \( \kappa \) rearrangement (10–12). In contrast, activation of the \( \lambda \) locus is controlled separately by two highly homologous enhancers: EA3-1 and EA2-4 (13). A number of transcription factor binding sites have been identified in the L chain enhancers, but only the transcription factor E2A has been shown to be critical for \( \kappa \) rearrangement. When the E2A binding sites located within the Exi were mutated, \( \kappa \) rearrangement is significantly impaired (14).

IFN regulatory factor (IRF)\(^4\), also known as pip and NF-EM5, was originally identified as a transcription factor that binds to \( \kappa \) 3’ enhancer and \( \lambda \) enhancers (15, 16). It has been shown that IRF4 or IRF8 interact with Ets family transcription factor PU.1 or its close relative Spi-B, to regulate the activities of Ig \( \kappa \) 3’ enhancer and \( \lambda \) enhancers (16, 17). IRF4 and Spi-B, when expressed in Abelson transformed pro-B cells, are sufficient to activate germline \( \kappa \) transcription (18). IRF4,8 have been found to interact with the E2A family of transcription factors to regulate activities of both Ig H chain intron enhancer and \( \kappa \) 3’ enhancer (19, 20). It has been shown that the interaction of IRF4 and E2A enhances binding affinity of E2A for the Ex3’ enhancer. Interestingly, a knockdown of IRF4 expression in a pre-B cell line reduces not only the binding of E2A to \( \kappa \) Ex3’ but also the histone acetylation at both Exi and Ex3’ (21). These results suggest that IRF4 may also regulate the activity of Exi.

Previous studies have demonstrated that B cell development is blocked at the cycling pre-B cell stage in IRF4,8\(^{-/-}\) mice (22), IRF4,8\(^{-/-}\) pre-B cells display a high proliferation index and fail to rearrange L chain. In this study, the molecular mechanism by which IRF4,8 control pre-B cell development was further investigated. We show that the defect in B cell development can be recapitulated in transplanted host mice receiving IRF4,8\(^{-/-}\) bone marrow, indicating that IRF4,8 function in a B cell intrinsic manner to control pre-B cell development. We also provide evidence that the defect in pre-B cell development in IRF4,8\(^{-/-}\) mice is not due to an impairment in cell survival. We further show that forced cell cycle exit by IL-7 withdrawal promotes the development of control but not IRF4,8\(^{-/-}\) pre-B cells, suggesting that withdrawal from cell cycle alone is not sufficient to rescue the development of...
IRF4,8\(^{-/-}\) pre-B cells. Using retroviral mediated gene transfer, we demonstrate that IRF4,8 function redundantly to control pre-B cell maturation. Finally, molecular analysis reveals that IRF4 expression leads to histone modifications, enhances V(D)J rearrangement activity, and promotes \(\kappa\) chain rearrangement and transcription at the \(\kappa\) locus.

Materials and Methods

Mice

C57B6 mice deficient for IRF4 and IRF8 (IRF4,8\(^{-/-}\)) have been previously described (22). Eμ-Bcl2 transgenic mouse was obtained from The Jackson Laboratory and were bred with IRF4,8\(^{-/-}\) mice to generate IRF4,8 heterozygous offspring expressing a copy of Bcl2 tumor suppressor (IRF4\(^{-/-}\)Bcl2), which were mated subsequently to generate IRF4,8\(^{-/-}\)Bcl2-2 mice. All mice were maintained under specific pathogen-free conditions. Experiments were performed according to the guidelines from the National Institutes of Health and with an approved protocol from the University of Nebraska Medical Center Institutional Animal Care and Use Committee. The mice from 6 to 10 wk of age were used for this study.

Bone marrow transplantation

Bone marrows were isolated from both hind limbs of wild-type (wt) mice or IRF4,8\(^{-/-}\) mice. Lineage marker negative progenitor cells (Lin\(^{-/-}\)) were isolated using a deletion protocol. The cells were injected via retro-orital sinus into sublethal-irradiated mice that were deficient for both common \(\gamma\)-chain and Rag2 (Rag2\(^{-/-}\)γ\(^{-/-}\)) (23). The B cell population in the bone marrow and spleen of recipient mice was analyzed by FACS 5 wk after transplantation.

Pre-B cell culture

B220\(^{+}\) cells were isolated from bone marrow of IRF4,8\(^{-/-}\) mice using a MACS separation column (Miltenyi Biotec) and were overlaid on top of an irradiated S17 stromal cell layer. The cells were cultured in Opti-MEM (Invitrogen Life Technologies) medium containing 5% FBS, 50 μM 2-ME, 2 mM L-glutamine, 100 U of penicillin-streptomycin, and 5 ng/ml IL-7 (R&D Systems). The pre-B cells were passaged every 3 days on to a new S17 stromal layer. Cells with fewer than five passages were used for the experiments.

Retroviral gene transduction

MigR1 is a bicistronic retroviral vector that links the target gene with a red-shifted GFP (24). This vector allows rapid and specific identification of successful gene transfer and expression in living cells based on GFP expression by FACS. MigR1 vector expressing IRF4 and IRF8 were generated. To infect cultured pre-B cells, MigR1 empty vector or MigR1 vector containing the genes of interest were transfected into the ecotropic retroviral packaging cell line Plat-E using FuGene 6 (Roche). The cell-free supernatants are collected at 24 and 48 h after transfection. The virus was concentrated by centrifugation at 20,000 × g for 1 h and was typically used the same day to infect target cells via spin infection. The infection was conducted in a 24-well plate at 1700 rpm for 1 h in the presence of 10 μg/ml polybrene. The cells were analyzed by FACS 48 h later. The infection efficiency was typically around 40%. When higher infection efficiency is desired, an amphotropic helper plasmid was used for the initial transfection and the target cells were infected twice within 24 h. The infection efficiency in the latter case reached >80%.

PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies). RNA was reverse transcribed using a first-strand cDNA synthesis kit (Amersham Biosciences). PCR primers used to analyze B cell lineage gene expression have been previously described (22). Serial diluted cDNA templates were used for semiquantitative analysis.

Jk1 rearrangement analysis

Genomic DNA was isolated from either control or IRF4 infected cells. Jk1 rearrangement analysis was performed essentially as described (25, 26). Serial dilutions of cDNA or genomic DNA were used for semiquantitative purpose. The PCR products were visualized after ethidium bromide staining.

Quantification of Jk1 signal end break

Jk1 signal end break in developing B cell was quantified using a ligation-mediated PCR (LM-PCR) as previously described (27). Briefly, IRF4,8\(^{-/-}\) pre-B cells were infected with control or IRF4 virus and were cultivated in the absence of IL-7 for 36 h. Genomic DNA was isolated, and 1 μg of DNA is subject to linker ligation and PCR amplification with previously described locus-specific primers and a linker primer. PCR product will be described after Southern blot with an internal probe and quantified by phosphor imaging.

FACS analysis

Cells were preincubated with either 2% rat serum or Fc block (2.4G2), and stained with optimal amounts of specific Abs, either biotinylated or directly fluorophore-conjugated. Abs against B220 (RA3-6B2), CD19 (ID3), CD2, and CD25 were purchase from BD Pharmingen; anti-IgM and κ were obtained from Southern Biotechnology Associates. FACS analysis was performed with a FACS Calibur flow cytometer.

Chromatin immunoprecipitation (ChiP)

The ChiP assay was performed essentially as described before (22). Briefly, 20 million cells in culture medium were fixed in 1% paraformaldehyde, lysed, and sonicated to generate chromatin fragment around 500 bp. Chromatin fractions (equivalent to 4 million cells each) were immunoprecipitated with indicated Abs. The 1 μg of Abs against acetylated histone H3, H4, and dimethylated histone H3 lysine 4 (H3-K4, Upstate Biotechnology) was used for each immunoprecipitation.

Quantitative real-time PCR analysis was conducted using SYBR Green PCR Core Reagents (Applied Biosystems). Each immunoprecipitate were typically dissolved in 40 μl of H2O and 2 μl was used for each PCR analysis. All samples were tested in triplicate, and average threshold Ct values were calculated. The enrichment of specific sequences was determined by the ratio of specific immunoprecipitation signal/input signal. Enrichment of previously described housekeeping gene GAPDH and the silent tryosinogen locus (T4D) were used as positive and negative control, respectively (21). The primer sequences used for Erk3, Eri, G6PD, and T4D have been described (21, 22).

Results

IRF4,8\(^{-/-}\) act in a cell autonomous fashion to control pre-B cell development

We have demonstrated previously that B cell development is blocked at the pre-B stage in IRF4,8\(^{-/-}\) mice. The fact that B cell development is arrested at the pre-B stage in IRF4,8-deficient mice might reflect either a cell autonomous or a cell nonautonomous defect. To address this question, we isolated Lin\(^{-/-}\) progenitor cells from wt and IRF4,8\(^{-/-}\) mice and injected them into sublethal-irradiated Rag2\(^{-/-}\)γ\(^{-/-}\) mice. B cell populations were analyzed in transplanted recipient mice 5 wk after transplantation. As shown in Fig. 1, Lin\(^{-/-}\) progenitor cells from wt mice were able to generate

![FIGURE 1](http://www.jimmunol.org/)
a significant number of B220<sup>+</sup> IgM<sup>+</sup> cells in the spleen (44%) and bone marrow (15%) of Rag2<sup>−/−</sup> γ<sup>−/−</sup> host mice; in contrast, very few B220<sup>+</sup> IgM<sup>+</sup> B cells are observed in the spleen (3%) and bone marrow (1%) of Rag2<sup>−/−</sup> γ<sup>−/−</sup> host mice injected with IRF4,8<sup>−/−</sup>−/− Bcl2 mice. This result indicates that the defect in pre-B cell development associated with IRF4,8 deficiency is cell autonomous.

**Bcl-2 transgene fails to efficiently rescue B cell development in IRF4,8<sup>−/−</sup> mice**

IRF4 and IRF8 have been implicated in the regulation of cell apoptosis and survival (28–31). B cell development is blocked at the pre-B stage in IRF4,8<sup>−/−</sup> mice. One possible explanation for the defect is that IRF4,8 are necessary for the survival of pre-B cells. To test this possibility, we crossed IRF4,8<sup>−/−</sup> mice with Exp-Bcl2 transgenic mice to generate IRF4,8<sup>−/−</sup> Bcl2 mice expressing the Bcl-2 transgene (IRF4,8<sup>−/−</sup> Bcl-2). In this system, expression of the Bcl-2 transgene is driven by Ig H chain intron enhancer and, therefore, is mainly found in B cell. As shown in Fig. 2, expression of the Bcl-2 transgene fails to rescue pre-B cell development in the bone marrow. In the spleen, Bcl-2 transgene expression leads to the slight increase in the frequency of IgM<sup>+</sup> B cells, from 1 to 3%. The slight increase of IgM<sup>+</sup> B cells in the spleen of IRF4,8<sup>−/−</sup> Bcl2 mice is likely a result of enhanced cell survival in the presence of Bcl-2 transgene. The high levels of Bcl-2 expression likely will prolong the survival of B cells, including the few IgM<sup>+</sup> B cells in IRF4,8<sup>−/−</sup> mice, thereby resulting in their accumulation in the spleen. We believe a much greater number of IgM<sup>+</sup> B cells would be generated in IRF4,8<sup>−/−</sup> Bcl2 mice should the lack of survival signals play a major role in the defect of pre-B cell development. The finding that Bcl-2 transgene expression fails to efficiently rescue pre-B cell development suggests that the defect of pre-B cell development of IRF4,8<sup>−/−</sup> mice is not due to a lack of survival signals.

**IL-7 withdrawal fails to rescue the development of IRF4,8<sup>−/−</sup> pre-B cells**

DNA rearrangement activity occurs at higher frequency in G<sub>0</sub>-G<sub>1</sub> than in other phases of cell cycle (32). Pre-B and pre-B cells both require IL-7 to proliferate in vitro, and in the absence of IL-7, cells exit from the cell cycle and up-regulate Rag gene expression, which subsequently promotes L chain rearrangement (33). Previous studies have demonstrated that IRF4,8<sup>−/−</sup> pre-B cells are highly proliferative, fail to exit from the cell cycle, and do not rearrange L chain genes. Thus the defect in L chain rearrangement in the IRF4,8<sup>−/−</sup> pre-B cells could stem from the continued responsiveness to IL-7 or from an intrinsic defect in L chain rearrangement, or from both. As an initial attempt to examine those possibilities, IL-7 was withdrawn from cultured IRF4,8<sup>−/−</sup> pre-B cells. IL-7 withdrawal leads to cell cycle exit of both the control and the IRF4,8<sup>−/−</sup> pre-B cells (data not shown). The effect of IL-7 withdrawal on pre-B cell development was analyzed in IRF4,8<sup>−/−</sup> pre-B cells. IRF4<sup>+/−</sup>IRF8<sup>−/−</sup> pre-B cells were used as a control.

The results show that withdrawal of IL-7 significantly increases the frequency of κ expressing cells in IRF4<sup>+/−</sup>IRF8<sup>−/−</sup> pre-B cells (Fig. 3A), suggesting that IL-7 withdrawal promotes L chain rearrangement and transcription. In contrast, IL-7 withdrawal fails to promote the generation of κ expressing cells in IRF4,8<sup>−/−</sup> pre-B cells. The result of the RT-PCR is also consistent with FACS analysis (Fig. 3B). The germline and mature κ transcripts are dramatically induced upon IL-7 withdrawal in control cells but not IRF4,8<sup>−/−</sup> pre-B cells. Importantly, the control pre-B cells and IRF4,8<sup>−/−</sup> pre-B cells express similar amount of Rag transcripts, suggesting that the inability of IRF4,8<sup>−/−</sup> pre-B cells to rearrange the κ locus is not due to a defect in Rag expression. Together, these results demonstrate that forced cell cycle exit, by itself, is not sufficient to rescue the development of IRF4,8<sup>−/−</sup>−/− pre-B cells and that there is an intrinsic defect in L chain rearrangement and transcription in the absence of IRF4,8.

**IRF4 and IRF8 function redundantly to control pre-B cell maturation**

B cell development appears normal in IRF4<sup>−/−</sup> and IRF8<sup>−/−</sup> single mutant mice but is blocked at the pre-B cell stage of B cell maturation. The finding that Bcl-2 transgene expression fails to efficiently rescue pre-B cell development in the bone marrow (1%) of Rag2<sup>−/−</sup> pre-B cells was analyzed by FACS under a lymphocyte gate. The frequency of IgM<sup>+</sup> cells was expressed as a percentage of all cells within the gated lymphocyte. The data are representative of three independent experiments.
IRF4 and IRF8 are redundant transcription factors that control overlapping events in pre-B cell development. IRF4,8−/− pre-B cells were infected with IRF4, IRF8, or control-expressing retrovirus and were subsequently maintained in Opti-MEM medium in the absence of IL-7 for 36 h. The infection efficiency for the control, IRF4−/− and IRF8−/− infected cells are 35, 41, and 43%, respectively. The cells were stained with Abs against CD19, CD2, CD25, and IRF4,8 and analyzed by FACS (A). The numbers are percentage of cells within GFP+ population. The result is a representative of at least three independent experiments. B, Western blot analysis of IRF4 and IRF8 expression in the infected IRF4,8−/− pre-B cells. IRF4,8−/− pre-B cells were infected twice within 24 h (83% infection efficiency). The infected cells were cultivated in the absence of IL-7 and were lysed 36 h later. Cultured IRF4+/− IRF8+/− pre-B cells were also lysed after IL-7 withdrawal and were used as a reference on expression levels of endogenous IRF4,8. Western blot analysis was performed to determine IRF4,8 expression in the infected and reference cells. The expression of β-actin was measured and used as an internal loading control.

IRF4 expression induces κ germline transcription, enhances V(D)J rearrangement activity, and promotes L chain rearrangement

Because neither enhanced survival by Bcl-2 transgene expression nor forced cell cycle exit is sufficient to rescue the development of IRF4,8−/− pre-B cells, we wanted to test the possibility that IRF4,8 may directly regulate L chain rearrangement and transcription. Rearranging gene segments at Ig loci are flanked by a recombination signal sequence. During V(D)J recombination, recombinase will induce a dsDNA break at the junctions between the recombination signal sequence and the coding region. The presence of dsDNA breaks at recombination signal sequence elements has been used as an indicator of on-going V(D)J recombination. The dsDNA breaks can be quantified by ligation-mediated PCR and used to reflect relative V(D)J rearrangement activity at the locus (27). To determine whether IRF4 promotes V(D)J rearrangement activity at the κ L chain locus, the accumulation of Jk1 signal end was quantified by PCR. IRF4,8−/− pre-B cells were infected with either control or IRF4 containing virus and were cultivated in the absence of IL-7 for 36 h. Genomic DNA was isolated and ligation-mediated PCR was performed. We were able to detect signal end break in control vector infected cells, suggesting that IRF4,8 cells are not absolutely required for V(D)J rearrangement at the κ locus (Fig. 5A). However, in the presence of IRF4, there is a 4-fold increase in end break signal when compared with the control cells, suggesting that IRF4 expression leads to enhanced V(D)J rearrangement activity. Consistent with this result, Jk1 rearrangement is also significantly increased in the IRF4-infected cells (Fig. 5B).

The fact that expression of IRF4 enhances V(D)J rearrangement activity and induces κ rearrangement suggests that IRF4 may regulate the κ locus activation. It has been shown that κ germline transcription is significantly elevated before rearrangement; therefore, expression of germline transcript has been used as an indicator of L chain locus activation (34). RT-PCR analysis shows that κ germline and mature transcript are induced significantly in the IRF4 but not the control infected IRF4,8−/− pre-B cells (Fig. 5C). This result suggests that IRF4 may regulate the activation of κ locus. It is worth pointing out that we were able to detect low level Jk1 rearrangement but not the transcription of mature κ transcript in control infected cells, suggesting that the transcription of rearranged κ gene may also be regulated by IRF4,8. Taken together, our results suggest that V(D)J rearrangement activity at κ locus occurs at low levels in the absence of IRF4,8, but is significantly induced in the presence of IRF4.

FIGURE 4. IRF4,8 are redundant transcription factors that control overlapping events in pre-B cell development. IRF4,8−/− pre-B cells were infected with IRF4, IRF8, or control-expressing retrovirus and were subsequently maintained in Opti-MEM medium in the absence of IL-7 for 36 h. The infection efficiency for the control, IRF4−/− and IRF8−/− infected cells are 35, 41, and 43%, respectively. The cells were stained with Abs against CD19, CD2, CD25, κ, and IgM and analyzed by FACS (A). The numbers are percentage of cells within GFP+ population. The result is a representative of at least three independent experiments. B, Western blot analysis of IRF4 and IRF8 expression in the infected IRF4,8−/− pre-B cells. IRF4,8−/− pre-B cells were infected twice within 24 h (83% infection efficiency). The infected cells were cultivated in the absence of IL-7 and were lysed 36 h later. Cultured IRF4+/− IRF8+/− pre-B cells were also lysed after IL-7 withdrawal and were used as a reference on expression levels of endogenous IRF4,8. Western blot analysis was performed to determine IRF4,8 expression in the infected and reference cells. The expression of β-actin was measured and used as an internal loading control.
IRF4 expression leads to histone modifications at the κ locus

The activation of L chain enhancers triggers chromatin remodeling, resulting in an increase in the accessibility of L chain loci to V(DJ) recombinase (35). Our results suggest that IRF4 regulates κ locus activation. Therefore, we asked whether IRF4 expression also leads to chromatin modifications that increase the accessibility of κ locus. It has been shown that the increases in histone H3 and H4 acetylation and H3-K4 methylation are associated with κ locus activation (36–38). To determine the effect of IRF4 on histone modifications at the κ locus, IRF4,8−/− pre-B cells were infected with either control or IRF4 expressing retrovirus. After infection, the cells were cultivated in the absence of IL-7 for 36 h. ChIP analysis was conducted to examine the histone modifications status in κ enhancer region and Jκ1 region. The housekeeping gene G6PD and the silent loci T4D were used as controls. C. Total RNA was also isolated from the infected cells and RT-PCR analysis was conducted to determine the expression level of the indicated genes.

Compared with the silent locus T4D, histones at Ex3′ and Exi are acetylated, whereas H3-K4 is methylated in control-infected IRF4,8−/− pre-B cells, suggesting that κ locus is accessible even in the absence of IRF4,8 (Fig. 6). However, in the presence of IRF4, there is a further increase in H3/H4 acetylation and H3-K4 methylation at Ex3 enhancer. IRF4 expression also leads to a further increase in H3 acetylation and H3-K4 methylation at Exi. Our result shows that κ germine transcript and Jκ1 rearrangement are induced in the presence of IRF4. The histone modifications at the Jκ1 locus therefore were also analyzed. Histones H3 and H4 at the Jκ1 region are hypoacetylated in control-infected cells but in the presence of IRF4, H3 became hyperacetylated. In addition, H3-K4 methylation is also dramatically elevated at the Jκ1 region in the presence of IRF4. This result suggests that IRF4 expression not only leads to histone modifications at the enhancer region but also in other regions of the κ locus. As a control, a nonrelated rabbit IgG Ab was subjected to the ChIP analysis, but no significant enrichment of κ locus sequences was noted (data not shown). Taken together, our results suggest that IRF4,8, although not essential for the basal activation of κ locus, can dramatically enhance κ locus activation.

Discussion

In this study, we have investigated the molecular mechanism by which IRF4,8 control pre-B cell development. The proper development of B cells in the bone marrow and spleen requires an intact microenvironment. It has been shown that IRF8−/− mice develop a chronic myelogenous leukemia (CML)-like syndrome (39). IRF4,8−/− mice also manifest a CML-like syndrome accompanied by a dramatic expansion of the granulocyte population (data not shown). The CML-like syndrome together with other unknown factors might indirectly affect the microenvironment that is critical for B cell development. To address this question, we transplanted the Lin−/− progenitor cells isolated from bone marrow of IRF4,8−/− mice into Rag2−/−γ−/− mutant host mice. We show that pre-B cell development defect can be recapitulated in the host mice, indicating that IRF4,8 function intrinsically to control pre-B cell development.

B cell development also requires a proper integration of differentiation and survival signals. The defect in B cell development in IRF4,8−/− pre-B cells could be a result of reduced cell survival in the absence of IRF4,8. To examine this possibility, we crossed IRF4,8−/− mice with a Bcl-2 transgenic mice. However, Bcl-2 transgene fails to efficiently rescue the development of pre-B cells in IRF4,8−/− mice. In addition, ectopic expression of another member of the Bcl-2 family, Bcl-xL, also fails to rescue the development of IRF4,8−/− pre-B cells (data not shown). This result suggests that simply prolonging cell survival is not sufficient to rescue the defect in pre-B cell development in IRF4,8−/− mice.

V(DJ) rearrangement activity is cell cycle dependent. It occurs at a higher frequency in G2–M than in other phases of cell cycle, at least in part because Rag expression is regulated in a cell cycle-dependent manner (32). Previous studies have suggested that IRF4,8 negatively regulate pre-B cell proliferation. In their absence, pre-B cells are highly proliferative in vivo and in culture
IRF4,8/−/− pre-B cells can be expanded in culture in the presence of IL-7. Using a retroviral gene transduction system, we reconstitute the expression of IRF4 and IRF8 in mutant pre-B cells. Our results show that IRF4 or IRF8, when expressed in mutant pre-B cells, induces expression of pre-B cell maturation makers CD2 and CD25 and the generation of IgM+ B cells. These results demonstrate that reconstituted expression of either IRF4 or IRF8 is sufficient to rescue the development of IRF4,8/−/− pre-B cells and that IRF4 and IRF8 are redundant with respect to their roles in pre-B cell development. It is worth pointing out that simply restoring IRF4 or IRF8 expression in IRF4,8/−/− pre-B cells in culture is not sufficient to induce cell cycle exit, nor can they rescue the pre-B cell development (our unpublished observation). Only a combination of IRF4 or IRF8 expression and IL-7 withdrawal can efficiently rescue the development of IRF4,8/−/− pre-B cells. This observation would argue that the function of IRF4,8, at least in the in vitro system we use, is not to induce cell cycle exit, but rather to directly promote pre-B cell differentiation.

Our result suggests that IRF4,8 promote κ locus activation. κ germline transcripts have been used as an indicator of κ locus activation. We show that κ germline transcript is expressed at a low level in IRF4,8/−/− pre-B cells but its expression is dramatically induced in the presence of IRF4. Consistent with this result, we show that IRF4 expression in mutant pre-B cells leads to histone modifications at the κ locus as evidenced by increased histone H3/H4 acetylation and H3-K4 methylation in the κ enhancer region as well as the Jκ1 region. The amount of signal end breaks in the absence of IRF4 is also significantly increased in mutant pre-B cells, suggesting that the chromatin modifications triggered by the IRF4 expression likely enhance the accessibility of κ locus to V(DJ) recombinases.

Our studies cannot distinguish whether IRF4,8 act directly to promote κ locus activation or act indirectly by simply promoting pre-B cell maturation. However, previous studies from several groups have clearly demonstrated that IRF4,8 directly bind Eκ3 and regulate its activity through interacting with transcription factors PU.1, Spi-B, and E2A (16, 17, 19, 20, 42, 43). The activation of κ locus is controlled by two redundant enhancers Exi and Ex3. The finding that PU.1−/− Spi-B−/− pre-B cells have intact κ germ-line expression and rearrangement suggests that PU.1/Spi-B are not essential for κ locus activation (44). IRF4,8 have also been found to interact with E2A (19, 20). More recently, knockdown of IRF4 expression in pre-B cells was shown to lead to reduced κ germ-line transcription and decreased binding of E2A to Ex3 (21). Intriguingly, ChIP analysis further revealed that knockdown of IRF4 expression reduced histone H3/H4 acetylation at both Ex3 and Exi, suggesting that IRF4 may be important for the activation of both κ enhancers. Our results, which are consistent with their previous findings, also show that IRF4 expression leads to enhanced histone modifications at both κ enhancers. It is still not clear at present how IRF4 regulates the activity of Exi. It is possible IRF4 may enhance binding of other transcription factors such as E2A to Exi. Other possibilities are that IRF4 may bind to other regulatory regions at the κ locus that indirectly regulate Exi activity or that IRF4 may regulate the expression or activity of other factors, which in turn regulate Exi activation.

We show that histones H3 and H4 are acetylated, whereas H3-K4 is methylated in the κ enhancer region in the absence of IRF4,8, suggesting that κ locus is active in the absence of IRF4,8. Consistent with this statement we are also able to detect low levels of V(DJ) recombination activity in the absence of IRF4,8. However, our data also indicate that in the presence of IRF4, κ locus activity is dramatically induced. It has been shown that IRF4 expression is up-regulated at the pre-B stage (18). Thus, we hypothesize that IRF4,8, although not absolutely required for κ rearrangement, act to further enhance κ rearrangement and transcription at the pre-B stage of B cell development. Our finding is also consistent with previous reports showing that Eκ3 is already accessible at the pre-B stage but the binding of transcription factors such as IRF4 can only be detected at the pre-B stage, an event that is correlated with increased chromatin accessibility at the κ locus (45, 46).

The block in pre-B cell development in IRF4,8/−/− mice is likely the result of the requirements for IRF4,8 at the two key stages of pre-B cell development. Previous studies have indicated that IRF4,8 negatively regulate pre-B cell expansion and are required for pre-B cell to exit from cell cycle, an important function which, by itself, would indirectly facilitate L chain rearrangement. In this study, we provide evidence that IRF4,8 also directly promote the activation of κ locus. Thus, IRF4,8 function at multiple steps to promote pre-B cell differentiation.

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Disclosures
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References


