

IRF4 and IRF8: governing the virtues of B lymphocytes

Vipul SHUKLA (✉), Runqing LU (✉)

Department of Genetics, Cell Biology & Anatomy, University of Nebraska Medical Center, Omaha, NE 68198, USA

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Abstract Interferon regulatory factor 4 (IRF4) and IRF8 are critical regulators of immune system development and function. In B lymphocytes, IRF4 and IRF8 have been shown to control important events during their development and maturation including pre-B cell differentiation, induction of B cell tolerance pathways, marginal zone B cell development, germinal center reaction and plasma cell differentiation. Mechanistically, IRF4 and IRF8 are found to function redundantly to control certain stages of B cell development, but in other stages, they function nonredundantly to play distinct roles in B cell biology. In line with their essential roles in B cell development, deregulated expressions of IRF4 and IRF8 have been associated to the pathogenesis of several B cell malignancies and diseases. Recent studies have elucidated diverse transcriptional networks regulated by IRF4 and IRF8 at distinct B cell developmental stages and related malignancies. In this review we will discuss the recent advances for the roles of IRF4 and IRF8 during B cell development and associated diseases.

Keywords IRF4, IRF8, B lymphocytes, transcriptional regulation, leukemia and lymphoma, B cell development

Introduction

Interferon regulatory factor 4 (IRF4) and interferon regulatory factor 8 (IRF8) are highly homologous proteins that belong to the interferon regulatory factor (IRF) superfamily of transcription factors. Physiologically, IRFs are important mediators of anti-viral responses (Tamura et al., 2008). In addition to their role in antiviral responses, IRF4 and IRF8 also act as critical regulators of immune system development and function. This suggests that IRF4 and IRF8 have presumably arisen as a result of divergent evolution from a common ancestor belonging to the IRF superfamily. IRF4 and IRF8 were initially thought to be exclusively expressed in cells of immune lineages. However, recent reports have also identified IRF4 and/or IRF8 expression in melanocytes, adipocytes, smooth muscles, cardiac muscles and neurons where they perform diverse functions (Eguchi et al., 2011; Jiang et al., 2013; Praetorius et al., 2013; Guo et al., 2014; Jiang et al., 2014a; Xiang et al., 2014; Yoshida et al., 2014; Zhang et al., 2014).

IRF4 is induced in response to pathways activating NF- κ B

signaling while IRF8 is induced by type II interferon (Tamura and Ozato, 2002; Saito et al., 2007). Structurally, IRF4 and IRF8 are similar to other IRFs in having a tryptophan pentad containing DNA binding domain (DBD) and an interferon association domain (IAD) through which they can homo- or hetero-dimerize with other members of the family. To perform their transcriptional regulatory functions, IRF4 and IRF8 can form homo- or hetero-dimers with each other and with other members of the family. These homo- or hetero-dimers bind DNA with low affinity at canonical interferon-stimulated response elements (ISRE) represented as GAAANNGAAA. Besides their interaction with other IRFs, IRF4 and IRF8 can also form heterodimers with members of Ets family or AP-1 family of transcription factors (Escalante et al., 2002; Glasmacher et al., 2012; Li et al., 2012; Tussiwand et al., 2012). The heterodimers formed between IRF4/8 and Ets members, PU.1 and Spi-B bind DNA at Ets interferon composite elements (EICE) represented as GGAANN(N)GAAA. The EICE motifs were initially identified in immunoglobulin (Ig) light chain 3' κ enhancer and λ enhancer regions mediating Ig light chain locus activation (Brass et al., 1996; Brass et al., 1999). The IRF4/8-Ets hetero-dimers bind to DNA at EICE motifs with much greater affinity than ISRE motifs (Ochiai et al., 2013). More recently, IRF4 and IRF8 have been identified to co-bind DNA with AP-1 family members on AP-1-IRF composite elements (AICE) represented as GAAATGAGTCA or GAAANNNTGAGTCA in

Received April 4, 2014; accepted May 28, 2014

Correspondence: ^aVipul SHUKLA; ^bRunqing LU

E-mail: ^avipul.shukla@unmc.edu; ^bRlu@unmc.edu

a variety of immune cell subsets (Glasmacher et al., 2012; Li et al., 2012; Tussiwand et al., 2012). The formation of complexes between IRF4/IRF8 with either Ets or Ap-1 transcription factors depends on the cell type and cellular context. For example, the Ap-1-IRF complexes are predominantly known to regulate cellular functions in T cells and dendritic cells while Ets/IRF complexes are critical for B cell development and functions. The cooperative binding of IRF4 and IRF8 to DNA with members of IRF, Ets and AP-1 families represents evolutionary conserved mechanisms to integrate diverse signaling inputs during immune system development and function (Glasmacher et al., 2012). Moreover, IRF4 and IRF8 have been shown to interact with transcription factors NFATs and E2A to regulate transcription in different cell types (Rengarajan et al., 2002; Hodawadekar et al., 2012).

IRF4 and IRF8 are important regulators for generation, differentiation and functions of several immune cell subsets. IRF4 play key roles in generation and functions of T follicular helper cells (Tfh), Th1 cells, Th2 cells, Th9 cells, T regulatory cells, CD8⁺ T cells, Th17 cells, macrophages and dendritic cells (Mittrücker et al., 1997; Lohoff et al., 2002; Brüstle et al., 2007; Kwon et al., 2009; Zheng et al., 2009; Satoh et al., 2010; Staudt et al., 2010; Cretney et al., 2011; Bollig et al., 2012; Tussiwand et al., 2012; Gao et al., 2013; Man et al., 2013; Persson et al., 2013; Schlitzer et al., 2013; Vander Lugt et al., 2014). Similarly, IRF8 is important for Th1, Th2, Th17, macrophage and dendritic cell development and function (Giese et al., 1997; Wu et al., 1999; Ouyang et al., 2011; Becker et al., 2012; Xu et al., 2012; Schönheit et al., 2013). In B cells specifically, IRF4 and IRF8 are expressed at multiple stages to control important decisions affecting their differentiation, function and transformation (Lu, 2008) (Fig. 1). At early stages of B cell development IRF4 functions redundantly with IRF8 to coordinate pre-B cell differentiation (Lu et al., 2003) (Fig. 1). On the other hand, at later stages of B cell development, IRF4 and IRF8 have been shown to function non-redundantly to regulate follicular versus marginal zone cell fate decisions, germinal center reaction (GC), class switch recombination (CSR) and plasma cell differentiation (Klein et al., 2006; Sciammas et al., 2006; Lu, 2008; Feng et al., 2011; Ochiai et al., 2013; Simonetti et al., 2013) (Fig. 1). In this review we will focus on describing the recent advances on the roles of IRF4 and IRF8 in B cell development and associated diseases.

IRF4 and IRF8 in early B cell development

Early pro-B cells arise from multi-potent hematopoietic progenitors in bone marrow upon coordinated expression of transcription factor E2A, Ebf1 and the B cell commitment gene Pax5 (Busslinger, 2004). An early study investigating the role of IRF8 in HSCs identified defects in early

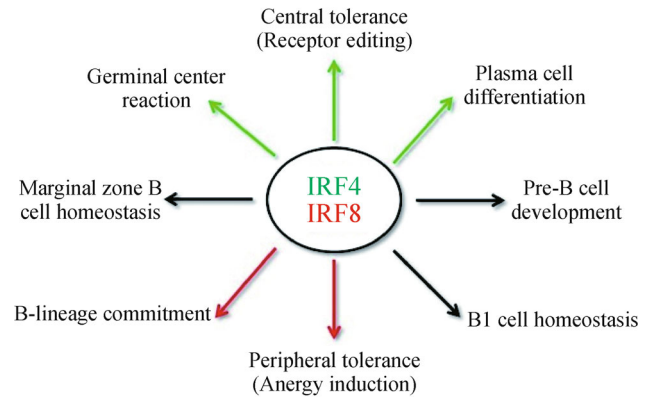


Figure 1 Regulation of B cell development by IRF4 and IRF8. The stages of B cell development regulated jointly by IRF4 and IRF8 are indicated by black arrows. Other stages of B cell development regulated exclusively by IRF4 or IRF8 are indicated by green or red arrows, respectively.

commitment to B lineage in IRF8 deficient HSCs (Wang et al., 2008). IRF8 deficiency leads to a skewed development of HSCs toward myeloid lineages at the expense of B cells (Wang et al., 2008). Furthermore, IRF8 was shown to directly bind and repress PU.1 which is known to be critical for myeloid development (Wang et al., 2008). Additionally, to reinstate B cell fate decisions IRF8 was shown to directly activate Ebf1 transcription (Wang et al., 2008). Nevertheless, HSCs can still differentiate into B lineage even in the absence of IRF8, indicating that IRF8 only modulates but is not essential for these cell fate decisions. Pro-B cells undergo immunoglobulin (Ig) heavy chain rearrangements as a first step to generate B cell receptors (BCRs). These events are mediated by several transcription factors including Pax5. IRF4 and IRF8 were initially identified as direct targets of Pax5 in early B cell development at the pro-B cell stage (Pridans et al., 2008). Intriguingly, IRF4 and IRF8 along with PU.1 have been recently identified to bind a putative enhancer region in Pax5 locus to regulate its expression (Decker et al., 2009).

At the pre-B cell stage, developing B cells undergo light chain rearrangements to generate functional B cell receptors (BCR) as they transition to the immature B cell stage. Early studies identified IRF4 and IRF8 as transcription factors that bind the 3' κ enhancer and λ enhancer to regulate the rearrangement and expression of immunoglobulin light chain at the pre-B cell stage (Brass et al., 1996; Brass et al., 1999; Ma et al., 2006). IRF4 and IRF8 are required for differentiation of pre B cells to immature B cells as B cell development is blocked at the pre-B cell stage in IRF4 and IRF8 (IRF4,8^{-/-}) double deficient mice. Furthermore, IRF4,8^{-/-} pre-B cells were shown to have a hyper-proliferative phenotype. Further analysis showed that the defects in the IRF4,8^{-/-} pre-B cells can be rescued by reconstitution of either IRF4 or IRF8 (Ma

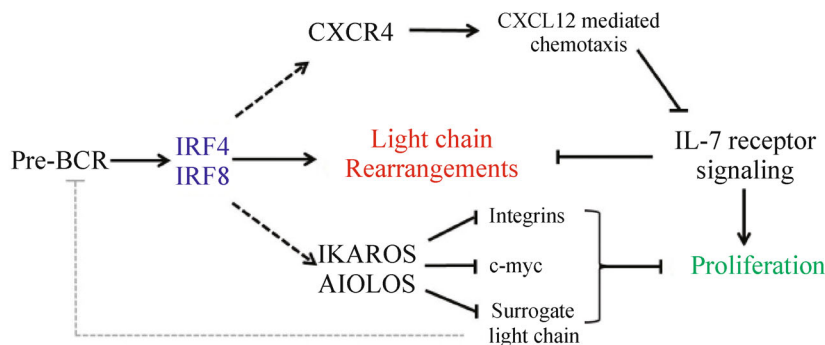


Figure 2 IRF4 and IRF8 utilize multiple mechanisms to control pre-B cell Development. Pre-BCR signaling leads to expression of IRF4 and IRF8 that directly promotes light chain rearrangement. IRF4 and IRF8 also induce Ikaros and Aiolos to inhibit pre-B cell proliferation. Ikaros directly suppresses expression of c-myc, surrogate light chain and integrins. Moreover, IRF4 regulates the expression of CXCR4, which leads to migration of pre-B cells toward CXCL12 expressing stromal cells and away from IL-7 expressing stromal cells. This leads to attenuation of IL-7 signaling.

et al., 2006). These results indicate that IRF4 and IRF8 function redundantly to orchestrate pre-B cell development.

Pre-B cell development can be further divided into two distinct stages; the cycling large pre-B cells that transition to generate resting small pre-B cells. In cycling pre-B cells a primitive BCR called the pre-B cell receptor (pre-BCR) is assembled, that functions to mediate initial expansion and eventual differentiation of large pre-B cells to small pre-B cells (Clark et al., 2014). Interestingly, IRF4 has been shown to be induced downstream to pre-BCR signaling (Thompson et al., 2007). Large pre-B cells also depend on IL-7 receptor signaling for their proliferation *in vivo*. Moreover, IL-7 has also been shown to impede pre-B cell differentiation by directly inhibiting light chain rearrangements (Mandal et al., 2011). We and others have shown that IRF4 and IRF8 limits pre-B cell expansion by negatively regulating both pre-B cell receptor and IL-7 receptor signaling (Johnson et al., 2008; Ma et al., 2008).

Expression of IRF4 and IRF8 at the pre-B cell stage occurs downstream to pre-B cell receptor signaling (Fig. 1). Upon induction, IRF4 and IRF8 further induces the expression of transcription factors Ikaros and Aiolos that functions as negative regulators of pre-B cell receptor signaling and cell cycle progression (Ma et al., 2008) (Fig. 2). Ikaros and Aiolos repress the expression of surrogate light chain (SLC), which is an essential component of the pre-B cell receptor complex (Ma et al., 2008). We further showed that Ikaros and Aiolos inhibit large pre-B cell expansion by directly binding and repressing c-myc (Fig. 2) (Ma et al., 2010). Notably, Ikaros deficient pre-B cells fail to undergo growth arrest even upon IL-7 withdrawal (Heizmann et al., 2013). These results indicate a direct role of Ikaros in inhibiting the pre-B cell expansion. Intriguingly, two recent reports have identified a novel mechanism for inhibition of cell cycle by Ikaros in pre-B cells (Joshi et al., 2014; Schwickert et al., 2014). Ikaros directly suppresses the expression of several integrins (Fig. 2) including Itga1, Itga5 and Itgb1 as well as components

mediating focal adhesions like Ptk2, Dock1 and Vcl. Therefore, increased expression of integrin and focal adhesion components in Ikaros deficient pre-B cells allow them to strongly adhere to stromal cells. The adhesion of Ikaros deficient pre-B cells to stromal cells in turn provides them with essential growth factors including IL-7 and SCF, promoting their survival and proliferation. These results suggest an indirect role for Ikaros in limiting pre-B cell expansion via inhibiting their adhesion to the stromal cells. In summary, Ikaros employs multiple mechanisms to promote pre-B cell differentiation and these mechanisms may function concurrently and may not be mutually exclusive.

IRF4 has also been shown to limit the pre-B cell expansion by attenuating IL-7 receptor signaling (Johnson et al., 2008). IRF4 was shown to regulate the expression of chemokine receptor CXCR4 in pre-B cells (Fig. 2) (Johnson et al., 2008). CXCR4 induction by IRF4 drives pre-B cells toward CXCL12 expressing stromal cells and away from the IL-7 secreting stromal cells (Tokoyoda et al., 2004). Importantly, IL-7 signaling is known to inhibit pre-B cell differentiation by directly repressing light chain rearrangements (Mandal et al., 2011). Therefore, the chemotaxis of pre-B cells to niches bearing low levels of IL-7 would be important to limit their expansion and to initiate productive light chain rearrangements (Johnson et al., 2008; Mandal et al., 2011) (Fig. 2). These results support an indirect role for IRF4 in limiting the pre-B cell expansion and promoting their differentiation.

IRF4 and IRF8 in regulation of B cell tolerance

Upon assembly of a functional B cell receptor (BCR), immature B cells exit the bone marrow and enter into the peripheral lymphoid organs. However, prior to their migration to the peripheral lymphoid organs, the BCRs of the newly generated B cells are tested for their self-reactivity. The entry of the self-reactive B cells into the periphery is abolished by

Central and Peripheral tolerance mechanisms that function at the immature and transitional B cell stages. The central tolerance mechanism in bone marrow is primarily constituted of Receptor Editing, wherein the self-reactive BCRs on newly developed B cells are edited by secondary Ig rearrangements. Self-reactive B cells that fail to surmount self-reactivity following receptor editing undergoes eventual deletion, as a default pathway. The peripheral tolerance mechanisms may render a self-reactive B cell unresponsive to antigen stimulation by a process called anergy. These tolerance mechanisms help curtail self-reactive B cells which may otherwise lead to development of systemic autoimmune diseases.

We have shown that IRF4 is involved in central tolerance mechanisms by promoting receptor editing (Pathak et al., 2008). Using a BCR transgenic mice and a membrane bound antigen we demonstrated that IRF4 is critical for secondary rearrangements at the immature B cell stage (Pathak et al., 2008). We showed that secondary rearrangement is impaired in IRF4 deficient mice. Moreover, we found IRF4 to be more critical for λ rearrangements than κ rearrangements. Consistent with our findings, a recent study also showed that elevated levels of IRF4 in pre-B cells leads to more efficient activation and rearrangements at λ locus (Bevington and Boyes, 2013). Remarkably, premature induction of IRF4 using the surrogate light chain promoter in pro-B cells was capable of triggering the entire cascade of events required for light chain rearrangements (Bevington and Boyes, 2013). Another recent report described activated NF- κ B signaling as a feature of cells engaged in receptor editing (Cadera et al., 2009). Importantly, the cells undergoing receptor editing in this study also expressed high levels of IRF4 (Cadera et al., 2009). These studies have identified a critical role for IRF4 in central tolerance mechanisms as a regulator of receptor editing.

Unlike during pre-B cell development, IRF8 cannot compensate for loss of IRF4 in receptor editing (Pathak et al., 2008). However, we have recently identified a novel role for IRF8 in regulating peripheral tolerance by maintaining the anergic state of self-reactive B cells. Using a double transgenic mouse model expressing a transgenic BCR and the secreted cognate auto-antigen, we showed that B cell anergy is breached in IRF8 deficient mice (Pathak et al., 2013). We further showed that both germline and B cell specific IRF8 deficient mice produce anti-dsDNA antibodies (Pathak et al., 2013). It is known that anergic B cells fail to undergo complete maturation and are stalled at the transitional stage. Intriguingly, we showed that IRF8 negatively regulates the survival of these anergic B cells at transitional B cell stage (Pathak et al., 2013). These studies identified IRF8 as a novel regulator of B cell anergy. However, the underlying molecular events controlled by IRF8 in maintenance of B cell anergy remain unclear. Collectively, these studies have demonstrated that IRF4 and IRF8 are critical for regulating different arms of B cell tolerance induction pathways.

IRF4 and IRF8 in Follicular, Marginal Zone and B1 B cell development

Mature B cells in mouse can be subdivided into two main subsets: the major B2 cells and the minor B1 cells. B2 cells in murine spleen can either differentiate to a predominant Follicular B cell population (FO B) or can give rise to a minor Marginal Zone B cell population (MZ B). FO B cells are primarily localized to B cell follicles situated in the splenic white pulp and are responsible for T cell dependent humoral responses. MZ B cells on the other hand, are located at the border of splenic white pulp and are required for rapid T cell independent responses against blood borne pathogens and particulate antigens. These cell fate decisions are primarily influenced by BCR specificity, BCR signaling strength and Notch signaling (Pillai and Cariappa, 2009). Both IRF4 and IRF8 have been identified as transcriptional regulators known to affect these alternative cell fate decisions in mature B cells.

In a recently published report, IRF4 was found to restrict the MZ B cell pool in a B cell intrinsic manner. IRF4 deficiency in mature B cells lead to retention of mature B cells preferentially to the splenic MZ. These defects were mechanistically attributed to the elevated levels of Notch2 protein in IRF4 deficient mature B cells (Simonetti et al., 2013). Importantly, activated notch signaling is one of the pathways obligatory required for generation and maintenance of MZ B cells (Pillai and Cariappa, 2009). Furthermore, inhibition of Notch2 by an inhibitory antibody reverses the MZ B cell defects in IRF4^{-/-} mice. Interestingly, regulation of Notch proteins by IRF4 occurs at a post-transcriptional level and is linked to the reduced levels of an E3 ubiquitin ligase Fbxw7 (Simonetti et al., 2013). Additionally, IRF4 deficiency also lead to altered expression of integrins and chemokine receptors known to mediate migration and retention of mature B cells to specific anatomical sites (Simonetti et al., 2013). It is noteworthy that the expansion of MZ B cells in IRF4^{-/-} mice can only be detected by immunohistochemistry staining but not by conventional flow cytometry analysis (Simonetti et al., 2013). This suggests that the expanded B cell population in the marginal zone of IRF4^{-/-} mice is not comprised of bona fide MZ B cells and may simply represent FO B cells that are aberrantly mislocalized.

IRF8 was also identified as a regulator of FO or MZ cell fate decisions (Tailor et al., 2008; Wang et al., 2008; Feng et al., 2011). Both germline and B cell specific deficiency of IRF8 cause an expansion of MZ B cells with a concomitant decrease in the frequency of FO B cells (Tailor et al., 2008; Feng et al., 2011). Interestingly, a BXH2 mouse harboring a point mutation in the interferon association domain (IAD) of IRF8 (R294C) phenocopies the IRF8^{-/-} mice in their MZ expansion features (Tailor et al., 2008). Although, these studies have identified a role for IRF8 in MZ B cell development, the molecular mechanism by which IRF8 restricts MZ B cell pool is still unclear.

B1 cells represent a minor B cell subset that primarily

occupies peritoneal and pleural cavities in rodents. Additionally, a small fraction of B1 cell population is also present in murine spleen. B1 cells recognize natural antigens and spontaneously differentiate to plasma cells without requiring T cell help. B1 cells are also unique in displaying properties to self-renew themselves (Hardy, 2006). On the basis of CD5 expression, B1 cells are divided into the CD5 (+) subset called B1a cells and the CD5 (-) B1b cells. IRF8 was shown to regulate the B1 cell numbers in a B cell intrinsic manner. B1b cells particularly, undergo an expansion in peritoneal cavity in the absence of IRF8 (Feng et al., 2011). Whether IRF8 regulates B1 cell numbers in peritoneal cavity by altering their self-renewal or differentiation still remains unknown. On the other hand, we have recently shown that New Zealand Black (NZB) mice expressing low levels of IRF4 (IRF4^{+/-}) exhibit defects in B1 cells. NZB IRF4^{+/-} B1 cells exhibit enhanced proliferation and survival while having decreased ability to differentiate to plasma cells (Ma et al., 2013). All of these defects cumulatively contribute to an accumulation of B1a cells in peritoneal cavities of these mice. Further studies are needed to identify the molecular events regulated by IRF4 and IRF8 in limiting the B1 cell pool.

Germinal center reaction, class switch recombination and plasma cell differentiation

Germinal centers represent specialized sites in secondary lymphoid organs that are induced during T cell dependent immune responses. Anatomically, germinal centers are constituted of a peripheral dark zone and an inner light zone. Centroblasts present in dark zone of germinal centers (GCs) represent the early arriving B cells that eventually give rise to the more mature centrocytes in the light zone. B cells in GCs undergo somatic hypermutation (SHM) to generate high affinity B cell receptors. B cells expressing high affinity BCRs bind to their cognate antigens present on antigen presenting cells (APCs) and to co-stimulatory molecules on T cells to differentiate to antibody secreting plasma cells and memory B cells. IRF4 is well defined as a transcription factor obligatory required for terminal differentiation of B cells to plasma cells (Klein et al., 2006; Sciammas et al., 2006). In GCs, IRF4 and IRF8 follow a reciprocal expression pattern (Cattoretti et al., 2006). IRF8 is shown to be highly expressed in centroblasts of GCs that are negative for IRF4 expression (Fig. 3). On the other hand, the more mature centrocytes differentiating toward plasma cells are positive for IRF4 expression (Cattoretti et al., 2006). These findings initially supported the notion that IRF4 may be dispensable for early stages of GC reaction while IRF8 may be obligatory required. In line with the notion, an early report identified severely impaired GC formation in mice globally lacking IRF8 (Lee et al., 2006). However, a recent report from the same group identified normal GC formation in mice lacking IRF8 specifically in B cells (Feng et al., 2011). These latest

findings indicate that IRF8 deficiency specifically in B cells is dispensable for GC reaction.

Contrary to IRF8, studies have shown that IRF4 is indispensable for early stages of GC reaction (Ochiai et al., 2013; Willis et al., 2014). Mice lacking IRF4 specifically in B cells fail to form GCs due to insufficient induction of Bcl6, Obf1 and AID (Fig. 3) (Ochiai et al., 2013). Bcl6 is a master regulator for GC reaction while, AID is critical for somatic hypermutation and class switch recombination (CSR) in GC B cells. Interestingly, using an inducible IRF4 transgene it was shown that a short pulse of IRF4 (for 2 days) was both required and sufficient for GC induction in IRF4^{-/-} mice (Ochiai et al., 2013). Furthermore, a recent report also identified a B cell intrinsic role for IRF4 in GC formation in response to a wide variety of antigens (Willis et al., 2014). T follicular helper (Tfh) cells represent a T cell subset that is critical for GC formation (Ramiscal and Vinuesa, 2013). Interestingly, IRF4 is recently described to be important for Tfh cell differentiation as well (Kwon et al., 2009; Bollig et al., 2012). IRF4 is shown to be critical for induction of Bcl6 that is required for generation of Tfh cells (Bollig et al., 2012). These results suggest that regulation of GC reaction may require both B cell intrinsic and extrinsic activities of IRF4.

Studies have shown that IRF4 levels undergo dynamic changes during germinal center reaction (Cattoretti et al., 2006; Ochiai et al., 2013). To rationalize these dynamic changes, Ochiai et al. have proposed a “kinetic control” model to explain how distinct stages of GC reaction are controlled by IRF4 expression (Sciammas et al., 2011; Ochiai et al., 2013). According to the “kinetic control” model, differential levels of IRF4 allow the regulation of mutually antagonistic GC and plasma cell programs in GC B cells (Ochiai et al., 2013). IRF4 at low levels co-operate with Ets and AP-1 family members to co-bind EICE and AICE motifs respectively to initiate GC program (Fig. 3). At later stages high levels of IRF4 cause a shift in binding to low affinity ISRE motifs to execute plasma cell differentiation program (Fig. 3). Several lines of evidences support the “kinetic control” model. First, in plasma cells containing low levels of Ets family members, IRF4 was shown to regulate Blimp-1 expression by binding to its locus at sites with ISRE motifs (Sciammas et al., 2006; Ochiai et al., 2013). Secondly, in plasma cells expressing high levels of IRF4, AID expression is repressed by IRF4; although IRF4 was not shown to bind AID locus (Sciammas et al., 2006). Thirdly, in GC B cells and Diffuse large B cell lymphoma cell (DLBCL) lines, IRF4 was shown to suppress Bcl6 expression by primarily binding to regions rich in ISRE motifs (Saito et al., 2007). Presumably, at lower levels in early GC B cells; IRF4 co-operate with Ets and Ap-1 family members to induce Bcl6 and AID whereas, at later stages IRF4 predominantly binds to ISRE motifs to shut down their expression. This would also explain the seemingly contradictory findings on role of IRF4 on Bcl6 expression in B cells (Saito et al., 2007; Ochiai et al., 2013).

IRF4 is induced in B cells upon BCR engagement to their

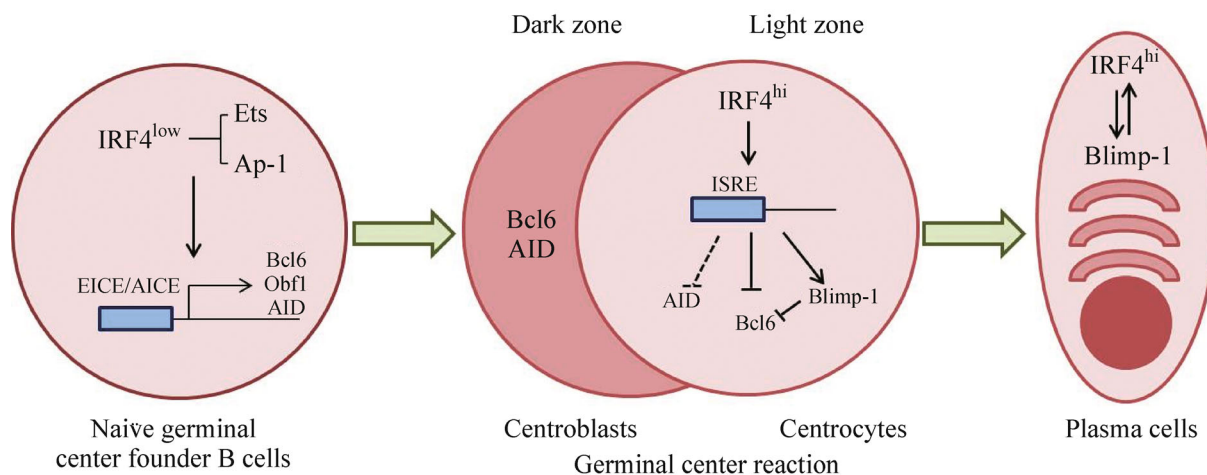


Figure 3 Role of IRF4 in germinal center (GC) reaction. At low levels, IRF4 (IRF4^{low}) binds cooperatively with Ets and AP-1 family members at EICE/AICE motifs and is critical for induction of Bcl6, Obf1 and AID to initiate GC reaction. In centrocytes located in light zone, high levels of IRF4 (IRF4^{hi}) is induced by antigen binding to high avidity BCRs. High levels of IRF4 cause a shift in binding of IRF4 from EICE/AICE motifs to low affinity ISRE motifs. IRF4 binding to ISRE induces Blimp-1 and represses Bcl6 to end GC reaction and to initiate plasma cell differentiation program.

cognate antigens and binding to co-stimulatory receptor on T cells. Presumably, the BCR signaling strength will directly determine the expression levels of IRF4. Therefore, naïve B cells expressing high avidity BCRs capable of inducing high levels of IRF4 can suppress conventional GC reaction and spontaneously differentiate to plasma cells. On the other hand, in naïve B cells expressing low avidity BCRs, a GC program is initiated due to low levels of IRF4. Low levels of IRF4 in these cells cooperate with Ets and Ap-1 family members to directly induce Bcl6 and AID expression and give rise to GC founder cells (Fig. 3). Upon ingress to the GCs, the GC founder cells in Dark Zone (centroblasts) undergo SHM events to generate high avidity BCRs. The downstream signals from the high avidity BCRs and co-stimulatory receptors on T cells can now sufficiently induce IRF4 in centrocytes to then execute a plasma cell differentiation program. To initiate a plasma cell differentiation program, high levels of IRF4 induces the expression of Blimp-1 that further suppresses Bcl6 expression to terminate GC program (Fig. 3). Thus, IRF4 levels in GC cells represent a critical checkpoint that determines the exit from a GC program and initiation of a plasma cell differentiation program. Therefore, IRF4 plays deterministic roles in initiating GC program, executing the exit from GCs and eventual plasma cell differentiation. The plausible flow of events summarized here are similar to the model proposed by Sciammas et al. in which IRF4 functions as a determinant of BCR signaling strength to direct these B cell fate decisions (Sciammas et al., 2011). It is worth noting that although initial studies identified IRF4 expression in centrocytes, subsequent studies have notably failed to detect IRF4 expression in any B cell population undergoing GC reaction (Willis et al., 2014).

Hence, it appears that IRF4 is expressed at high levels in B cells only upon exit from GCs. However, it is also possible that IRF4 is expressed only transiently or at a low level in centrocytes making it difficult to detect.

B cell malignancies and diseases

Consistent with critical involvement of IRF4 and IRF8 in B cell development, deregulated expression of IRF4 and IRF8 is associated with pathogenesis of several B cell malignancies and diseases. IRF4 is known to play distinct roles in B cell malignancies. In early B cell derived acute lymphoblastic leukemia (B-ALL) and mature B cell derived Chronic lymphocytic leukemia (CLL), IRF4 functions as a tumor suppressor (Fig. 4) (Pathak et al., 2011; Shukla et al., 2013). However, in multiple myeloma (MM) originated from plasma cells, it acts as a survival factor (Fig. 4) (Shaffer et al., 2008). The role of IRF8 in B cell related anomalies is not well studied. IRF8 is implicated as a tumor suppressor in myeloid lineage derived neoplasms while, in B cells it has been recently linked to the pathogenesis of Follicular lymphoma (FL) and CLL (Fig. 4) (Koniczna et al., 2008; Slager et al., 2011; Bouamar et al., 2013; Li et al., 2014).

B cell acute lymphoblastic leukemia (B-ALL)

B-ALL is a precursor B cell derived malignancy, predominantly affecting children and having an aggressive clinical course. Although, clinical studies have not demonstrated a clear correlation between IRF4 and B-ALL, the role of IRF4

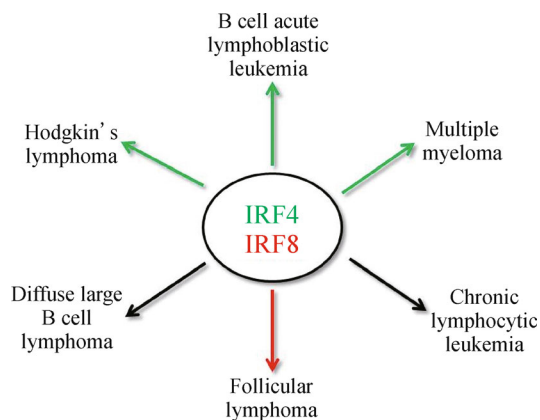


Figure 4 Role of IRF4 and IRF8 in B cell Malignancies. Green arrows indicate malignancies associated with deregulated IRF4 expression. Red arrows indicate malignancies associated with deregulated expression of IRF8. Black arrows indicate malignancies associated with deregulated expression of both IRF4 and IRF8, however; their roles in these malignancies are not essentially redundant.

has been well-studied in murine models of the disease. Consistent with its role in pre-B cell differentiation, IRF4 acts as tumor suppressor in murine models of B-ALL. Using an E μ -myc model of ALL, we have recently highlighted the importance of IRF4 in the development of ALL. IRF4^{+/-} E μ -myc mice develop ALL with an extremely short disease latency (~8 weeks) compared to the IRF4^{+/+} E μ -myc mice (~20 weeks) (Pathak et al., 2011). Similarly, IRF4 was shown to suppress the proliferation BCR-ABL1 derived mouse B-ALL clones (Acquaviva et al., 2008). Furthermore, IRF4 but not IRF8 was induced upon treatment of B-ALL cells with imatinib that inhibits the oncogenic fusion protein BCR-ABL1 (Acquaviva et al., 2008). Therefore, IRF4 functions in impeding the development and progression of B-ALL in mouse models of the disease. However, the exact molecular mechanism for tumor suppressive role of IRF4 in B-ALL needs further investigation.

Diffuse large B cell lymphoma (DLBCL)

DLBCL represents a diverse group of B cell malignancy that can be divided into several subgroups including germinal center B cell type DLBCL (GCB type) and activated B cell type DLBCL (ABC type) (Alizadeh et al., 2000). IRF4 expression is high in ABC type DLBCL, while GCB type DLBCL is negative for IRF4 expression (Cattoretti et al., 2006). However, the functional significance of IRF4 expression in ABC type DLBCL is ambiguous. Initial studies identified IRF4 as a suppressor of Bcl6 in GC B cells as well as DLBCL cell lines, where Bcl6 functions as an oncogene (Saito et al., 2007). Strikingly, DLBCL cell lines were shown to harbor mutations in the IRF4 binding sites, generating Bcl6 alleles that do not respond to IRF4 mediated suppression

(Saito et al., 2007). Consequently, these mutations allow high expression of Bcl6 in the presence of IRF4. Furthermore, the plasma cell differentiation regulator Blimp-1 is inactivated by multiple mechanisms in ABC type DLBCL (Mandelbaum et al., 2010). These findings indicate that the genetic alterations carried by ABC type DLBCL leads to an abortive plasma cell differentiation program.

IRF4 and Spi-B have been identified as survival factors for ABC type DLBCL. In these studies IRF4 functions with Ets family member Spi-B to repress type I interferon responses in DLBCL cell lines (Yang et al., 2012). IRF4 and Spi-B co-bind to EICE motifs to suppress IRF7 causing subsequent inhibition of interferon β production and interferon response mediated cell death (Yang et al., 2012). Furthermore, the drug lenalidomide was shown to inhibit the survival of ABC DLBCL cells by downregulating Spi-B and IRF4 levels. It is noteworthy that Spi-B is amplified and overexpressed in ~25% of DLBCL cases (primarily ABC type) (Lenz et al., 2007; Lenz et al., 2008). Moreover, the ABC DLBCL cell lines are shown to be sensitive to knock down of Spi-B (Lenz et al., 2008). Furthermore, IRF4 is shown to execute normal plasma cell differentiation without cooperative binding with Spi-B (Ochiai et al., 2013). Therefore, Spi-B overexpression due to genetic alterations may derail the transcriptional network critical for differentiation of activated B cells. Since IRF4 interacts with Spi-B, it is reasonable to speculate that Spi-B overexpression may alter the DNA binding landscape of IRF4 and obstruct its normal functions in plasma cell differentiation. Hence, the pro-survival effects of IRF4 observed in these studies may result from modulation of IRF4 DNA binding activity by Spi-B. IgH-IRF4 translocations are recently identified in ~4.5% of pediatric DLBCL cases further indicating an active role of IRF4 in pathogenesis of DLBCL (Salaverria et al., 2011). Surprisingly, the cases with IRF4 translocations have favorable outcomes. Nevertheless, further mechanistic insights are needed to fully delineate the role of IRF4 in the etiology of different subgroups of DLBCL.

Consistent with their GC B cell origin, IRF8 is expressed in GCB type DLBCL. A recent report identified IRF8 mutations in ~6% of follicular lymphoma cases. The mutations identified predominantly mapped to the c-terminus region of IRF8 with still unidentified functional consequences (Li et al., 2014). Similar to IRF4, IRF8 is also identified as a fusion partner with IgH locus in small subgroup of DLBCL patients (Bouamar et al., 2013). However, the precise role of IRF8 in these malignancies is still ambiguous. The identification of genetic alterations targeting IRF8 in DLBCL warrant further studies in order to elucidate its role in pathogenesis of DLBCL.

Chronic lymphocytic leukemia (CLL)

CLL is a mature B cell derived malignancy marked by

progressive accumulation of CD5⁺ CLL cells. An initial study identified higher IRF4 expression to correlate with better prognosis in CLL patients (Chang et al., 2002). More recently a genome wide association study (GWAS) identified single nucleotide polymorphisms (SNPs) in the 3' untranslated region (UTR) of IRF4 locus in sporadic and familial CLL cases (Di Bernardo et al., 2008). The SNPs in the IRF4 locus are associated with strongest susceptibility for developing CLL. The risk alleles harboring the SNPs are linked to downregulation of IRF4 and poor patient outcomes (Di Bernardo et al., 2008; Allan et al., 2010; Crowther-Swanepoel et al., 2010). Another recent study identified mutations in the DNA binding domain of IRF4 in a small subset (~1.5%) of CLL patients (Havelange et al., 2011). However, the functional consequences of these mutations in B cells remain undetermined. Recently, using two distinct genetic models we have shown that low levels of IRF4 promote CLL development. New Zealand Black (NZB) mice are natural occurring, late onset mouse model of CLL. Interestingly, low levels of IRF4 dramatically accelerated CLL development in the NZB IRF4^{+/-} mice (Ma et al., 2013). Vh11 knock-in (Vh11 KI) mice contains a pre-arranged Vh11 family Ig heavy chain inserted into the heavy chain locus and Vh11 KI mice have an expanded B1 cell population (Wen et al., 2005). Since CLL cells are derived from B1 cells in rodents; we also used Vh11 KI mice to study the effect of IRF4 on CLL development. Strikingly, IRF4 deficiency mice expressing Vh11 transgene (IRF4^{-/-} Vh11) developed spontaneous CLL with 100% penetrance (Shukla et al., 2013). Importantly, neither the IRF4 deficient nor the Vh11 KI mice develops CLL independently. These studies have established a causal relationship between low levels of IRF4 and CLL development. Further studies are needed to elucidate the molecular mechanism through which IRF4 controls CLL development.

A recent GWAS study identified SNPs in the IRF8 locus to be associated with the risk of developing CLL (Slager et al., 2011). However, unlike IRF4 the SNPs in the IRF8 locus are linked to an increased expression of IRF8 among CLL patients. Future studies are needed to fully decipher the functions of IRF8 in the etiology of CLL.

Hodgkin's lymphoma (HL)

HL is a B cell malignancy presumably derived from GC B cells. Classical HL patients accumulate Hodgkin Reed-Sternberg (HRS) tumor cells that have been shown to express IRF4, whereas the expression of IRF8 has not yet been reported (Tsuboi et al., 2000; Aldinucci et al., 2010). Contrary to the role of IRF4 in CLL, HL cells require IRF4 as a survival factor and HL cell lines are sensitive to IRF4 knockdown (Aldinucci et al., 2011). Furthermore, in HRS cells IRF4 is upregulated in response to survival signals while, IRF4 is repressed upon activation of apoptotic pathways (Aldinucci et al., 2010). A recent study categorized HL cases into two

molecular subgroups based on their strength of Myc, Notch1 and IRF4 activation (Tiacchi et al., 2012). Interestingly, the HL cases with myc activation signature were also enriched for upregulation of Notch1 and IRF4 target genes. These studies indicate a myc, Notch1 and IRF4 target genes to be active in a subgroup of HL cases potentially contributing to HL pathobiology (Tiacchi et al., 2012). Moreover, according to these studies IRF4 mediated survival of HRS cells may be important for at least a subset of HL cases. Surprisingly, a recent study identified SNPs associated with the risk for developing CLL to be also associated with HL susceptibility (Broderick et al., 2010). However, the effect of these SNPs on IRF4 expression in HL cells has not yet been evaluated. Nevertheless these studies collectively point to a pro-survival role for IRF4 in IRF4 HL however; further studies are required to elucidate the mechanistic details for the functions of IRF4 in the pathobiology of HL.

Multiple myeloma (MM)

MM is an aggressive malignancy derived from plasma cells. Consistent with their plasma cell origin, MM cells express high levels of IRF4 while their IRF8 expression is low. IRF4 was initially identified to be translocated to the IgH locus and overexpressed in MM for which it was named multiple myeloma oncogene 1 (Mum1) (Iida et al., 1997). More recently, an immunomodulatory drug lenalidomide known to target IRF4 is shown to inhibit the survival and proliferation of MM cells (Lopez-Girona et al., 2011). Given the essential role of IRF4 in plasma cell differentiation, it is rather paradoxical that IRF4 functions as a survival factor in MM. Shaffer et al. have put forward the concept of "non-oncogene addiction" to explain the role of IRF4 in MM (Shaffer et al., 2008; Shaffer et al., 2009). "Non-oncogene addiction" of IRF4 describes the unusual abilities of IRF4 to direct plasma cell differentiation on one end and to function as a survival factor for MM cells on the other end. Studies aiming to identify the molecular basis for IRF4 addiction in MM have identified myc as a direct target of IRF4 (Shaffer et al., 2008). Intriguingly, myc was also shown to positively regulate IRF4 expression in MM cells thereby establishing an autoregulatory loop between the two proteins (Shaffer et al., 2008). Other IRF4 target genes in MM cells include genes involved in glucose metabolism and ATP production that are also known to be regulated by myc (Shaffer et al., 2008). This suggests that IRF4 may also be involved in metabolic adaptations associated with myc induced transformation of MM cells. Myc was initially identified to be translocated and amplified in 16% of MM cases however, a recent report has identified ~50% of MM cases to harbor myc rearrangements (Affer et al., 2014; Shou et al., 2000). These studies indicate that myc deregulation is central to the pathogenesis of MM and IRF4 in part functions as a survival factor by directly regulating myc expression. It is possible that MM may

represent a condition in which normal plasma cell differentiation program is disrupted due to sustained myc expression. It is also worth noting that myc is overexpressed by multiple mechanisms in MM cells that do not involve IRF4 (Affer et al., 2014). This means that myc functions as an oncogene in MM cells by IRF4 independent mechanisms as well.

Besides regulating myc expression, IRF4 has been recently shown to tweak the autophagy pathways in MM cells (Lamy et al., 2013). Using a RNA interference screen, caspase-10 was found to be essential for viability of MM cells irrespective of their underlying genetic alteration landscapes. Caspase-10 along with caspase like protein (cFLIP) was demonstrated to partially cleave and inhibit the Bcl2 interacting protein (Bclaf1). Furthermore, Bclaf1 functions as an inducer of autophagy by directly binding and displacing Bcl2 from Beclin1. Interestingly, IRF4 was shown to induce the expression of caspase-10 and cFLIP in MM cells (Lamy et al., 2013). Therefore, IRF4 attenuates hyperactive autophagy induction which promotes apoptosis in MM cells. Intriguingly, the same study identified a dependence of MM cells on a basal level of autophagy for their survival. Inhibition of the basal level autophagy by targeting essential autophagic machinery in MM cells leads to spontaneous decrease in viability (Lamy et al., 2013). Therefore it appears that IRF4 only inhibits the hyperactivation of autophagy pathways in MM cells but not the basal autophagy activity.

Viral infections and associated malignancies

Kaposi Sarcoma associated Herpes Virus (KSHV) infection is associated with primary effusion lymphoma (PEL) and Epstein Barr Virus (EBV) infection is associated with several B cell malignancies including B lymphoblastoid lymphoma (LBL). Similar to the induction of IRF4 by NF- κ B signaling in normal B cells, viral proteins modulate NF- κ B signaling to regulate IRF4 expression (Forero et al., 2013). For example, the KSHV encoded viral FLICE inhibitory protein (vFLIP) induces IRF4 expression by activation of NF- κ B signaling (Forero et al., 2013). During KSHV replication, the cellular form of IRF4 (cIRF4) is inhibited by viral IRF4 (vIRF4) which subsequently suppresses c-myc expression to induce viral lytic cycle (Lee et al., 2014). Furthermore, IRF4 is expressed in PEL however; the functional significance of IRF4 expression in KSHV associated PEL is still ambiguous (Arguello et al., 2003).

A recent study has identified a role of EBV encoded EBV associated nuclear antigen 3C (EBNA3C) in counter-regulating IRF4 and IRF8 in EBV transformed cell lines. EBNA3C was found to stabilize IRF4 by direct interaction causing subsequent degradation of IRF8 in a proteasome dependent manner (Banerjee et al., 2013). Furthermore, EBV transformed lymphoblastoid cell lines showed reduced proliferation and enhanced apoptosis upon IRF4 knock-down

(Banerjee et al., 2013). IRF4 was further shown to be phosphorylated in a c-src dependent manner which interfered with the DNA binding ability of IRF4 in EBV transformed cells (Wang and Ning, 2013). Furthermore, EBNA3C was shown to bind AICE and EICE sites in p14 (ARF)/CDKN2A locus, to repress its expression (Portal et al., 2013; Jiang et al., 2014b). These studies collectively indicate that viral proteins mediated phosphorylation of IRF4 may lead to suppression of its normal cellular functions. Additionally, the EBV viral proteins may bind DNA at usual IRF4 and IRF8 binding sites to perform their oncogenic functions.

Concluding remarks

IRF4 and IRF8 act as quintessential regulators at several stages of B cell development. IRF4 and IRF8 are co-opted to execute specific transcriptional programs at certain B cell stages while, at others they independently and distinctively govern critical developmental decisions. Interactions of IRF4 and IRF8 with Ets family members, NFATs, E2A, and recently identified AP-1 family members have highlighted them as molecular rheostats integrating diverse signaling inputs (Rengarajan et al., 2002; Glasmacher et al., 2012; Hodawadekar et al., 2012; Li et al., 2012; Tussiwand et al., 2012). Moreover, the “kinetic control” model provides the molecular basis for understanding diverse effects of IRF4 on B cell development and function (Ochiai et al., 2013). Similar themes highlighting IRF4 as a determinant of T cell receptor (TCR) signaling strength involved in mediating effector T cell differentiation have also been described (Man et al., 2013; Yao et al., 2013). Recent studies have further identified genes involved in cellular metabolism and cell cycle as some of the direct targets of IRF4 in effector T cells (Shaffer et al., 2008; Man et al., 2013; Yao et al., 2013). Future studies aiming towards identifying key target genes regulated by IRF4 and IRF8 in diverse B cell malignancies will be useful to define disease pathogenesis and to design specific therapeutic interventions.

Acknowledgements

This work is supported by NIH Grant AI 107237.

Compliance with ethics guidelines

This manuscript is a review article and does not involve a research protocol requiring approval by the relevant institutional review board or ethics committee.

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