

## IRF8 regulates myeloid and B lymphoid lineage diversification

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**Abstract** Interferon regulatory factor 8 (IRF8) is a member of the IRF family of transcription factors whose members play critical roles in interferon (IFN) signaling pathways governing the establishment of innate immune responses by myeloid and dendritic cells. IRF8 is also expressed in lymphoid cells and recent studies have documented its involvement in B cell lineage specification, immunoglobulin light chain gene rearrangement, the distribution of mature B cells into the marginal zone and follicular B cell compartment, and the transcriptional regulation of critical elements of the germinal center reaction. Here we review the contributions of IRF8 to B cell development from hematopoietic stem cells in the bone marrow and its place in the hierarchical regulatory network governing specification and commitment to the B cell fate.

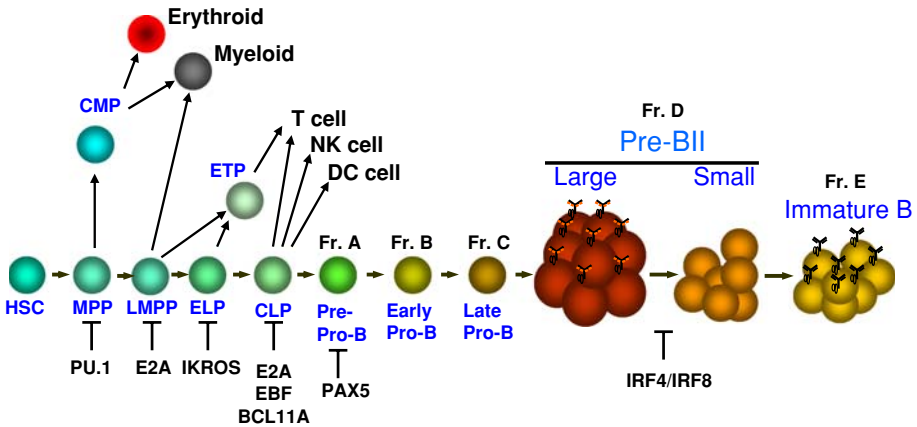
**Keywords** Hematopoietic stem cell · Common lymphoid progenitor · IRF8 · PU.1 · EBF · PAX5 · E2A · Pre-pro-B · Lineage · B cell

### Introduction

Hematopoietic stem cells (HSCs) differentiate into lymphoid and myeloid lineages under the influence of cytokine receptors and an unknown number of transcription factors. In the first phase of differentiation, HSCs give rise to progeny of at least three types—the common lymphoid progenitor (CLP), early T lineage progenitor (ETP), and common myeloid progenitor (CMP), with potentials to differentiate into predominantly the lymphoid lineage (B, T, NK, and dendritic cells), T cells, and myeloid lineage cells (megakaryocytic-erythroid cells, granulocytes, and macrophages), respectively. However, the plastic nature of these progenitors reflects a carefully balanced regulatory transcriptional network that is modulated to favor development of one lineage over another. Several key transcription factors

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**Fig. 1** Differentiation of HSC and their progeny in the bone marrow (BM). Targeted deletion of each transcription factor results in interrupted differentiation at a specific stage. HSC (hematopoietic stem cell), MPP (multipotent progenitor), LMPP (lymphoid-primed MPP), ELP (early lymphoid progenitor), CLP (common lymphoid progenitor), Frs. A to E (B lymphocytes of Hardy Fractions A–E). Modified from [1]

such as PU.1, EBF, ID2, E2A, and PAX5 have been demonstrated to control the flow of hematopoiesis into distinct pathways with varying quantities and kinetics (reviewed in [1]).

The stages of progression from HSC through commitment to the B cell lineage and generation of a functional BCR are controlled by multiple transcriptional factors and growth factor receptors [1–3]. The roles played by transcription factors in these processes have been starkly outlined by studies of gene knockouts. These analyses showed that IKAROS and PU.1 are involved in the generation of more primitive lymphoid progenitors, while E2A and EBF are critically involved in the process of B lineage specification (Fig. 1). Even though PU.1 was originally reported to be vital for B cell development, later studies have found that B cells can develop from *Sfp1*<sup>-/-</sup> fetal liver cells (*Sfp1* encodes PU.1) [4–6] suggesting that PU.1 may synergize with other factors to facilitate B cell differentiation. PAX5 is responsible for solidifying the B cell program by repressing lineage inappropriate genes and promoting expression of B cell-specific genes. Finally, IRF8 and IRF4 are uniquely required for Ig light chain gene rearrangement [7].

In this article, we review the most recent findings regarding the roles played by the transcription factor, IRF8, in the regulation of the B cell lineage and the myeloid lineage specification and differentiation.

## IRF8 biology

IRF8, otherwise known as interferon consensus sequence binding protein (ICSBP) [8], is a member of the IRF family of transcription factors that is induced by interferons (IFNs) in a variety of cell types. IRF8 itself is induced by IFN $\gamma$  in macrophages and T cells, with induction mediated by a gamma activation sequence (GAS) element in the IRF8 promoter. The family is characterized by a DNA binding domain in the N-terminal half of the proteins and an IRF association domain (IAD) in the C-terminus that is responsible for heterodimerization with other transcription factors.

IRF8 functions as a transcriptional activator or repressor depending on the formation of different heterodimeric DNA binding complexes with partners that include members of

ETS family (PU.1, TEL) [9, 10] and IRF family (IRF1, IRF2, and IRF4) [11–13] as well as E47 [14], NFATc1 [15], and MIZ1 [16]. Stable complexes of IRF8 and its partners can bind to any of a series of target sites termed IFN-stimulated response elements (ISRE), ETS/IRF response elements (EIRE), ETS/IRF composite elements (EICE), and the recently described IRF/ETS composite elements (IECE) [17]. Complexes bound to these elements regulate the expression of a large number of genes involved in cytokine signaling (*Il12*), cell cycle regulation (*Cdkn2b*), and host defense (*Slc11a1* [Nramp1] and *Nos2* [iNOS]), and differentiation (*Prdm1* [BLIMP1]) among many other activities. The levels of IRF8 protein are determined in part by CBL-mediated ubiquitylation and subsequent proteasomal degradation [18]. In addition, more recent studies have shown that the transcriptional activity of IRF8 for the *Il12p40* promoter is enhanced following ubiquitylation by the E3 ubiquitin ligase, TRIM21 [19].

Much of what is known about the biology of IRF8 has come from studies of mice bearing a null mutation of the gene (*IRF8*<sup>-/-</sup>) [20]. These mice exhibit a marked expansion of granulocytes and, to a lesser extent, macrophages and are markedly immunodeficient because of deficiencies in IFN $\gamma$  and IL-12 production [21]. Interestingly, BXH2 mice with a point mutation effecting a single amino acid change in the IAD domain of IRF8 exhibit an almost identical phenotype [22]. Since the IAD domain is responsible for the ability of IRF8 to heterodimerize, this finding demonstrates that almost all activities of IRF8 are dependent on its interactions with other proteins.

A large number of studies have demonstrated that IRF8 plays critical roles in the differentiation of myeloid cells, promoting monocyte over granulocyte differentiation [23]. It is also a crucial controller of many aspects of dendritic cell differentiation and function, thereby playing an essential role in the establishment of innate immunity [24, 25].

Until recently, little was known about the expression and function of IRF8 in peripheral B lineage cells or T cells. In the last several years it has been shown that IRF8 is expressed at relatively low levels in peripheral follicular (FOL) B cells and at high levels in germinal center (GC) B cells of both mice and humans, but is strikingly downregulated in plasma cells [26, 27]. In GC, IRF8 modulates the expression of BCL6 and AID [26]. Parallel studies of mouse and human B cell malignancies showed IRF8 was expressed in almost all neoplasms except for human plasmablastic plasmacytomas and multiple myeloma and mouse plasmacytomas [26, 27]. Interestingly, all GC type diffuse large B cell lymphomas of humans expressed IRF8 but only about half of the non-GC type cases [27].

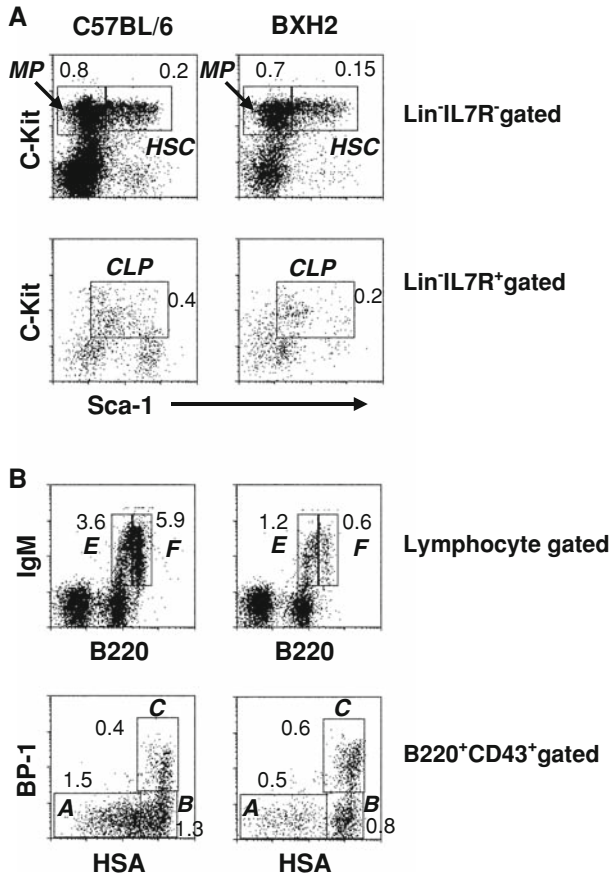
The focus of the remainder of this review will be on expression and function of IRF8 during earlier stages of hematopoietic differentiation with an emphasis on commitment to the B cell lineage and differentiation events that occur in the bone marrow (BM). Although the last several years have seen remarkable progress in defining stages of B cell differentiation that lie between the hematopoietic stem cell and cells expressing a mature B cell receptor (BCR), there is still not a standard nomenclature for this progression. Nonetheless, it appears that a general consensus is slowly being reached on the major pathway for becoming a B cell (Fig. 1). The nomenclature displayed gives the phenotypic “fraction” (Fr.) designations as described by Hardy and Hayakawa [28] together with the “Basel nomenclature” of Rolink et al. [29] as well as contributions from the Kincade group and others [2].

### **IRF8 regulates myeloid lineage progression**

IRF8 is expressed at low levels in HSC, but its expression is increased in Lin<sup>-</sup>Sca-1<sup>-</sup>IL-7R<sup>-</sup>cKit<sup>+</sup> cells including CMP, GMP (granulocyte-macrophage progenitor), and MEP

(megakaryocyte-erythrocyte progenitor) [30]. Deficiency in IRF8 in both traditional IRF8 knockout and BXH2 IRF8 mutant mice results in significant expansion of neutrophils at the expense of macrophages [20, 22]. We have observed increased number of myeloid progenitors in mice of both genotypes [30] (Fig. 2 and Table 1). While an increased sensitivity of IRF8<sup>-/-</sup> MPs to G-CSF and GM-CSF [31] may explain why the myeloid progenitor population is expanded in these mice, our studies suggest that altered expression of PU.1 in IRF8<sup>-/-</sup> MPs may be the molecular mechanism that drives this expansion [30]. Compared with MPs of wild-type mice, IRF8-deficient MPs express significantly higher levels of PU.1. This is consistent with the notion that high levels of PU.1 expression facilitate myeloid lineage differentiation [32, 33]. Moreover, IRF8 suppresses cell cycle progression [23, 30]. Thus, its absence may allow PU.1 to accelerate the outgrowth of MPs.

Regulation of PU.1 expression is complex with controls exerted by both a proximal promoter and by an upstream regulatory element (URE) ~15 kd upstream of the transcription start site [34, 35]. There is ample evidence indicating that IRF8 negatively regulates PU.1



**Fig. 2** Impaired B cell development in the BM of BXH2 and control mice. (a) BM cells from BXH2 and C57BL/6 mice were stained with antibodies against Lineage panel (Lin), IL-7R $\alpha$ , c-Kit, and Sca-1. Numbers represent the percentage of total events. (b). BM cells from the same mice were stained for B220, IgM, CD43, BP-1, and HSA. The percentage of B cells falling within each gate is given. Hardy Frs. A to F are indicated. Data are representative of three mice per group. The results from multiple analyses are summarized in Table 1

**Table 1** B cell deficiency in IRF8-deficient mice

	IRF8 <sup>-/-</sup>	BXH2 <sup>a</sup>
HSC	Down 2×	Similar
CLP	Down 2×	Down 20%
MP	Increased 20%	Increase 67%
Fr.A	Down 5×	Down 1×
Fr.B/C	Down 35%	Down 1×
Fr.D (L)	Down 33%	Down 1×
Fr.D (S)	Down 2×	Down 2×
Fr.E	Down 3×	Down 3×
Fr.F	Down 10×	Down 7×

Bone marrow cells were analyzed by flow cytometry. The definition of each subset was described in [30]. Data indicate changes in mutant mice relative to wild-type controls. 3–8 mice (6–8 weeks) were analyzed per group. Fr.D (L), large pre-BII; Fr.D (S), small pre-BII

<sup>a</sup> Male BXH2 mice were obtained from Jackson laboratory and backcrossed with female B6 mice to eliminate endogenous B-tropic murine leukemia virus (MuLV) that are passaged to offspring through the milk and contribute to the development of myeloid leukemias by proviral insertional mutagenesis of proto-oncogenes

expression at both the RNA and protein levels [30]. The proximal promoter of PU.1 harbors an IRF8 target sequence, which is occupied by IRF8 as shown by chromatin immunoprecipitation (ChIP) analyses. Studies examining knockdowns and overexpression of IRF8 showed that IRF8 levels were inversely correlated with the levels of PU.1 expression [30]. Therefore, IRF8 represses PU.1 to restrict production of myeloid lineage precursors. Very recently, Tenen and colleagues found that the transcription of non-coding antisense RNAs of *PU.1* negatively regulates PU.1 expression by modulating mRNA translation [36]. Interestingly, both the coding and antisense transcripts were strongly influenced by the URE. It will be interesting to determine whether IRF8 affects expression of the antisense as well as the sense transcripts of PU.1.

### IRF8 promotes B lineage specification

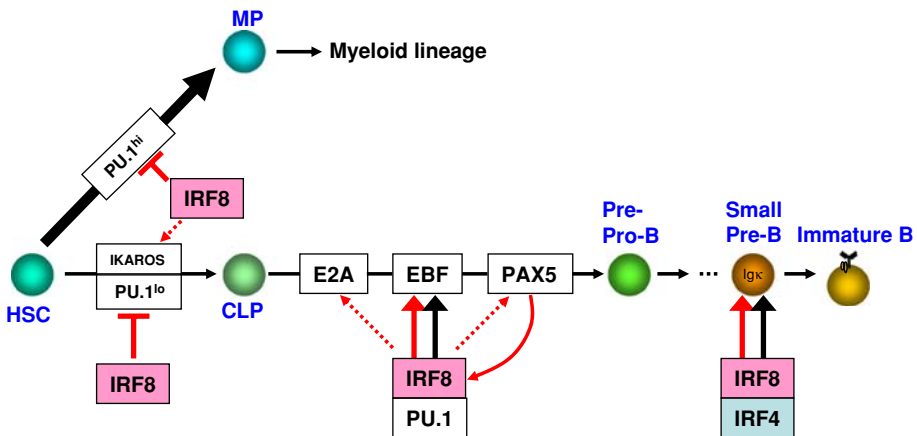
As noted above, numerous studies have demonstrated the importance of E2A, EBF, and PAX5 to B cell lineage specification and commitment in the BM. Careful analysis of early B cells in the BM of IRF8<sup>-/-</sup> and BXH2 mice revealed a previously unrecognized role for IRF8 in both of these activities (Table 1). The pre-pro-B cell compartments in the BMs of mutant mice were significantly reduced compared to wild-type mice [30] (Fig. 2 and Table 1). While the size of CLP population was also reduced in the BM of mutant mice, adoptive transfer experiments demonstrated that this effect was not cell autonomous and was possible due to the alterations in the size or activities of subsets of other BM cells [30].

The decreased commitment of CLP to the pre-pro-B and pro-B stages of differentiation in IRF8-deficient mice was associated with reduced expression of *E2A*, *EBF*, and *PAX5*. In contrast, for CLP of wild-type mice, commitment to the B cell lineage is marked by a dramatic increase in IRF8 expression [30]. Further analyses revealed that IRF8 directly regulates expression of EBF [30], which is responsible for the activation of several genes involved in B cell lineage commitment, such as *Pax5*, *Cd79a*, *Vpreb1*, and *Igll1* [37]. Interestingly, studies using an EBF promoter reporter construct demonstrated that IRF8 synergizes with PU.1 in regulating EBF expression [30]. This finding is consistent with earlier reports showing that siRNA-mediated knockdown of *PU.1* in ES cells induced expression of *Ebf* and *Pax5*, which correlated with enhanced B cell developmental potential [38].

The importance of IRF8 to regulation of B lymphopoiesis is supported by findings in aged mice and humans [39]. Hematopoietic progenitor cells from aged individuals express significantly lower levels of IRF8 than those from young individuals [39], which was associated with increased proliferative activity and biased myeloid differentiation [40], a condition similar to that found in IRF8<sup>-/-</sup> and BXH2 mice [20, 22].

### The transcriptional network regulating B lineage specification

To date, at least four transcription factors have been shown to contribute to lymphoid-lineage priming. These include E2A, IKAROS, PU.1, and EBF. IRF8 may represent a fifth regulator of B cell lineage priming (Fig. 3). The upregulated expression of IRF8 in CLP and pre-pro-B cells could downregulate PU.1 to suppress myeloid lineage potential. By synergizing with PU.1, IRF8 promotes EBF expression. The significantly reduced expression of E2A in IRF8<sup>-/-</sup> CLP and pre-pro-B cells hints that IRF8 may also control E2A expression through an as yet unidentified mechanism [30]. The similarly reduced levels of PAX5 expression in IRF8<sup>-/-</sup> CLP and pre-pro-B cells may be secondary to low levels of EBF [30]. As the promoter region of *Ikaros*, which encodes IKAROS, contains an IRF8 consensus binding sequence (unpublished observations), IRF8 may also directly contribute to the regulation of IKAROS expression [41]. These five transcription factors form a dynamic, mutual, and autoregulatory system that defines the optimal signaling strengths for lymphoid and myeloid lineage specification, commitment, and differentiation. For example, IRF8 has been found to be a target of PAX5 [42], whereas *Pax5* expression is directly regulated by EBF [3, 43], which itself is transcriptionally regulated by IRF8 [30], PAX5, and PU.1 [3, 44]. In addition, PU.1 and EBF have been shown to be autoregulatory [35, 44]. IRF8 may function as a factor that, early on, primes the lymphoid and myeloid lineage potential of progenitors by regulating the expression of PU.1, followed by E2A, EBF, and PAX5. Even though studies of PU.1 reporter mice did not reveal a gradient of expression between CMP and CLP [45], studies by us and others strongly support the idea that B lineage cell development requires low levels of PU.1 expression. From this perspective,



**Fig. 3** A model of IRF8 function in early B cell development. IRF8 modulates expression of PU.1 and together with PU.1 restricts myeloid lineage differentiation while promoting B lineage differentiation by activating expression of EBF, E2A, and PAX5. By associating with IRF4, IRF8 regulates Ig $\kappa$  gene rearrangement and facilitates generation of immature B cells. Modified from [1]

IRF8 can be seen to function as a rheostat, modulating the expression of PU.1 and the flow rate of cells into the lymphoid and myeloid lineages.

### **IRF8 and IRF4 act synergistically to drive immunoglobulin light chain rearrangement**

During the later stages of B cell development in the bone marrow, a productively rearranged heavy chain genes pairs with the surrogate light chain genes,  $\lambda 5$  and Vpre-B, that together with the signaling molecules, CD79A and CD79B, form the pre-BCR on the cell surface. Pre-BCR-induced signals results in active proliferation of large, cycling pre-B cells for several cycles before they exit the cell cycle and become small, resting pre-B cells that undergo light chain rearrangement and transcription (Fig. 1). It was previously shown that B cell development in mice deficient in both IRF8 and IRF4 is arrested at a stage that resembles the normal large pre-B cell stage and that fails to downregulate surrogate light chains and rearrange light chain genes [7]. Reintroduction of IRF8 and IRF4 into these cells was shown to rescue light chain rearrangement and transcription [46]. Consistent with these reports, our studies of IRF8<sup>-/-</sup> mice demonstrated that IRF8-deficient pre-B cells proliferated more vigorously than normal control cells in vitro [30]. Recently, it was reported that IRF8 and IRF4 downregulate surrogate light chain expression and pre-BCR levels by activating expression of IKAROS and AILOS [41]. These genes are critical for downregulation of the pre-BCR and exit from the cell cycle, thereby mediating the transition from large to small pre-B cells [41].

### **Conclusions and future prospects**

The studies described here solidify the identity of IRF8 as a member of the family of transcription factors that orchestrate the complex programs of B cell differentiation and function beginning with B cell lineage specification through generation of a functional BCR. Direct effects on the transcriptional regulation of PU.1 and EBF are likely to be of greatest importance. The fact that BXH2 mice with a mutation of IRF8 in the IAD domain so closely resemble mice with a conventional IRF8 knockout indicates that the vast majority of IRF8 functions are carried out as through heterodimerization with IRF4 and an unknown number of other partners.

A number of new tools, now nearing completion, should add quickly to the depth and clarity of these understandings. These include the development of a conditional knockout of IRF8, an IRF8 reporter mouse, a reporter mouse for TRIM21, which is responsible for IRF8 ubiquitylation, and a mouse with a knockin of IRF8-EGFP fusion protein that will permit assessment of IRF8-chromatin interactions in a variety of cell types with IRF8 expressed at physiologic levels. In addition, ChIP–ChIP analyses of IRF8 targets in pro-B cells using genome-wide promoter chip arrays will greatly enrich our understanding of IRF8 function in developing B lineage cells. Finally, powerful systems are being generated for identifying IRF8 partner proteins in early B lineage cells. We anticipate that these new approaches will define novel contributions of IRF8 to both innate and adaptive immune responses.

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