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A Signaling Pathway Mediating Downregulation of *BCL6* in Germinal Center B Cells Is Blocked by *BCL6* Gene Alterations in B Cell Lymphoma

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SUMMARY

The *BCL6* proto-oncogene encodes a transcriptional repressor necessary for the development of germinal centers (GCs) and directly implicated in lymphomagenesis. Post-GC development of B cells requires BCL6 downregulation, while its constitutive expression caused by chromosomal translocations leads to diffuse large B cell lymphoma (DLBCL). Herein we identify a signaling pathway that downregulates *BCL6* expression in normal GC B cells and is blocked in a subset of DLBCL due to alterations in the *BCL6* gene. Activation of the CD40 receptor leads to NF-κB-mediated induction of the IRF4 transcription factor, which, in turn, represses *BCL6* expression by binding to its promoter region. A subset of DLBCL displays chromosomal translocations or mutations that disrupt the IRF4-responsive region in the *BCL6* promoter and block its downregulation by CD40 signaling.

INTRODUCTION

The *BCL6* proto-oncogene encodes a transcriptional repressor of the POZ/BTB-zinc finger protein family, which binds to specific DNA sequences and, via recruitment of corepressor complexes, represses its target genes (Chang et al., 1996; Dhordain et al., 1997, 1998; Fujita et al., 2004; Parekh et al., 2007; Seyfert et al., 1996; Wong and Privalsky, 1998). Within the B cell lineage, BCL6 is expressed in the germinal centers (GCs) (Cattoretti et al., 1995), the structures in which B cells undergo somatic hypermutation (SHM) and class switch recombination (CSR) of immunoglobulin (Ig) genes and are then selected based on the production of antibodies with high affinity for the antigen (Rajewsky, 1996). BCL6 is a key regulator of GC development, as mice lacking BCL6 cannot form GCs and fail to mount secondary T cell-dependent responses due to the lack of antibody affinity maturation (Dent et al., 1997; Fukuda et al., 1997; Ye et al., 1997). Within the GC, BCL6 performs its biological function by modulating the transcription of genes controlling DNA damage responses, cell cycle arrest, apoptosis, cell activation, differentiation, and CSR (Baron et al., 1995; Harris et al., 1999; Niu et al., 2003; Parekh et al., 2007; Phan and Dalla-Favera, 2004; Phan et al., 2005; Ranuncolo et al., 2007; Shaffer et al., 2000).

In about 15%–40% of DLBCL cases and in 5%–10% of follicular lymphoma (FL) cases (Baron et al., 1993; Kerckaert et al., 1993; Ye et al., 1993, 1995), chromosomal translocations place the intact coding region of *BCL6* under the control of heterologous promoters derived from partner chromosomes, resulting in the deregulated expression of BCL6 by a mechanism called promoter substitution (Chen et al., 1998b; Ohno, 2006; Ye et al., 1995). In addition, the 5′ regulatory region of *BCL6* can be altered

SIGNIFICANCE

Chromosomal translocations and mutations of the *BCL6* promoter region are associated with ~40% of DLBCL and ~10% of follicular lymphoma, the two most frequent types of B cell lymphoma. These aberrations lead to BCL6 deregulation and cause DLBCL in transgenic mice, but the mechanism of deregulation remains obscure. The results herein identify a signaling pathway in which CD40 receptor signaling, normally induced by the CD40 ligand on T cells, leads to downregulation of *BCL6*. This pathway is blocked in a subset of DLBCL in which the *BCL6* promoter region is made unresponsive by structural alterations. Thus, these results identify a mechanism for BCL6 deregulation of direct relevance for the understanding of the pathogenesis of DLBCL.

by mutations introduced by the SHM mechanism in normal GC B cells (Pasqualucci et al., 1998; Shen et al., 1998); specific mutations found only in DLBCL (13% of cases) affect two BCL6-binding sites within the 5′ region of the *BCL6* gene and disrupt a negative autoregulatory circuit (Pasqualucci et al., 2003b; Wang et al., 2002). Recently, the role of BCL6 in the pathogenesis of DLBCL was confirmed by showing that mice engineered to express deregulated BCL6 develop lymphomas displaying features typical of human DLBCL (Cattoretti et al., 2005).

Consistent with its important role in the GC reaction and in lymphomagenesis, the expression and activity of BCL6 are tightly regulated. B cell receptor (BCR) engagement induces MAP-kinase-mediated phosphorylation of BCL6, leading to its degradation by the ubiquitin proteasome pathway (Niu et al., 1998). The activity of BCL6 is regulated through p300-mediated acetylation, which inhibits its transrepressive function (Bereshchenko et al., 2002). In addition, early observations suggested that engagement of the CD40 receptor leads to transcriptional downregulation of *BCL6* (Allman et al., 1996; Basso et al., 2004; Niu et al., 2003). This mechanism is potentially important since, in GC B cells, CD40 signaling occurs as the result of interaction with the CD40 ligand presented by T cells and is involved in multiple events in T cell-dependent antibody responses, including B cell survival and proliferation, GC and memory B cell formation, and CSR (van Kooten and Banchereau, 2000). CD40 signaling is not active in most normal GC B cells, while is detectable in a subset of centrocytes, suggesting that it may represent an important mechanism for the downregulation of *BCL6* and for post-GC differentiation of B cells (Basso et al., 2004). Furthermore, the fact that this mechanism acts on *BCL6* transcription suggests that it could be disrupted by chromosomal translocations and mutations affecting the *BCL6* promoter region, therefore representing an important pathogenetic mechanism in B cell lymphomagenesis.

To directly address these issues, this study has explored further the relationship between CD40 signaling and *BCL6* downregulation. The results show that CD40 signaling-induced downregulation of *BCL6* occurs through NF-κB activation and consequent activation of *IRF4*, a member of the interferon-regulatory factor family of transcription factors required for plasma cell differentiation and expressed in a subset of centrocytes and in ABC-type DLBCL (Alizadeh et al., 2000; Cattoretti et al., 2006; Falini et al., 2000; Iida et al., 1997; Klein et al., 2006; Matsuyama et al., 1995; Sciammas et al., 2006; Wright et al., 2003). IRF4, in turn, can repress *BCL6* transcription by binding to its promoter region. This pathway is disrupted by translocations and mutations in a subset of DLBCL and Burkitt's lymphoma (BL), thus preventing CD40-mediated downregulation of *BCL6*.

RESULTS

CD40-Induced *BCL6* Downregulation Is Mediated by NF-κB Activation

Since CD40 signaling is transmitted to the nucleus principally through the activation of NF-κB transcription factors (Berberich et al., 1994), we investigated whether NF-κB activation was involved in *BCL6* downregulation. In the Ramos (IκB-ER) cell line, which was engineered to express a mutant IκB-α protein fused to the estrogen receptor (ER) (Lee et al., 1999), NF-κB is inactivated via two mechanisms: (1) the mutant IκB-α cannot be phosphorylated and targeted for proteosomal degradation, thus preventing the nuclear translocation of NF-κB factors; (2) in the presence of estrogen, IκB-α-ER proteins can actively enter the nucleus, displace NF-κB from its binding sites, and transport it back to the cytoplasm, leading to an additional level of NF-κB inactivation. As expected, induction of CD40 signaling by coculture with fibroblasts expressing the murine CD40 ligand (Basso et al., 2004; Spriggs et al., 1992) (or with control fibroblasts) in the presence of estrogen leads to upregulation of *BFL-1* mRNA and protein, a known CD40 signaling target (Lee et al., 1999), and to *BCL6* downregulation in control Ramos cells (WT). However, this effect was lost in Ramos (IκB-ER) cells in which NF-κB is inactivated, indicating that NF-κB activation is required for *BCL6* downregulation by CD40 signaling (Figures 1A–1C). This result was confirmed in CD40-stimulated P3HRI cells in which NF-κB was pharmacologically inhibited by a specific NF-κB inhibitor (BAY11-7082) (Figure 1D). To explore the physiological significance of these findings, we examined whether the activation of NF-κB (identified by the nuclear translocation of its p50 and c-Rel subunits) was associated with downregulation of BCL6 in normal GC centrocytes by double immunofluorescence analysis of BCL6 expression and p50 or c-Rel subcellular localization. The results show that the small subset of centrocytes expressing nuclear p50 (Figure 1E) or c-Rel (Figure S1 in the Supplemental Data available with this article online) corresponds precisely to the few centrocytes lacking BCL6 expression, consistent with NF-κB activation causing *BCL6* downregulation. Together, these results indicate that CD40-mediated downregulation of *BCL6* requires NF-κB activation.

NF-κB Induces *IRF4* Transcription

Although required for *BCL6* downregulation, NF-κB activation appeared unlikely to act directly on *BCL6* transcription, since NF-κB complexes have been reported to act mainly as transcriptional activators (Pahl, 1999). Among possible intermediate molecules, IRF4 appeared as a strong candidate since: (1) its expression is mutually exclusive with BCL6 in GC B cells (Falini et al., 2000); (2) it can function as a transcriptional repressor on particular target genes (O'Reilly et al., 2003; Rosenbauer et al., 1999); and (3) it can be induced by NF-κB at least in human T cell leukemia virus-I (HTLV1)-transformed T cells (Sharma et al., 2002). Furthermore, CD40 stimulation induced upregulation of *IRF4* and downregulation of *BCL6* in transformed GC B cell lines, Ramos, P3HR1, Raji, Mutul, Ly1, Ly7, and SUDHL4 (Basso et al., 2004) (Figure S2A and data not shown). Finally, nuclear translocation of NF-κB was found associated with upregulation of IRF4 in normal GC B cells by immunofluorescence analysis (data not shown).

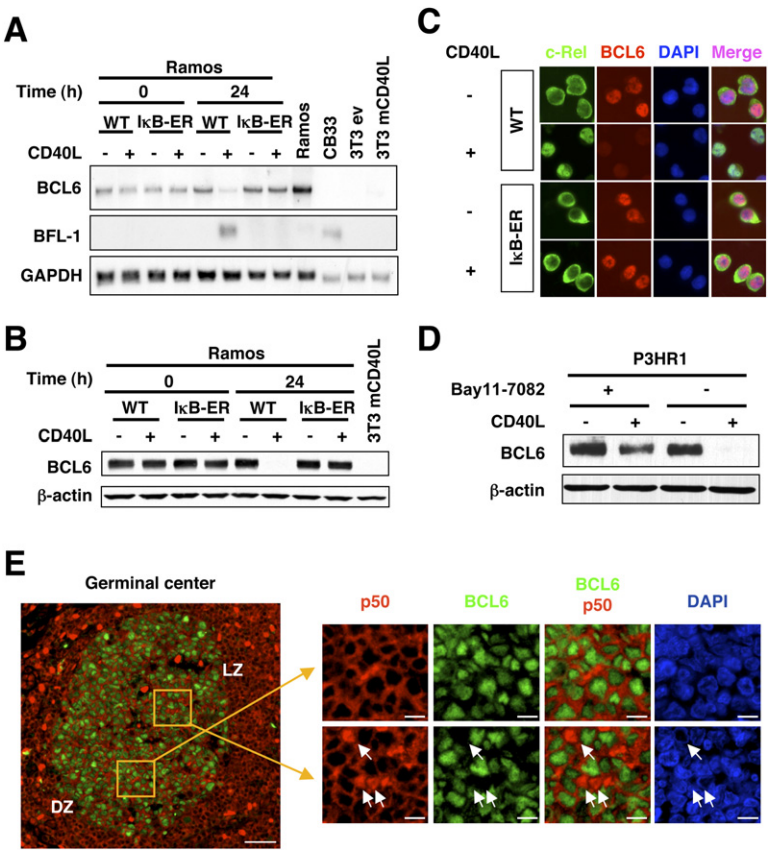

Figure 1. CD40-Induced *BCL6* Downregulation Is Mediated by NF-κB

(A) Northern blot analysis for *BCL6* expression in Ramos (WT) and Ramos (IκB-ER) cells upon CD40 stimulation by coculture with control fibroblasts (NIH 3T3 ev) or fibroblasts expressing CD40L (NIH 3T3 mCD40L) in the presence of estrogen for 24 hr. The expression of the NF-κB target *BFL-1* was used as positive control for NF-κB activation by CD40 signaling. The lymphoblastoid cell line CB33 was used as a negative and positive control for *BCL6* and *BFL-1* expression, respectively. *GAPDH* expression was used as loading control.

(B) Western blot analysis for BCL6 in Ramos (WT) and Ramos (IκB-ER) cells upon CD40 stimulation. Protein extracts were obtained from the same cells used for northern blot analysis (A). β-Actin was used as a loading control.

(C) Immunofluorescence staining of unstimulated and CD40-stimulated Ramos (WT) and Ramos (IκB-ER) cells with anti-c-Rel (green) and anti-BCL6 (red) antibodies. DAPI (blue) was used for the detection of nuclei.

(D) Western blot analysis for BCL6 in BAY11-7082-treated P3HR1 cells upon CD40 stimulation. P3HR1 cells were cocultured with NIH 3T3 ev or NIH 3T3 mCD40L cells for 12 hr with or without BAY11-7082. β-Actin was used as loading control.

(E) Immunofluorescence staining of GC B cells. A human tonsil section was stained with anti-p50 (red) and anti-BCL6 (green) antibodies. DAPI (blue) was used for the detection of nuclei. Enlargements of selected areas from the dark zone (DZ) and light zone (LZ) are displayed on the right (left panel scale bar, 100 μm; right panel scale bar, 30 μm). White arrows mark nuclear p50-positive and BCL6-negative cells.

Based on these observations, we examined whether the *IRF4* locus contained candidate NF-κB-binding sites and whether direct binding of NF-κB to these sites was detectable in vivo by chromatin immunoprecipitation (ChIP) analysis in unstimulated or CD40-stimulated P3HR1 cells. Of three potential NF-κB-binding sites on the *IRF4* promoter ("A," "B," and "C" in Figure 2A), regions A (−1232/−1032) and B (−605/−346) were immunoprecipitated in CD40-stimulated P3HR1 cells by NF-κB p50 and p65 antibodies, but not by c-Rel (see below), RelB, p52, and species- and isotype-matched (control) antibodies. Despite containing a potential NF-κB-binding site, region C could not be immunoprecipitated by any of the antibodies tested (Figure 2A). The binding of NF-κB was not detected in unstimulated P3HR1 cells or in the control region D (+6237/+6564), which does not contain NF-κB-binding sites. The lack of binding to the *IRF4* promoter for some NF-κB subunits was not due to inefficient ChIP analysis, since all five NF-κB subunits bound to the *IκB-α* promoter, a known CD40/NF-κB target (Basso et al., 2004; Ito et al., 1994; Verma et al., 1995) (Figure 2A, bottom panel). The binding of p50- and p65-containing complexes to the *IRF4* promoter was confirmed by electrophoresis mobility shift assays (Figure S2B). Together, these results indicate that NF-κB can bind to the *IRF4* promoter region in vivo. The selective binding of the p65 and p50 subunits suggests the involvement of the "canonical" NF-κB pathway (Xiao et al., 2006).

To confirm that NF-κB is involved in the activation of *IRF4* upon CD40 stimulation in B cells, we used small interference RNA to suppress the expression of p50, p65, and c-Rel. Western blot analysis showed that the protein levels of p50, p65, and c-Rel were significantly reduced in P3HR1 cells after transduction with lentiviruses expressing siRNAs specific for the three NF-κB subunits (Figure S2C). When p50 and p65 siRNA-expressing P3HR1 cells were stimulated with CD40L, IRF4 protein upregulation was significantly blocked as compared to P3HR1 cells expressing control siRNAs (Figure 2B). IRF4 upregulation was also blocked in the absence of c-Rel, despite the lack of direct binding in the ChIP assay, suggesting that c-Rel-containing NF-κB complexes act on *IRF4* transcription indirectly, most likely via induction of intermediate molecules or, alternatively, by direct binding to sequences not presently identified within the *IRF4* promoter region. Taken together, these data suggest that,

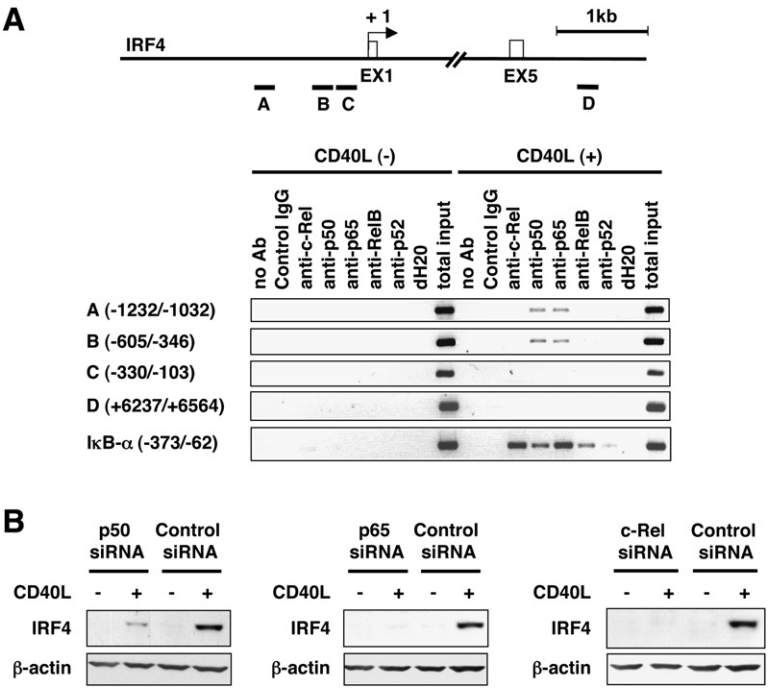

Figure 2. NF-κB Activates *IRF4* Expression via Binding to Its Promoter Region upon CD40 Stimulation

(A) Schematic representation of the *IRF4* locus. The DNA fragments (A, B, C, and D) amplified by PCR following ChIP assays are approximately positioned below the map. P3HR1 cells were cocultured with NIH 3T3 ev or NIH 3T3 mCD40L cells for 24 hr. Chromatin was immunoprecipitated with antibodies recognizing NF-κB subunits or an irrelevant antibody (rabbit IgG) as control. Test regions (A, B, and C) and negative control region (D) were amplified by PCR. Total chromatin before immunoprecipitation (total input) was used as positive control for PCR. Samples processed with no antibody (no Ab) and no DNA (dH_2O) were used as negative controls for ChIP assay and for PCR, respectively. The PCR amplification of the *IκB-α* promoter region (−373/−62) was used as positive control for the ChIP assay (bottom panel). Representative results from one of three independent experiments are shown.

(B) P3HR1 cells were infected with lentivirus expressing siRNA specific to p50, p65, or c-Rel and subjected to puromycine selection. After coculture with NIH 3T3 ev or NIH 3T3 mCD40L cells for 24 hr, P3HR1 p50 siRNA, p65 siRNA, c-Rel siRNA, and control siRNA clones were collected and analyzed by western blotting using anti-IRF4 and anti-β-Actin antibodies. Representative results from one of three independent experiments are shown.

upon CD40 stimulation, NF-κB complexes (p50/p65) activate the expression of *IRF4* by direct binding to its promoter.

IRF4 Suppresses *BCL6* Transcription

To investigate whether IRF4 can repress *BCL6* transcription, we first examined if the *BCL6* gene contains potential IRF4-binding sites. In fact, a total of 25 potential core IRF4-binding sites (Brass et al., 1999; Escalante et al., 2002a, 2002b; Furuita et al., 2006; Yoshida et al., 2005) are detectable within the highly conserved (mouse versus human) *BCL6* 5′ promoter region (−1 kb to +3 kb from transcription initiation site). Based on this observation, we examined whether IRF4 binds any of these sites in vivo using U266 multiple myeloma cells, which express IRF4 constitutively. ChIP assays for six regions ("A"–"F" in Figure 3) containing potential IRF4-binding sites within the 5′ flanking sequences and the first intron of the *BCL6* gene showed that IRF4 binds specifically to the B (−375/−80), C (+400/+696), E (+1452/+1718), and F (+2026/+2268) regions, but not to the A (−1562/−1244), D (+772/+1031), and G (+16039/+16301) regions (Figure 3). Identical results were obtained using three additional DLBCL cell lines expressing moderate levels of IRF4 upon CD40 stimulation (data not shown; Ly8, RCK8, and VAL; note that ChIP analysis cannot distinguish between normal and translocated *BCL6* alleles in lines that carry a translocated *BCL6* allele; see below). No IRF4 binding was observed in P3HR1 cells, which do not express IRF4 under basal conditions. Thus, regions B, C, E, and F of the *BCL6* promoter represent physiologic targets for IRF4 binding in vivo.

To examine the effect of IRF4 binding on *BCL6* gene transcription, a reporter gene driven by the native *BCL6* promoter region, pLA/B9 WT (−2913/+4211), was transiently cotransfected into 293T cells with vectors encoding a wild-type IRF4 molecule fused to a myc epitope tag (myc-IRF4) or a myc-IRF4 mutant (myc-IRF4 ΔDBD) lacking the IRF4 DNA-binding domain (Figure 4A and Figure 7C). The IRF4 expression vector, but not IRF4 ΔDBD, suppressed the expression of the *BCL6* reporter gene in a dose-dependent manner, indicating that IRF4 represses *BCL6* transcription and that DNA binding is required for this function (Figure 4B).

To determine whether IRF4 can influence the expression of endogenous BCL6 in native B cells, MutuI cells, which express BCL6 but not IRF4, were stably transfected with vectors expressing IRF4-HA or a control vector (MutuI IRF4-HA and MutuI control; P3HR1 cells could not be used for this experiment since we could not obtain stable expression of exogenous IRF4 in these cells). Western blot analysis of clones expressing IRF4 at levels comparable to those found in most post-GC B cell lines revealed that the expression of IRF4 leads to a significant decrease in BCL6 expression (>50%) in MutuI cells compared to control-transfected cells (Figure 5A). This result indicates that IRF4 can repress endogenous BCL6 expression in B cells.

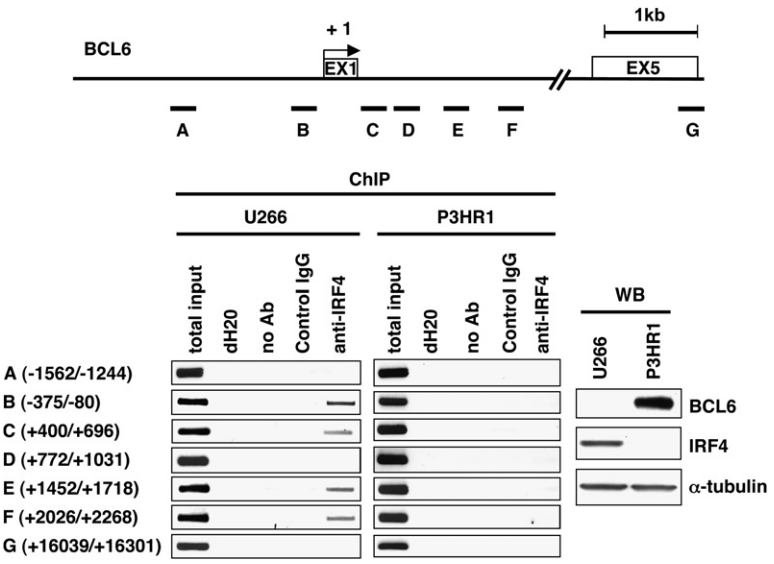

Figure 3. IRF4 Binds to the *BCL6* Promoter Region In Vivo

Schematic representation of the *BCL6* locus. The DNA fragments (A, B, C, D, E, F, and G) amplified by PCR following ChIP assays are approximately positioned below the map. The ChIP assays on U266 and P3HR1 cells were performed in parallel using equivalent number of cells. P3HR1 cells that do not express IRF4 were used as negative control for the ChIP assay. Chromatin was immunoprecipitated with anti-IRF4 antibody or an irrelevant antibody (control IgG) as control. Test regions (A–F) and negative control region (G) were amplified by PCR. Samples were processed as described in the Figure 2 legend. Representative results from one of three independent experiments are shown. The right panel (WB) shows IRF4 and BCL6 protein expression levels in U266 and P3HR1 cells. α-Tubulin was used as loading control.

To further confirm that *BCL6* is a target of IRF4 repression, we examined the effect of removing IRF4 on *BCL6* expression in P3HR1 clones stably transduced with either IRF4 siRNA or control siRNA. Western and northern blot analyses showed that, as expected, CD40-mediated upregulation of IRF4 is associated with downregulation of *BCL6* in P3HR1 control siRNA clones, whereas no induction of IRF4 and only a modest downregulation of *BCL6* (<20%) were detected in CD40-stimulated P3HR1 IRF4 siRNA clones (Figures 5B and 5C). These results indicate that IRF4 is required for the transcriptional suppression of *BCL6* in CD40-stimulated B cells.

Validation in Normal GC B Cells

The above-described experiments were performed in transformed GC B cells (Ramos, P3HR1, and Mutul) because normal GC B cells cannot be cultured and manipulated in vitro due to rapid mortality (Feuillard et al., 1995; Liu et al., 1989, 1991). However, the fact that the survival of these cells can be prolonged by CD40 stimulation allowed us to examine whether the CD40 signaling pathway, which induces *IRF4* and downregulates *BCL6*, was operative in normal cells. As shown in Figure 6A, GC centroblasts purified from human tonsils (~95% purity) express BCL6, but not IRF4. CD40 stimulation induces IRF4 and downregulates BCL6 with a rapid kinetic comparable to that observed in CD40-stimulated BL lines (Figure S2A; note that the more rapid downregulation of BCL6 protein compared to that of IRF4 induction is due to the fact that a fraction of GC B cells is already undergoing apoptosis prior to CD40-induced rescue). To confirm that CD40 signaling acts on *BCL6* and *IRF4* expression at the transcriptional level, the transcripts of these genes were examined in unstimulated and CD40-stimulated normal GC B cells by qRT-PCR. As shown in Figure 6B, substantial downregulation of *BCL6* and upregulation of *IRF4* were detectable in CD40-stimulated cells. To further confirm that CD40 signaling downregulates *BCL6* and upregulates IRF4 through activation of NF-κB in normal GC B cells, we examined the subcellular localization of p50 in CD40-stimulated normal GC B cells by immunofluorescence analysis. The results show nuclear localization of p50 and IRF4 expression in CD40-stimulated, but not in unstimulated normal GC B cells (Figure 6C). These results, together with the mutually exclusive expression of IRF4 or nuclear NF-κB and BCL6 in normal GCs (Figure 1E) (Falini et al., 2000), support the existence of the CD40-induced, NF-κB-IRF4-mediated pathway of *BCL6* downregulation in normal GC B cells.

Suppression of *BCL6* Is Blocked in Lymphoma Cells Carrying Chromosomal Translocations Involving *BCL6*

Analysis of *BCL6* chromosomal breakpoints indicates that the majority of them cluster in a region within the *BCL6* intron 1 overlapping with the identified major IRF4-binding domain (Figure 7C), suggesting that these breakpoints disrupt IRF4-mediated downregulation of *BCL6* by CD40 signaling. Thus, we first analyzed the expression of *BCL6* upon CD40 stimulation in DLBCL and BL cell lines carrying either *BCL6* alleles disrupted by translocations (Ly8, VAL, and RC-K8) or similar ones with nonrearranged *BCL6* loci (Ly1, Ly7, Ramos, P3HR1, Mutul, SUDHL4, and Raji). With the exception of RC-K8, which constitutively expresses *IRF4* due to a mutation in *IκB-α* causing constitutive activation of NF-κB (Kalaitzidis et al., 2002), the CD40-NF-κB-IRF4 pathway appeared to be functional in all cell lines, as shown by the nuclear translocation of NF-κB (data not shown) and upregulation of *IRF4* (see Figure 7B for representative data). However, *BCL6* downregulation was detectable in all seven cell lines carrying nondisrupted *BCL6* alleles (partial downregulation in Ly1; see below), while all three carrying chromosomal translocations at the *BCL6* locus continued to express both *BCL6* mRNA and protein, as shown by northern blot (Figure 7A) and western blot analysis (Figure 7B),

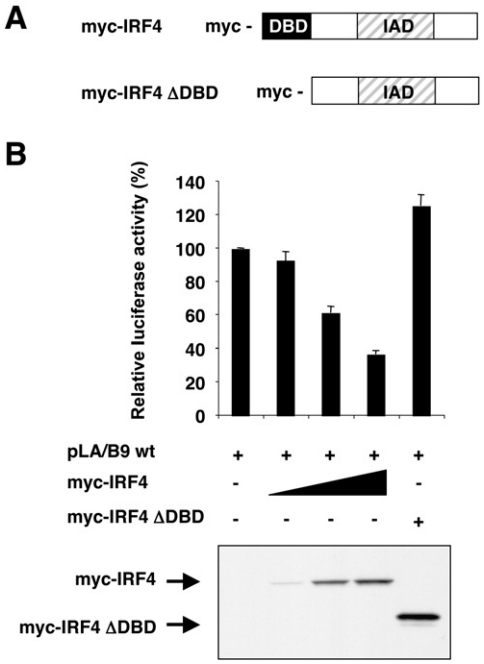

Figure 4. IRF4 Represses *BCL6* Promoter Activity

(A) Schematic representation of wild-type IRF4 (myc-IRF4) and mutant IRF4 (myc-IRF4 ΔDBD) constructs. IRF4 and IRF4 ΔDBD were fused to myc-tag at the N-terminus. The interaction activation domain (IAD) and DNA-binding domain (DBD) of IRF4 are represented in shaded and black rectangles, respectively.

(B) The *BCL6* promoter-driven luciferase reporter construct, pLA/B9 WT (−2913/+4211), was transiently cotransfected into 293T cells with increasing amounts of myc-IRF4 and myc-IRF4ΔDBD plasmids. Data are shown as mean ± SD of three independent experiments. The lower panel shows the protein expression levels of myc-IRF4 and myc-IRF4 ΔDBD.

respectively. Note that the lack of *BCL6* downregulation directly reflects the activity of the translocated *BCL6* allele, since the normal allele is silent in all lymphoma cases with *BCL6* translocations (Lossos et al., 2003; Ye et al., 1995).

The chromosomal breakpoints affect the *BCL6* intron 1 region in both the "CD40-resistant" Ly8 and VAL cell lines (Figure 7C). In VAL cells, the breakpoint separates most of the IRF4-binding sequences from the coding sequences of the *BCL6* gene and juxtaposes a heterologous *TTF* promoter region from chromosome 4 (Dallery et al., 1995; Kerckaert et al., 1993). In Ly8 cells, a similar alteration disrupts region C and eliminates region B, while juxtaposing the Iγ Ig promoter from chromosome 14 (Ye et al., 1995). In RC-K8 cells, the translocation juxtaposes an unidentified promoter region from chromosome 14, while leaving the IRF4-binding regions apparently intact. Notably, most breakpoints within the *BCL6* "major breakpoint region" (MBR) (Chen et al., 1998a; Pasqualucci et al., 2003a; Ye et al., 1993) disrupt the *BCL6* intron 1 region in large series of primary DLBCL cases (Akasaka et al., 2000; Yoshida et al., 1999) (Figure 7C). These observations indicate that chromosomal translocations can variably reduce the number of IRF4-binding regions in the *BCL6* promoter in the majority of DLBCL cases, thus impairing IRF4-mediated *BCL6* downregulation.

To examine the contribution of individual portions of the IRF4-binding domain to IRF4-mediated *BCL6* downregulation, we constructed luciferase-reporter genes driven by deletion mutants of the *BCL6* promoter region mimicking the loss of IRF4-binding domains observed in DLBCL (Figure 7C). We then tested the responsiveness of these reporters to IRF4-induced suppression in transient cotransfection assays in 293T cells. The pLA/B9 WT (−2913/+4211) reporter, as well as the 3′-truncated-pLA/S5 (−2913/+2082) and 5′-truncated-pLA/S5 (−121/+2082) reporter constructs displayed comparable repression (60%–65%) by IRF4, confirming that the IRF4-responsive domain is largely restricted to the intronic sequences identified by regions C–F, with a minimal contribution from region B (see slightly reduced suppression of pLA/S5 [−121/+2082]). On the other hand, a significant reduction in *BCL6* reporter downregulation was detectable in all constructs containing deletions that variably affect the C–F domains. In particular, the reporter genes pLA/S5 (−2913/+1777), pLA/S5 (−2913/+749), and pLA/S5 (−2913/+203), which lack one (F), two (E and F), or three (C, E, and F) IRF4-binding regions, were proportionately less suppressible (37%, 7%, and 0%, respectively). Notably, the last two plasmids, which more closely mimic the Ly8- and VAL-associated deletions, were virtually unresponsive to IRF4-induced suppression. These results indicate that chromosomal translocations frequently disrupt the IRF4-binding region in the *BCL6* locus, leading to the inability of IRF4 to downregulate *BCL6* transcription.

Suppression of *BCL6* Is Blocked in B Cell Lymphoma Cells Carrying *BCL6* Mutations

The region spanning ~2 Kb downstream of the *BCL6* transcription initiation site and including the 5′ portion of intron 1 is targeted by SHM in normal GC B cells and in GC-derived B cell lymphoma, including DLBCL (Migliazza et al., 1995; Pasqualucci et al., 1998; Shen et al., 1998). Notably, a subset of DLBCL-associated mutations (i.e., not found in normal cells) within the *BCL6* exon 1 was shown to disrupt two BCL6-binding sites and block an autoregulatory circuit (Pasqualucci et al., 2003b; Wang et al., 2002). Since numerous mutations can be found also in the IRF4-responsive domain, we examined whether some of these mutations could affect *BCL6* expression by an alternative mechanism, i.e., by preventing IRF4-mediated repression. To this end, we performed transient transfection/reporter assays using IRF4 expression vectors and a series of reporter constructs driven by mutated *BCL6* alleles deriving either from normal GC B cells (n = 7) or B cell lymphoma cases (five DLBCL and two BL). These alleles were previously characterized for their mutational status (number of mutations ranges from 2 to 4 in GC B cells, and 4 to 60 in B cell lymphoma cases) (Pasqualucci et al., 2003b). Partial but significant resistance to repression by IRF4 was observed in two of seven constructs derived from B cell lymphomas, but in none of seven alleles from normal GC

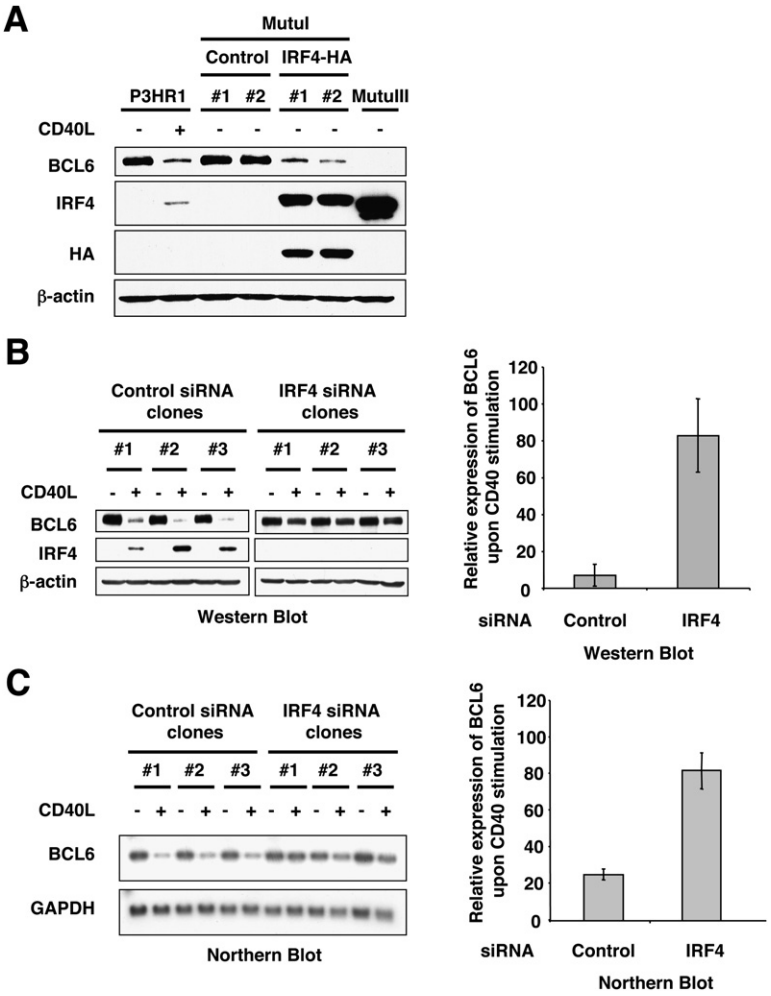

Figure 5. IRF4 Represses *BCL6* Expression in B Cells

(A) Expression of BCL6, IRF4, HA, and β-Actin proteins was analyzed by western blot in MutuI control and MutuI IRF4-HA cells. Unstimulated and CD40-stimulated P3HR1 and MutuIII cells were used as positive/negative controls for expression of IRF4 and BCL6, respectively. β-Actin was used as loading control.

(B) P3HR1 cells were transduced with lentivirus expressing luciferase siRNA (control) or IRF4 siRNA, and single-cell clones were selected. Western blot analysis for BCL6, IRF4, and β-Actin was performed on protein extracts of nine each P3HRI control siRNA and IRF4 siRNA clones after coculture with NIH 3T3 ev or NIH 3T3 mCD40L cells for 24 hr. The results obtained from three representative clones (out of nine tested) are shown. Quantification of BCL6 protein expression upon CD40 stimulation in P3HR1 control siRNA and IRF4 siRNA clones is shown on the right. The signal intensities of BCL6 and β-Actin proteins were measured by the ImageQuant 5.2 software. β-Actin-normalized BCL6 expression values are represented as mean ± SD of nine analyzed clones.

(C) Northern blot analysis for *BCL6* expression was performed on the same clones shown in Figure 5B. The signal intensities of *BCL6* and *GAPDH* mRNA were quantified by the ImageQuant 5.2 software. *BCL6* expression was normalized using *GAPDH*, and data are shown as mean ± SD of three clones (right panel).

B cells (Figure 8A). Both the partially resistant Ly1A and 1705B alleles displayed significant dose-dependent resistance when challenged with increasing amounts of IRF4 (Figure 8B). The Ly1A and 1705B alleles carry 60 and 16 mutations, of which 4 and 1 involve core IRF4-binding sites, respectively. In order to better define the region that causes the resistance to IRF4-mediated repression of *BCL6* in Ly1A allele, we constructed reporter genes in which Ly1A and wild-type regions were variably "swapped" (Figure 8C) and tested them for their response to IRF4 in transient transfection assays. The results show that the mutations in regions D, E, and F (reporters h, i, and j in Figure 8C) do not cause resistance to IRF4, whereas mutations in region C are critical for resistance (reporters a, e, and g). A minor effect of the *BCL6* exon 1 region (compare reporters e versus g) is compatible with the presence of a mutation in the BCL6 autoregulatory site (Pasqualucci et al., 2003b; Wang et al., 2002), which may also be recognized by IRF4 since it contains a core IRF4-binding motif. Ly1A region C contains 16 mutations, including two located, respectively, within and in proximity of a core IRF4-binding site (Figure S3). These mutations may affect responsiveness to IRF4 individually or in combination (see Discussion). These results indicate that a subset of *BCL6* alleles found in B cell lymphoma carry mutations that affect the IRF4-binding region and alter responsiveness to IRF4.

DISCUSSION

CD40-Induced, NF-κB-Mediated Expression of *IRF4* in GC B Cells

Our results identify a pathway in which CD40 receptor engagement leads to transcriptional activation of the IRF4 transcription factor. *IRF4* induction requires activation of the NF-κB transcription complex, a known mediator of CD40 signaling (Berberich et al., 1994; Lalmanach-Girard et al., 1993), since it can be blocked by NF-κB inactivation. The involvement of NF-κB in *IRF4* activation is consistent with previous observations showing that *IRF4* expression is induced by NF-κB in mitogen-stimulated or virus-infected T cells (Sharma et al., 2002), and that c-Rel is involved in the activation of *IRF4* by different stimuli in splenic naive B cells (Grumont and Gerondakis, 2000; Sharma et al., 2002). Our results demonstrate a direct role for the p50 and p65 subunits, which bind the IRF4

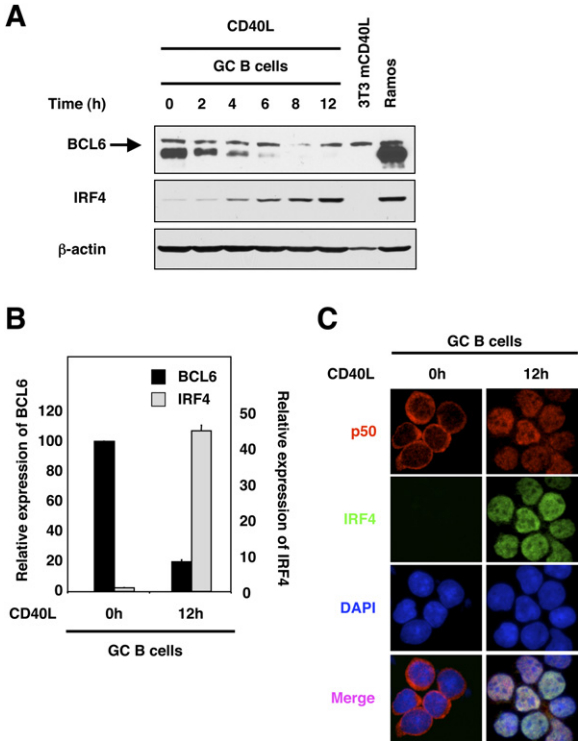

Figure 6. CD40 Stimulation Induces NF-κB Activation, *IRF4* Upregulation, and *BCL6* Downregulation in GC B Cells

(A) Centroblasts purified from human tonsils were cocultured with NIH 3T3 mCD40L cells and collected at different time points from 0 to 12 hr. Western blot analysis was performed using anti-BCL6, anti-IRF4, and anti-β-Actin antibodies. The Ramos cell line was used as positive control for BCL6 and IRF4 expression.

(B) qRT-PCR analysis for *BCL6* and *IRF4* expressions in unstimulated and CD40-stimulated GC B cells. *BCL6* and *IRF4* expressions were normalized using *HPRT1*, and data are shown as mean ± SD of three PCR reactions.

(C) Immunofluorescence staining of unstimulated and CD40-stimulated GC B cells with anti-p50 (red) and anti-IRF4 (green) antibodies. DAPI (blue) was used for the detection of nuclei.

promoter region and activate its transcription. In addition, our results suggest an indirect role for c-Rel in *IRF4* induction in GC B cells, although we cannot exclude direct binding by c-Rel to sequences of the *IRF4* locus more distant than the ones examined. The involvement of these NF-κB subunits is consistent with the activity of p50/p65 and p50/c-Rel NF-κB complexes, and thus indicate that the "canonical," as opposed to the "alternative" (mediated by p52/Rel-B), NF-κB pathway (Xiao et al., 2006) is involved in the CD40-mediated activation of *IRF4* in GC B cells. In addition, the observation that IRF4 is located in the cytoplasm in the Ly7 cell line and can translocate to the nucleus upon CD40 stimulation (data not shown) suggests the existence of additional mechanisms controlling IRF4 expression (Mamane et al., 2000).

IRF4-Mediated Downregulation of *BCL6*

The results herein indicate that the IRF4 transcription factor binds to the promoter region of the *BCL6* gene and directly represses its transcription. IRF4 is a member of the interferon regulatory factor (IRF) family of transcription factors, whose activity as transactivation or transrepression factor is in part determined by cofactors with which it interacts, as well as by DNA-binding motifs to which it binds (Marecki and Fenton, 2002). In particular, IRF4 has been shown to form a heterodimer with IRF8 and synergistically repress the transcription of target genes by binding to the IFN-stimulated response elements (ISRE) (Rosenbauer et al., 1999). Although the latter finding supports the notion that IRF4 can act as a transrepressor, IRF8 is unlikely to act as the IRF4 heterodimeric partner in suppressing *BCL6*, since IRF8 has been shown to be coexpressed with and to transcriptionally activate *BCL6* (Lee et al., 2006), and its distribution within the GC is mutually exclusive with IRF4 (Cattoretti et al., 2006). Thus, the mechanism by which IRF4 represses *BCL6* transcription remains to be investigated. The dense distribution of IRF4-binding sites in the *BCL6* promoter region and the requirement for multiple binding sites for full *BCL6* repression, demonstrated by the analysis of both DLBCL-associated and experimental mutations (Figures 7 and 8), suggest that oligomerization of IRF4 and possibly cooperative binding may be required for its function as a transrepressor (Figure S4).

Previous studies have suggested that CD40 signaling, as documented by NF-κB activation, occurs only in a subset of centrocytes that are located in the light zone of the GC (Basso et al., 2004) and may therefore represent B cells on their way to post-GC differentiation. The results herein show that CD40 signaling leads to *BCL6* downregulation in these centrocytes, as demonstrated by experiments in transformed GC B cells as well as by the validation in normal lymphoid tissues. Thus, the present results suggest that T cells/B cells interactions, known to occur within the light zone of the GC, may be responsible for CD40 ligand-receptor interactions leading to *BCL6* suppression.

Other signals functioning through NF-κB may also induce the *IRF4-BCL6* pathway. Indeed, transcriptional downregulation of *BCL6*, upregulation of *IRF4*, and nuclear translocation of p50 were also detected in B cell lines upon pharmacological induction of DNA damage (data not shown), which has also been shown to activate the "canonical" NF-κB pathway (Huang et al., 2003; Wu et al., 2006). In addition, other members of the TNF receptor family that, like CD40, function through NF-κB (Xiao et al., 2006), may induce the same pathway if their ligand is present in the GC environment. However, BAFF, a cytokine necessary for B cell survival (Schneider et al., 1999), did not induce downregulation of *BCL6* in B cell lines, perhaps consistent with the fact that its cognate TNF-type receptor, APRIL (Bossen and Schneider, 2006), functions mainly via the "alternative" NF-κB pathway (Xiao et al., 2006), while we show that *BCL6* responds to the "canonical" NF-κB signal. Furthermore, BCR signaling, which is physiologically induced by the antigen in GC B cells and which has been shown to downregulate BCL6 post-transcriptionally, can also activate NF-κB, implying its additional contribution to *BCL6* downregulation (Niu

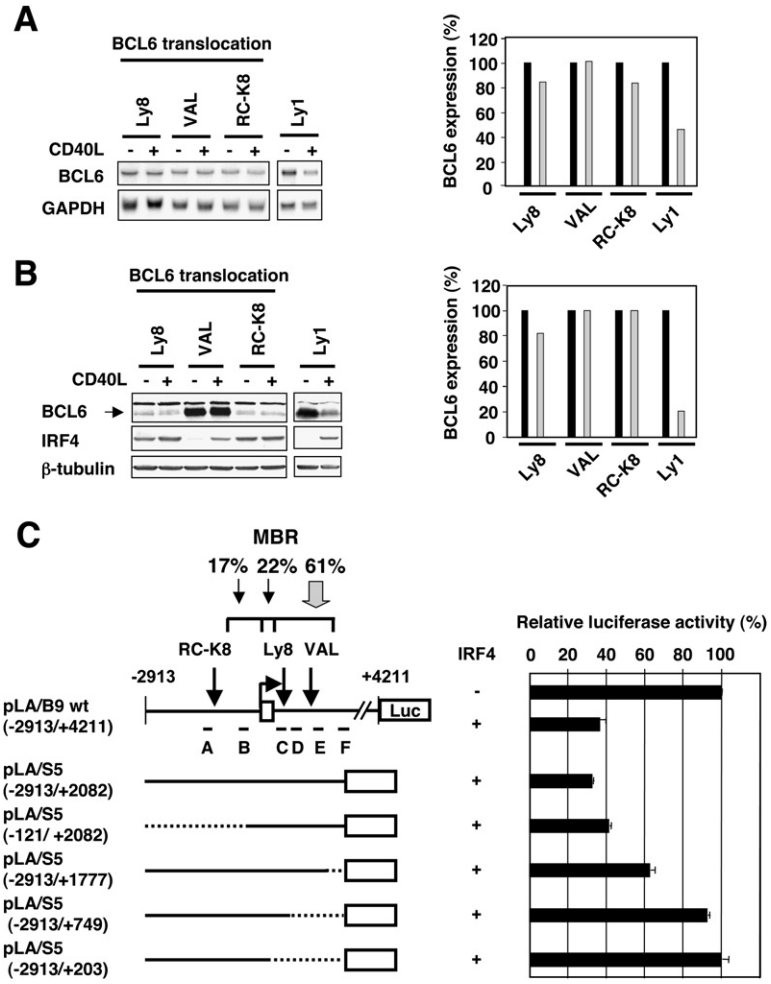

Figure 7. CD40-Induced *BCL6* Downregulation Is Abrogated in Cell Lines Harboring *BCL6* Translocation

(A) Northern blot analysis for *BCL6* was performed on DLBCL cell lines carrying a translocated (Ly8; VAL; RC-K8) or normal (Ly1) *BCL6* locus after coculture with NIH 3T3 ev or NIH 3T3 mCD40L cells for 24 hr. The signal intensities of *BCL6* and *GAPDH* mRNA were quantified by the ImageQuant 5.2 software. *BCL6* expression was normalized using *GAPDH* and compared between CD40-unstimulated cells (set as 100%; black bars) and CD40-stimulated cells (gray bars).

(B) Western blot analysis for BCL6 and IRF4 was performed on the same cells used for the analysis displayed in (A). The signal intensities of BCL6 and α-Tubulin were measured by the ImageQuant 5.2 software. BCL6 expression was normalized using α-Tubulin and compared between CD40-unstimulated cells (set as 100%; black bars) and CD40-stimulated cells (gray bars).

(C) Schematic representation of the *BCL6* major breakpoint region (MBR), the *BCL6* breakpoints in RC-K8, VAL, and Ly8 cell lines (arrows), and the *BCL6* reporter constructs. The DNA fragments analyzed by ChIP assays (see Figure 3) were positioned below the pLA/B9 WT map. Dashed lines indicate deletions. The luciferase activities of *BCL6* reporter constructs were compared between the control (set as 100%) and myc-IRF4-expressing samples. All experiments were performed in duplicate, and data are shown as mean ± SD of two independent experiments.

et al., 1998). Overall, these observations suggest that activation of NF-κB may represent a common pathway by which multiple stimuli can induce *IRF4* and suppress *BCL6* during the late stages of the GC reaction.

By simultaneously leading to *IRF4* induction and *BCL6* downregulation, CD40 or other NF-κB-mediated signaling may contribute to: (1) termination of the GC reaction via suppression of *BCL6*, which is required to maintain the GC phenotype (Dent et al., 1997; Fujita et al., 2004; Ye et al., 1997); (2) licensing CSR, which requires IRF4 (Klein et al., 2006; Sciammas et al., 2006); and (3) induction of plasma cell differentiation, both via upregulation of IRF4, a required inducer of this process (Klein et al., 2006; Sciammas et al., 2006), and via suppression of BCL6, which leads, in turn, to the release of the transcription of *Blimp1*, a BCL6 target gene (Tunyaplin et al., 2004; Vasanwala et al., 2002) and also a required factor for plasma cell differentiation (Angelin-Duclos et al., 2000; Shaffer et al., 2002; Shapiro-Shelef et al., 2003).

Implications for the Pathogenesis of DLBCL

The resistance to IRF4-mediated downregulation may represent one critical mechanism of BCL6 deregulation in tumors carrying *BCL6* alleles mutated within the IRF4-responsive domain. In fact, in these cases, the *BCL6* gene is in its normal chromosomal location, and its transcription is driven by its physiologic promoter (Pasqualucci et al., 2003b). Vice versa, most chromosomal translocations juxtapose heterologous promoter regions to the *BCL6* coding domain and, thus, *BCL6* expression is regulated by the specific pattern of expression of the various juxtaposed promoters. Thus, it is conceivable that translocations may affect *BCL6* expression by preventing IRF4-mediated downregulation as well as by juxtaposing heterologous promoter regions with regulatory properties different from those of *BCL6*.

Based on the position of the chromosomal breakpoints (Figure 7C), complete or partial lack of responsiveness to CD40-IRF4 may be present in approximately 60% of the DLBCL carrying chromosomal translocations affecting *BCL6* (~30% of all DLBCL), while chromosomal breakpoints located outside of the IRF4-responsive region, such as is the case in the RC-K8 cell line, may affect *BCL6* expression by different mechanisms. Among the large fraction of DLBCL cases carrying hypermutated *BCL6* alleles (>70%), it is presently unclear which

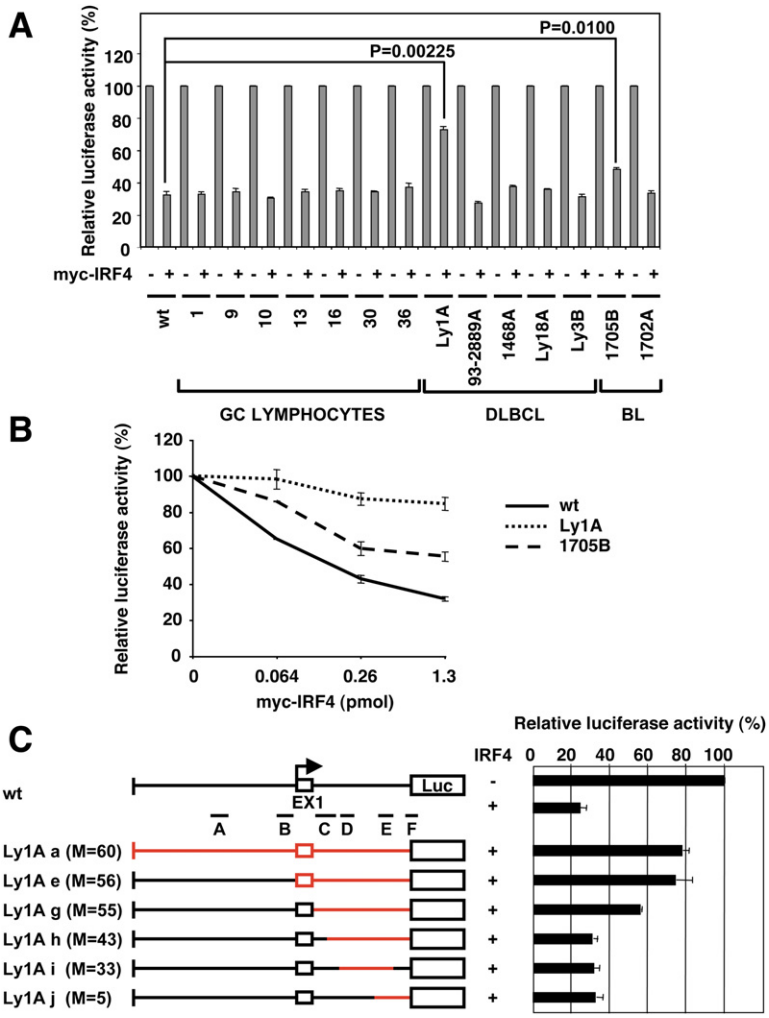

Figure 8. Somatic Mutations Inhibit IRF4-Mediated *BCL6* Downregulation in B Cell Lymphomas

(A) Seven *BCL6* promoter-driven luciferase reporter constructs derived from normal GC lymphocytes and seven reporter constructs derived from lymphomas (five DLBCL and two BL) were transiently cotransfected with control or myc-IRF4 plasmids. Data are shown as mean ± SD of three independent experiments. p values were calculated using the Student's t test.

(B) The luciferase activities of *BCL6* reporter constructs pLA/S5 WT, pLA/S5 Ly1A, and pLA/S5 1705B were analyzed upon cotransfection with increasing amounts of myc-IRF4. Data are shown as mean ± SD of three independent experiments.

(C) The luciferase activities of pLA/S5 WT, pLA/S5 Ly1A a, and pLA/S5 Ly1A mutants (e, g, h, i, and j) were analyzed upon coexpression with myc-IRF4. Data are shown as mean ± SD of two independent experiments.

percentage would display CD40-IRF4 unresponsiveness, due to the fact that the single allele functional analysis (Figure 8) is not suitable for the screening of large panels of cases. Nonetheless, the fact that two of seven non-Hodgkin's lymphoma tested displayed an altered response suggests that the fraction of involved cases is sizable. It should also be noted that the lack of responsiveness to CD40-IRF4 may only be relevant to the ABC subtype of DLBCL, since high levels of *IRF4* expression, as well as evidence of constitutive NF-κB activation, are preferentially observed in this subtype (6/6 and 4/6 ABC-DLBCL cell lines, respectively) (Alizadeh et al., 2000; Davis et al., 2001; Wright et al., 2003) (data not shown).

Based on present knowledge of the biological program controlled by BCL6, it is conceivable that the lack of BCL6 suppression may contribute to lymphomagenesis by enforcing the constitutive suppression of BCL6 target genes controlling the responses to DNA damage (Phan and Dalla-Favera, 2004; Ranuncolo et al., 2007), cell cycle arrest (Phan et al., 2005), and plasma cell differentiation (Angelin-Duclos et al., 2000; Shaffer et al., 2002; Shapiro-Shelef et al., 2003). The identification of the intermediate molecules of CD40-induced *BCL6* downregulation will allow further testing of the role of this pathway in lymphomagenesis and, possibly, the design of strategies for its therapeutic correction.

EXPERIMENTAL PROCEDURES

GC B Cell Purification

GC centroblasts were purified from human tonsils as previously described (Klein et al., 2003) and maintained in IMDM supplemented with 20% FBS. Human tonsils were obtained with approval from the Columbia University Institutional Review Board as discarded leftovers from tonsillectomies performed at the New York-Presbyterian Hospital.

qRT-PCR Analysis

Polymerase chain reaction with reverse transcription (RT-PCR) analysis was performed as described previously (Niu et al., 2003). Quantitative real-time RT-PCR was performed with SYBR Green using the 7300 Real Time PCR systems (Applied Biosystems) according to the manufacturer's instructions. The oligonucleotide primers are described in Table S1.

Transient Transfection and Reporter Assays

293T cells were transiently transfected by using the calcium-phosphate precipitation method, and luciferase reporter assays were performed as previously described (Bereshchenko et al., 2002; Chang

et al., 1996). Each transfection was done in duplicate, and luciferase activities were measured 48 hr posttransfection using a dual-luciferase reporter assay kit (Promega) according to the manufacturer's protocol.

ChIP

ChIP assays were performed as described (Niu et al., 2003; Pasqualucci et al., 2003b). The oligonucleotides used for PCR amplification of immunoprecipitated chromatin fragments are listed in Table S1.

Cell Lines, Plasmids, Lentivirus Vectors, Immunofluorescence, and Northern and Western Blot Analysis

These procedures are described in the Supplemental Experimental Procedures.

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, four supplemental figures, and one supplemental table and can be found with this article online at http://www.cancercell.org/cgi/content/full/12/3/280/DC1/.

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