Title: BLIMP-1 is insufficient to induce antibody secretion in the absence of IRF4 in DT40 cells

Short title: IRF4 is required for antibody secretion

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Abstract

Differentiation of B cells into antibody secreting cells (ASCs), plasmablasts and plasma cells, is regulated by a network of transcription factors. Within this network factors including PAX5 and BCL6 prevent ASC differentiation and maintain the B cell phenotype. In contrast, BLIMP-1 and high IRF4 expression promote plasma cell differentiation. BLIMP-1 is thought to induce immunoglobulin secretion whereas IRF4 is needed for the survival of ASCs. The role of IRF4 in the regulation of antibody secretion has remained controversial. In order to study the role of IRF4 in the regulation of antibody secretion, we have created a double knockout (DKO) DT40 B cell line deficient in both IRF4 and BCL6. Although *BCL6*-deficient DT40 B cell line had upregulated BLIMP-1 expression and secreted antibodies, the DKO cell line did not. Even enforced BLIMP-1 expression in DKO cells or IRF4 deficient cells could not induce IgM secretion while in WT DT40 cells it could. However, enforced IRF4 expression in DKO cells induced strong IgM secretion. Our findings support a model where IRF4 expression in addition to BLIMP-1 expression is required to induce robust antibody secretion.

Introduction

High affinity antibodies arise to fight infections and as a response to vaccinations, therefore they are an essential part of our immune system. Affinity of antibodies mature in germinal centers (GCs) of secondary lymphoid follicles, where accumulative cycles of somatic hypermutation (SHM) and selection of proliferating activated B cells eventually lead to improved antibodies with higher affinity for antigen [1]. Upon antigen encounter, B cells differentiate into antibody secreting cells (ASCs). ASCs consist of short-living plasmablasts, created during the early phase of immune response, as well as of long-lived plasma cells in the bone marrow [2]. Due to coupling of mutational processes and rapid proliferation in GCs, many lymphomas originate from GC B cells [3]. Given the importance of high affinity antibody secreting cell generation, and the risks associated with this process, it is important to understand how the transition of B cells into ASCs is regulated at genetic level.

Coordinated differentiation of B cells into ASCs is controlled by a complex hierarchical network of transcription factors. Within this network transcription factors PAX5, BCL6, IRF8 and BACH2 promote B cell gene-expression program in GC and prevent premature differentiation into ASCs [1, 2, 4, 5]. IRF4 and BLIMP-1 promote the initiation of ASC gene-expression program, and later during the differentiation, XBP1 is needed for unfolded protein response and ELL2 for the expression of secretory form of IgH [1, 2, 6-8].

IRF4 appears to have a dual role, as a low level of IRF4 expression induces *BCL6* expression and GC reaction whereas high level of IRF4 promotes the expression of *PRDM1* (the gene encoding BLIMP-1) [9]. High IRF4 expression together with Blimp-1 suppresses B cell phenotype [9, 10]. Studies with *Irf4*-deficient mice suggested

that restoration of *Prdm1* expression in activated *Irf4*$^{-/-}$ B cells is sufficient to promote immunoglobulin (Ig) secretion [9]. However, another study indicated that *Prdm1* expression in the absence of IRF4 was not sufficient for plasma cell differentiation or Ig secretion, and that the roles of IRF4 and BLIMP-1 were non-redundant for antibody secretion [11]. Recent studies in mice with a conditional deletion of *Prdm1* in plasma cells have shown that BLIMP-1 regulates various genes involved in antibody secretion but was unable to reverse the plasmacytic identity [12, 13]. Conditional *Irf4* deletion in plasma cells resulted in cell death and IRF4 was concluded to be mostly needed for survival of plasma cells rather than for Ig secretion phenotype [13].

Considering that many aspects regarding the role of IRF4 in the regulation of antibody secretion still remain unclear, and that *Irf4*-deficient mouse plasma cells are nonviable [13], we used another model to study the role of IRF4 in antibody secretion in DT40 B cell line. We reasoned that immortalized DT40 cells overexpressing MYC would allow to more precisely dissect the roles of IRF4 and BLIMP-1 in antibody secretion. This cell line is derived from chicken bursal follicles, where BCL6 and BACH2 are required for somatic hypermutation and gene conversion [14, 15]. We have previously shown that deletion of PAX5 or BCL6 in these cells leads to spontaneous upregulation of *PRDM1* and differentiation into early ASC phenotype [4, 16].

In this study, we tested whether IRF4 is needed for antibody secretion and *PRDM1* expression. Therefore, we created *IRF4*$^{-/-}$/*BCL6*$^{-/-}$ double knockout cell line (DKO). We found that in the absence of IRF4, the deletion of *BCL6* did not induce *PRDM1* expression and antibody secretion. Surprisingly, the overexpression of *PRDM1* was able to induce antibody secretion only in WT cells and not in IRF4KO or DKO cells.

Thus, our results demonstrate that BLIMP-1 is not sufficient to induce antibody secretion in the absence of IRF4 in DT40 B cells.

Materials and Methods

Generation of IRF4$^{-/-}$/BCL6$^{-/-}$ double knockout (DKO) cell line and culture conditions

To obtain DKO cell line, the *BCL6* encoding alleles were removed with previously described targeting vectors [4] from *IRF4*-deficient cell line (IRF4KO) [17]. The DT40 cell line carries three alleles of *IRF4* due to trisomy of *IRF4* harboring chromosome 2 [18]. Prior to the targeting of *BCL6*, the expression vector Mer-Cre-Mer-Puro [19] was stably transfected into IRF4KO cells to allow p-lox mediated removal of Bsr and Neo selection cassettes. The IRF4KO cells were induced with 1.29 mM (Z)-4-Hydroxytamoxifen (Sigma) for 3 days as described in [20]. After that, cells were single cell subcloned and clone with removed Bsr, Neo and Puro selection cassettes was used for further experiments. The absence of IRF4 and BCL6 expression was confirmed by western blot analysis (Fig. 1A) and by RT-qPCR (Fig. 1B), respectively.

DT40 WT, BCL6KO, IRF4KO and DKO cells were cultured in RPMI 1640 (Sigma life technologies) supplemented with 10% fetal calf serum (Hyclone), 1% chicken serum (BioWest), 50 μM β-mercaptoethanol, 1 x GlutaMax-I (Gibco) and 1 x Penicillin Streptomycin (Gibco) in a humidified atmosphere with 5% CO_2 at +40 ^{o}C.

Western blot analysis

DT40 cells were lysed with RIPA buffer (150 mM NaCl, 5 mM EDTA (pH 8.0), 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS) supplemented with 1x protease inhibitor cocktail (cOmplete tablets, Roche). The whole cell lysates were separated on SDS-PAGE gels, transferred onto nitrocellulose membrane with *e*Blot Protein

Transfer system (Gene Script) and detected by Odyssey FC system (Li-Cor Biosciences). Antibodies used in study: anti-IRF4 (M-17) (Sc-6059, Santa Cruz Biotechnology), anti-GAPDH mAb (4G5, Hytest), anti-chicken IgM (A30-102A, Bethyl Laboratories, Inc.), Li-COR IRDye 800CW donkey anti-goat (#926-32214) and goat anti-mouse (#926-32210).

RT-qPCR analysis

Total RNA was extracted from 5 x10^6 cells with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) with the RNAse-free DNaseI (Qiagen) treatment according to the manufacturer's instructions. 1 μg of RNA was used for cDNA synthesis using qScript. RT-qPCR analysis was done with Sybr No-Rox Kit (Bioline) with Light Cycler 480 (Roche). The data is an average (+ SEM) of at least two independent RNA extractions from separate cultures with two technical replicates. The values were compared to WT levels or WT/GFP Mock or WT/mC Mock which were given value 1. The expression levels of individual genes were normalized to *GAPDH* expression for each cDNA sample. The obtained results were analyzed with Biogazelle qbase PLUS 2.4 software. The following primers were used: *GAPDH* (forward GAGGTGCTGCCCAGAACATCATC, reverse CCCGCATCAAAGGTGGAGGAAT), *IRF4* (forward GTATGCGCACAGCTTTGTCAAG, reverse GTTGCTCTTGTTC-AAAGCACACCTCAATCT), *BCL6* (forward GAGAAGCCATACCCCTGTGA, reverse TGCACCTTGGTGTTTGTGAT), *PRDM1* (forward GGCAGCCTGTCAGAATGGAAT, reverse GCTCCTTCTTTGGGACGCTCT), *PAX5* (forward GAACGAGTGCGATAA-CGACA, reverse TCGCGACCTGTTACGATAGGAT), *BACH2* (forward TCATCC-ATGACGTTCGCCG, reverse GCTCCTTCTTTGGGACGCTCT), *IRF8* (forward GGCACATCACCACCTCGTAT, reverse CGTGTCTGGGAACTCCTCTC), μS (forward GGAGAACCCCGAAAATGAGT, reverse GCCAACACCAAGGAGACATT),

µM (forward GGAGAACCCCGAAAATGAGT, reverse GTTGGATGTCGTCGTCCTCT) and *ID3* (forward CGTCATCGATTATATCTTCGACCTG, reverse GACACAAACTTCTCTCGTCTTTGGA).

Measurement of cell proliferation

The cell proliferation was performed using carboxyfluorescein succinimidyl ester (CFSE) staining as previously described [15]. Briefly, WT, IRF4KO, DKO and BCL6KO cells were stained with 0.5 µM (CFSE; Invitrogen). CFSE labelled cells were cultured for 65 hours and followed with flow cytometer at time points indicated in Supporting Information Fig. 1.

ELISA analysis

For the ELISA assay, 10^6 cells were grown in 1 ml of DT40 medium without chicken serum for 24h in humidified atmosphere with 5% CO_2 at +40^oC. The medium containing secreted IgM was collected from two independent cultures (two biological repeats) per sample and used in a dilution of 1:10 or 1:100 in duplicates for the analysis with the chicken IgM ELISA kit (E33-102, Bethyl Laboratories, Inc.) in accordance to the manufacturer's instructions. The absorbance was measured on an ELISA plate reader (Multiscan EX, Thermo Electron Corporation) at 450 nm and the amount of chicken IgM in unknown samples was calculated from standard curve with curve fitting software GraphPad Prism version 7.01.

Gene expression array

Total RNA was isolated as described in RT-qPCR section. RNAs were isolated from 2 (BCL6KO) or 3 (WT, IRF4KO, DKO) independent cultures. Sample preparation

was performed with Agilent's Low Input Quick Amp Labeling Kit (one-color) using 200 ng of total RNA as starting material and samples were hybridized onto Agilent's 4x 44K Chicken V2 chip according to the manufacturer's instructions. Arrays were scanned with Agilent technologies Scanner and numerical results extracted with Feature Extraction version 10.7.1. The data were analyzed using R/Bioconductor tool (Bioconductor version 2.7, R version 2.12.0). Data were quantile normalized and a comprehensive quality analysis was performed. Limma package was used for statistical testing and cut off values of false discovery rate below 0.01 and absolute fold change above 2 used to filter differentially expressed genes. The Venn diagrams were created by Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Comparison was made based on gene names. Hierarchical clustering of gene expression profiles of BCL6KO, IRF4KO and DKO cells was done using Euclidean distance with an average linkage. Clustering analysis was performed with R/Limma. Hierarchical clustering of differentially expressed genes for Fig. 2B and 2D was done with heatmap.2 function in R (default settings: Euclidean distance, R version 3.3.2). The microarray data has been deposited in the NCBI Gene Expression Omnibus and is accessible through GEO accession number GSE56165 (IRF4KO and WT) and GSE94836 (DKO, BCL6KO and WT).

PRDM1 and IRF4 expression constructs

Schematic presentation of expression constructs is presented on Fig. 3A and Fig. 4A and Supporting Information Fig. 3A. Chicken *PRDM1* coding sequence was amplified from BCL6KO DT40 cDNA using primers Blimp1_F ATTCACCACAGCTAGATGAAGGCTGCTACACGGTGTAGC and Blimp1_linker_R CTACTCCCAGCTGCACTACCTCCAGGGTCCATTGGTTCAACTGT or Blimp1_R TCCAGGGTCCATTGGTTCAACTGTT for B1-GFP or B1-T2A-GFP expression

construct, respectively. The primers create linker sequence GGTAGTGCAGCTGGGAGTAGCGGT between *PRDM1* and EGFP coding sequence in B1-GFP construct. Chicken *IRF4* was amplified from WT DT40 cDNA using primers IRF4_F ATTCACCACAGCTAGATGAACTTGGAGCCGGG-TGAG and IRF4_R TTCTTGAATAGAGGAATGGCGAATAGATCTGTGA.

Infection of cells by lentiviral constructs

293T cells were co-transfected with the psPAX2 and pMD2.G packaging vectors and B1-GFP (p-lenti-RSV-B1/GFP-IRES-PURO), GFP Mock, IRF4^{mC} (p-lenti-RSV-IRF4-T2AmCherry-IRES-GPT) or mC Mock expression plasmids using X-treme GENE HP DNA transfection Reagent (Roche) according to the manufacturer's instructions. Three days after transfection, viral supernatants were harvested. 2x10^{6} DT40 cells suspended in 200 µl of RPMI 1640 (Sigma) were infected with 200 µl of viral supernatant in dark for 30 minutes at room temperature. Two days after infection, cells were grown in selective DT40 medium. After at least a week of selection, B1-GFP or IRF4^{mC} positive cells were sorted (FACSAria IIu Cell Cell Sorter, BD) and kept in selective medium. Cells infected with B1-GFP or GFP Mock were grown in the presence of 0.5 µg/ml puromycin (Gibco) and cells infected with IRF4^{mC} or mC Mock were grown in the presence of 15 µg/ml mycophenolic acid (Millipore), 250 µg/ml xanthine and 20 µg/ml hypoxanthine (Sigma).

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 7.01 for Mac (GraphPad Software, La Jolla California USA).

Results

IRF4$^{-/-}$/BCL6$^{-/-}$ DKO cells are incapable of PRDM1 upregulation and antibody secretion

In order to test whether IRF4 is required for antibody secretion we created *IRF4$^{-/-}$ /BCL6$^{-/-}$* DKO cell line by disrupting *BCL6* in IRF4KO cells. The resulting DKO cells were verified to lack both the IRF4 and BCL6 expression (Fig. 1A and B). BCL6 and IRF4 single knockouts were described previously [4, 17]. The DKO cell line had the same growth characteristics as IRF4 single knockout and WT cell lines (Supporting Information Fig. 1). Since BCL6KO cells secrete antibodies [4], we analyzed whether DKO cells lacking both IRF4 and BCL6 also secrete. DT40 cells do not undergo isotype switching. Therefore, we measured IgM from supernatants of WT, IRF4KO, DKO and BCL6KO cell cultures. DKO cells did not secrete more antibodies than WT cells (Fig. 1C). Thus, removal of *BCL6* is insufficient to induce antibody secretion in the absence of IRF4.

Since BLIMP-1 drives the antibody secretion [12, 13], and that IRF4 upregulates *PRDM1* expression [9], we analyzed the expression of *PRDM1* (Fig. 1D). *PRDM1* expression was not upregulated in either IRF4KO or DKO cells while BCL6KO cells had high *PRDM1* expression. This indicates that *PRDM1* expression correlates with antibody secretion.

We also analyzed whether the BCL6 and IRF4 deficiency would affect the expression levels of *PRDM1* repressors *BACH2, IRF8* and *PAX5* [16, 21, 22]. Removal of *IRF4* alone increased their expression. Furthermore, IRF4 was found to bind to *IRF8* promoter region (Supporting Information Fig. 2), suggesting direct repression of *IRF8* by IRF4. The deletion of *BCL6* in *IRF4$^{-/-}$* cells reduced the expression of *BACH2*, left *IRF8* expression upregulated and *PAX5* expression

unchanged when compared with WT cells (Fig. 1D). It seems logical that since several *PRDM1* downregulators were still expressed, *PRDM1* could not be upregulated. Together these findings suggest that DKO cells have not fully adopted the ASC phenotype due to absence of IRF4 and *PRDM1* upregulation.

Gene expression profiling of BCL6KO, IRF4KO and DKO cells

To take a deeper look into genes regulating the secretory phenotype, we characterized the transcriptome of the DKO and individual knockout cells using a gene expression array. We compared the expression profiles of BCL6KO, IRF4KO and DKO cells relative to WT cells (absolute fold change ≥2, p ≤0.01, Supporting Information Table 1). We found that in DKO cells 546 genes were dependent on BCL6 expression, whereas 837 genes were IRF4-dependent. Approximately 35% of upregulated genes and 31% of downregulated genes in DKO cells were not similarly altered in either one of the single knockout cells (Fig. 2A). Hierarchical clustering (Euclidian distance) of ASC signature genes showed that BCL6KO cells clustered separately from IRF4KO and DKO cells (Fig. 2B). Gene expression profiles of all of the differentially expressed genes clustered in a similar manner (data not shown). Thus, deletion of both factors together seems to have a unique effect on the gene expression profile.

We then compared gene expression profiles of each knockout cell line with genes of mouse antibody-secreting plasma cells identified previously [23]. The gene expression pattern of BCL6KO cells had the most overlap with the 301 ASC genes (Fig. 2B and Supporting Information Table 2) [23]. This analysis suggests that DKO cells do not obtain a gene expression profile of ASCs.

To reveal genes regulating the secretory phenotype, we analyzed the array data further. We selected the differentially expressed genes from IRF4KO and DKO cells that have not been changed in the same direction in BCL6KO cells (Fig. 2A). We next subtracted genes oppositely regulated (upregulated in BCL6KO and downregulated in IRF4KO and DKO and vice versa) in BCL6KO from IRF4KO and DKO (Fig. 2C dark purple region). We compared the resulting list of 138 genes (Fig. 2D, Supporting Information Table 3) with the list of genes of plasma cell signature [23] and found that only *PRDM1* and *IRF4* overlapped with it. This verifies independently that BLIMP-1 and IRF4 are both needed for secretory program. Interestingly, *SPI1* was among these 138 genes. The expression of *SPI1* was upregulated in IRF4 deficient cells and depleted in antibody secreting BCL6KO cells (Fig. 2D).

BLIMP-1 is insufficient to induce antibody secretion in IRF4KO and DKO cells

The absence of IgM secretion in IRF4-deficient cells could result from the lack of *PRDM1* expression. To test whether *PRDM1* expression can overcome the blockade of antibody secretion, we introduced forced expression of BLIMP-1 in the IRF4 deficient cells (IRF4KO and DKO). We expressed BLIMP-1 from a lentiviral vector B1-GFP that produces a BLIMP-1-GFP fusion protein (Fig. 3A) in WT, IRF4KO and DKO cells. As a parallel approach validating the functionality of BLIMP-1-GFP fusion protein, we generated another BLIMP-1 expression construct that did not produce potentially interfering fusion partner. This construct codes for a BLIMP1-T2A-GFP fusion protein (B1-T2A-GFP) where T2A self-cleaving peptide mediates the cleavage of BLIMP-1 and GFP during translation (Supporting Information Fig. 3A) yielding two separate proteins: BLIMP-1 and GFP. B1-GFP, B1-T2A-GFP and mock (GFP-mock) transduced cells were sorted for GFP expression and expression of *PRDM1* (from

endogenous and lentiviral sources) was measured by RT-qPCR (Fig. 3B and Supporting Information Fig. 3B). We chose cell populations that had substantial overexpression of *PRDM1* to make sure the *PRDM1* expression was not the limiting factor for antibody secretion in further analysis.

Overexpression of BLIMP-1 in WT cells (both WT/B1-GFP and WT/B1-T2A-GFP) induced a robust IgM secretion (Fig. 3C and Supporting Information Fig. 3C) as expected of functional BLIMP-1 over-expression. However, over-expression of BLIMP-1 in IRF4KO (IRF4/B1-GFP) or DKO (DKO/B1-GFP) cells did not increase IgM secretion when compared to mock transduced cells (Fig. 3C and Supporting Information Fig. 4). The increased secretion was accompanied by a increased transcript levels of secretory (μS) form of μ heavy chain in BLIMP-1 transduced cell lines (Fig. 3D). These data suggest that in addition to BLIMP-1, IRF4 plays an essential role in the pathway leading to a secretory phenotype. Given that transcription factors PAX5, BCL6, IRF8 and BACH2 are downregulated in ASCs [1, 2], we also analyzed their expression. Despite the enforced BLIMP-1 expression, the expression of *PAX5*, *BACH2* and *IRF8* was unaltered in all of the *PRDM1* transduced cell lines (Fig. 3D and E). We also analyzed the expression levels of *EBF1*, *SPI1* (encoding PU.1) that are downregulated in ASCs [23] as well as in BCL6KO cells (Supporting Information Table 1 and Fig. 3F). The expression of *EBF1 and SPI1* remained unaltered in *PRDM1* transduced IRF4KO and DKO cells.

The expression of *ELL2*, which is highly expressed in BCL6KO cells and is required for post-transcriptional expression switch to a secretory form of IgH, remained unaltered in each BLIMP-1 transduced cell line (Fig. 3F). In antibody secreting BCL6KO cells, where *PRDM1* expression is upregulated (Fig. 1D) the expression of *ELL2* was upregulated (Fig. 3E). The only difference we observed with *PRDM1*

overexpression was the reduction of *SPI1* and the surprising reduction of *IRF4* expression (Fig. 3E and F). Together these results indicate that BLIMP-1 is incapable of suppressing B cell phenotype and promoting antibody secretion in the absence of IRF4.

Enforced IRF4 expression in DKO cells induces IgM secretion

To clarify our observation that IRF4 has a major contribution to antibody secretion, we expressed IRF4 in knockout cell lines. We made a lentiviral vector where the IRF4 expression cassette has a T2A excision sequence between the IRF4 encoding sequence and mCherry (mC) fluorescence marker encoding sequence (IRF4^{mc}) (Fig. 4A). The expression constructs IRF4^{mc} and the mock control vector (mC Mock) were introduced into the WT, IRF4KO and DKO cells similarly to *PRDM1* expression vector before. We verified that IRF4 was over-expressed in IRF4KO/IRF4^{mC} and DKO/IRF4^{mC} cells using RT-qPCR and western blot (Fig. 4B and D).

In contrast to the enforced BLIMP-1 expression in IRF4KO and DKO cells (Fig. 3B and C), the over-expression of IRF4 resulted in a moderate increase of IgM secretion in IRF4KO (IRF4KO/IRF4^{mC}) cells but in a strong increase in DKO (DKO/IRF4^{mC}) cells (Fig. 4C). This is in line with increased transcript levels of secretory form of µ heavy chain (Fig. 4D). This result indicates that IRF4 promotes antibody secretion especially in the absence of BCL6.

To characterize the gene expression changes in IRF4 transduced cell lines we analyzed the expression levels of *IRF4*, *PRDM1*, *ELL2, EBF1, IRF8, SPI1, BACH2, BCL6* and *PAX5* by RT-qPCR analysis. *IRF4* over-expression was able to induce *PRDM1* expression only in DKO/IRF4^{mC} cells (Fig. 4D). In accordance with ASC

phenotype of DKO/IRF4^{mc} cells, also the expression of *PAX5, EBF1, IRF8, SPI1* and *BACH2* were downregulated in these cells (Fig. 4E)

Surprisingly, IRF4 over-expression in WT and IRF4KO cells was unable to induce *PRDM1* expression or consequent downregulation of B cell identity genes (*PAX5, BCL6, EBF1, IRF8, SPI1* and *BACH2*) suggesting that *BCL6* has a major role in the regulation of these genes. The expression of *ELL2*, however, remained unaltered. *SPI1* and *IRF8* expressions were increased with IRF4 over-expression in WT but not in IRF4KO cells. On the contrary, *BCL6* and *BACH2* showed increased expression with IRF4 over-expression in IRF4KO and showed the same trend in WT background, although the difference did not reach statistical significance. Together these results indicate that both IRF4 and BLIMP-1 are non-redundant for the antibody secretion. Furthermore, IRF4 can induce BLIMP-1 expression and consequent downregulation of B cell identity genes only in the absence of BCL6.

Discussion

Our data indicates that IRF4 together with BLIMP-1 is needed to induce antibody secretion in DT40 B cells. Ectopic expression of BLIMP-1 in mature B cells can induce maturation into immunoglobulin-secreting cells [24]. However, here we demonstrate that ectopic BLIMP-1 expression is not sufficient to induce secretion when IRF4 is not present as well as that IRF4 can induce robust secretion only in the absence of BCL6 (Supporting Information Fig. 5).

BLIMP-1 is the key factor upregulating genes needed for antibody secretion [12, 13]. The incapability of DKO cells to secrete antibodies could be explained by the lack of

BLIMP-1 upregulation. However, the over-expression of BLIMP-1 in DKO cells did not lead to IgM secretion. Since enforced BLIMP-1 expression was able to induce IgM secretion only in WT cells, but not in IRF4KO or DKO cells, our findings strongly support the idea that BLIMP-1 in the absence of IRF4 is insufficient to initiate the antibody secretion. Furthermore, it has been shown that BLIMP-1 accounts only for a subset of gene expression program of plasma cells [13]. Therefore, our results are in line with the previous study by Klein et al. (2006), suggesting that IRF4 and BLIMP-1 are non-redundant for the induction of antibody secretion [11].

The function of IRF4 in terminal B cell differentiation is likely not limited to the upregulation of *PRDM1* expression but may contribute to turning off the B cell gene expression program as well. Our results also indicate that IRF4 can repress *BACH2* and directly *IRF8*. Xu et al. showed that in activated B cells IRF4 in high doses inhibits *IRF8* expression and also directly represses *BACH2*, *SPIB*, and *EBF1* [25].

SPIB is not present in the chicken genome, is highly related to *SPI1* and both have a complementary function in B cells [26, 27], it is plausible that *SPI1* fulfills the function of *SPIB* in chicken B cells. Furthermore, the downregulation of *SPIB* in ASCs is among the plasma cell signature [23]. Similarly, *SPI1* was not expressed in antibody secreting chicken BCL6KO cells. Moreover, BLIMP-1-induced Ig secretion in DT40 cells occurred only when IRF4 was present and *SPI1* expression downregulated. IRF4 has a dual role during germinal center formation and induction of plasma cell differentiation. IRF4 is required for GC formation via upregulation of BCL6 and OBF1 [10]. On the other hand, a high expression of IRF4 is needed for the induction of BLIMP-1 and ASC differentiation as well as for the downregulation of *BCL6* [9, 28]. The difference in the regulatory outcome is due to the selection of different targets based on IRF4 concentration and involvement of cofactors. High affinity binding of

IRF4 together with PU.1 to EICE motif regulates genes during B cell activation and GC formation, whereas at high doses, IRF4 can bind alone to low affinity ISRE motif present in *PRDM1* inducing the ASC differentiation [10]. Here we showed that *SPI1* (the gene encoding PU.1) is upregulated in IRF4-deficient cells (IRFKO and DKO). This suggests at least two mutually non-exclusive options: the B cell program is upregulated in the absence of IRF4 or the cells try to compensate for the loss of IRF4 by upregulating *IRF8* and *SPI1*. IRF8 together with PU.1 is known to repress plasma cell differentiation via modulation of *BCL6* expression [21, 29].

Recently it has been shown that downregulation of *ID3* expression is essential for antigen-induced B cell differentiation into plasma cells [13, 23, 30]. As seen in microarray results (Supporting Information Table 1) and Supporting Information Fig. 6, the expression of *ID3* is significantly downregulated in all the knockout cell lines (IRF4KO, DKO and BCL6KO) even in ones that do not secrete. We cannot conclude whether IRF4 or BLIMP-1 downregulate ID3 in secreting cells. However, in non-secreting cells *ID3* is downregulated in the absence of IRF4 and BLIMP-1. Thus we conclude that ID3 is not the key factor regulating secretion in this model system.

Stability of BACH2 protein has been previously shown to be dependent on BCL6 [31]. Our results suggest that BCL6 is also a major factor for maintaining *BACH2* transcription since, in cells deficient in BCL6 (BCL6KO and DKO), *BACH2* expression drops dramatically and independently of BLIMP-1 action (DKO cells did not express BLIMP1). Hence BCL6 regulates BACH2 likely through direct binding to *BACH2* gene [4].

When BCL6 was present, the over-expression of IRF4 enhanced expression of B cell factors. This resembles the situation during the initiation of GC reaction [10] and may

be due to the fact that in these cells PU.1 was also expressed. The presence of BCL6 might dictate the outcome of IRF4 action, as IRF4 only induced secretion in the absence of BCL6 in DT40 B cells. Therefore, our data support the model in which BCL6 degradation induced by signals from B cell receptor [32] would need to occur first to change IRF4 binding preference for upregulation of *PRDM1* (through low affinity sites) and consequent initiation of antibody secretion.

Expression of IRF4 in DKO cell should restore the BCL6KO phenotype. Indeed all the transcription factors measured (*PAX5*, *BACH2*, *IRF8*, *SPI1*, *EBF1* and *PRDM1*) were restored to the level observed in single BCL6KO cells. The only exception was the expression of *ELL2* that was not restored. This might be the underlying reason behind the 10-fold lower IgM secretion by DKO/IRF4^{mC} cells than BCL6KO cells.

Overexpression of *PRDM1* did not have any impact on *BCL6* and *PAX5* expression in any of the cell lines tested here. Even though BLIMP-1 is essential for the terminal differentiation of ASCs [33], the upregulation of BLIMP-1 is not an initiative event and pre-plasmablasts can develop in the absence of BLIMP-1 [34]. In fact, it appears that when B cells differentiate to ASCs, *PAX5* is transcriptionally downregulated before BLIMP-1 upregulation [16, 34]. It remains to be solved how *PAX5* downregulation is achieved.

The absence of *BCL6* downregulation in the BLIMP-1 transduced cell lines might seem surprising. However, it was previously observed that human primary B cells with induced BLIMP-1 expression started antibody secretion while having high *BCL6* expression [35]. Furthermore, another study found BCL6 not to be regulated by BLIMP-1 despite BLIMP-1 binding to *BCL6* enhancers [12]. Thus the regulation of *BCL6* expression seems to be more complex than expected.

Taken together, our data support a model in which BCL6 would need to be downregulated before IRF4 expression can induce BLIMP-1 expression and consequent initiation of antibody secretion.

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CONFLICT OF INTEREST

The authors declare no financial or commercial conflict of interest.

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Figure Legends

Figure 1. IRF4 deficient cells are incapable of antibody secretion. A) Western blot analysis of IRF4 protein levels in WT, IRF4KO, DKO and BCL6KO cells. GAPDH is used as a loading control. B and D) The mRNA levels of *BCL6* (B) and *PRDM1, BACH2, IRF8* and *PAX5* (D) in cell types indicated along the x axis; quantified by RT-qPCR and normalized to GAPDH, shown in comparison to WT, set as 1. Mean + SEM of at least 3 independent RNA preparations. C) Analysis of the IgM secretion in WT, IRF4KO, DKO and BCL6KO cells by ELISA. Mean + SEM of 2 biological replicates which were done in duplicates. B-D) Statistical significance was analyzed by one-way ANOVA with Holm-Sidak's post-test where each cell type was compared to WT cell line. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$; ns, not statistically significant; nd, not detected.

Figure 2. Comparison of gene expression profiles of IRF4KO, DKO and BCL6KO cells. A) Venn diagrams showing differentially upregulated and downregulated genes in IRF4KO, BCL6KO and DKO cells relative to WT (absolute fold change ≥2, $p \leq 0.01$). Microarray analysis was done from 2 (BCL6KO) or 3 biological replicates (other cell lines). B) Genes belonging to the previously published ASC gene signature [23] in BCL6KO, DKO and IRF4KO cells relative to WT cells. Colors represent log Fold change in which 1 logFC = 2 Fold change. Genes with absolute logFC < 1 and/or $p \leq 0.01$ are shown as white. Dendrogram shows the clustering of genes (Hierarchical clustering, Euclidean distance). C) Genes expressed in opposite direction in BCL6KO and IRF4 deficient cell lines. Up and down refers to upregulated and downregulated genes, respectively as relative to WT. D) A heatmap of 138 genes from overlapping region (dark purple region) of all cell lines in C.

Figure 3. Enforced BLIMP-1 over-expression in WT, IRF4KO and DKO cells. A) Schematic presentation of lentiviral vector constructs B1-GFP which was used to create the Blimp-1 overexpressing cell lines and GFP Mock used as a control. B, D, E and F) The mRNA levels of *PRDM1* (B), secretory (μS) and membrane (μM) form of the immunoglobulin μ heavy chain, *PAX5*, and *BACH2* (D), *IRF8, IRF4* and *BCL6* (E), *SPI1, EBF1*, and *ELL2* (F) in cell types indicated along the x axis; quantified by RT-qPCR and normalized to GAPDH, shown in comparison to WT GFP Mock, set as 1. Mean + SEM of 2 independent RNA preparations of GFP Mock transduced cell lines and 3 independent RNA replicates of BCL6KO and B1-GFP transduced cell lines. C) Analysis of the IgM secretion in BLIMP-1 overexpressing cells by ELISA. Mean + SEM of 2 biological replicates analyzed in duplicates. B-F) Statistical significance was analyzed by unpaired two-tailed Student's t-test. In F) BCL6KO cells are compared with WT/GFP Mock cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$; ns, not statistically significant; nd, not detected.

Figure 4. Enforced IRF4 over-expression in WT, IRF4KO and DKO cells. A) Schematic presentation of lentiviral vector constructs $IRF4^{mC}$ which was used to create the IRF4 overexpressing cell lines and mC Mock used as a control. B) Western blot analysis of IRF4 protein levels in WT, IRF4KO, IRF4KO/IRF4mC, DKO and DKOmC cells. GAPDH is used as a loading control. The protein in which the mCherry was not excised from IRF4 is indicated by an arrow. C) Analysis of the IgM secretion in IRF4 over-expressing cells by ELISA. Mean + SEM of 2 biological replicates with the exception of WT/$IRF4^{mC}$ (n=4) and DKO/$IRF4^{mC}$ (n=3) analyzed in duplicates. D and E) The mRNA levels of secretory (μS) and membrane (μM) form of the immunoglobulin μ heavy chain, *IRF4*, *PRDM1*, *ELL2* (D), *EBF1*, *IRF8*, *SPI1*, *BACH2*, *BCL6* and *PAX5* (E) in cell types indicated along the x axis; quantified by

RT-qPCR and normalized to GAPDH, shown in comparison to the WT/mC Mock, set as 1. Mean + SEM of 2 independent RNA preparations of mC Mock transduced cell lines and at least 3 independent RNA replicates of IRF4^{mC} transduced cell lines. C-E) Statistical significance was analyzed by unpaired two-tailed Student's t-test. * $p<0.05$, ** $p<0.01$, *** $p<0.005$, **** $p\leq0.0001$; ns, not statistically significant; nd, not detected.

Supporting Information Figure 1. Proliferation of WT, IRF4KO, DKO and BCL6KO cells. Cell proliferation measured by reduction of CFSE signal over time. Mean ± SD of three replicate cultures; CFSE, carboxyfluorescein succinimidyl ester.

Supporting Information Figure 2. IRF4 binding to the promoter region of *IRF8*. The binding was examined by chromatin immunoprecipitation (ChIP) with anti-IRF4 antibody (α-IRF4) and unrelated polyclonal goat antibody (IgG) used as a control. The ChIP was performed as previously described [18]. Input and immunoprecipitated DNA were amplified by PCR with primers (IRF8-f GGGAGAATGAGGAGAAAACCATGTTCC and IRF8-r CTGCAAACTGCAGATCCTGCTGACAG) specific for the promoter region of IRF8. The PCR products were quantified and presented as % of input. Mean + SD of 5 independent experiments. *** $p=0.0001$; two-tailed Student's t-test.

Supporting Information Figure 3. Enforced BLIMP-1 over-expression in WT cells with B1-T2A-GFP construct. A) Schematic presentation of the lentiviral vector construct B1-T2A-GFP. B) The mRNA levels of *PRDM1* in WT/GFP Mock and WT/B1-T2A-GFP induced cells; quantified by RT-qPCR and normalized to GAPDH, shown in comparison to WT/GFP Mock, set as 1. Mean +SEM of 2 independent RNA preparations. C) Analysis of the IgM secretion in BLIMP-1 over-expressing cells by ELISA. Mean +SEM of 2 biological replicates analyzed in duplicates.

Supporting Information Figure 4. Western blot analysis of immunoglobulin µ heavy chain (µH) expression in whole cell lysates and culture supernatants. The µH was detected with anti-chicken IgM antibody. GAPDH is used as a loading control for whole cell lysates and as an indicator of potential leakage of intracellular components from dead cells into culture supernatants. Equivalent of 0.5 $x10^6$ cells/ lane were loaded on the gel from whole cell lysates and a 10 µL/ lane aliquot of culture supernatants used in ELISA. The blot is a representative of 2 Western blots.

Supporting Information Figure 5. The schematic presentation of induction of antibody secretion in DT40 cell lines by BLIMP-1 or IRF4 over-expression. IgM secretion in DT40 cell lines can be induced by ectopic over-expression of BLIMP-1 only in the presence of IRF4, or by over-expression of IRF4 when BLIMP-1 is de-repressed in the absence of *BCL6* (both situations are indicated by pink background).

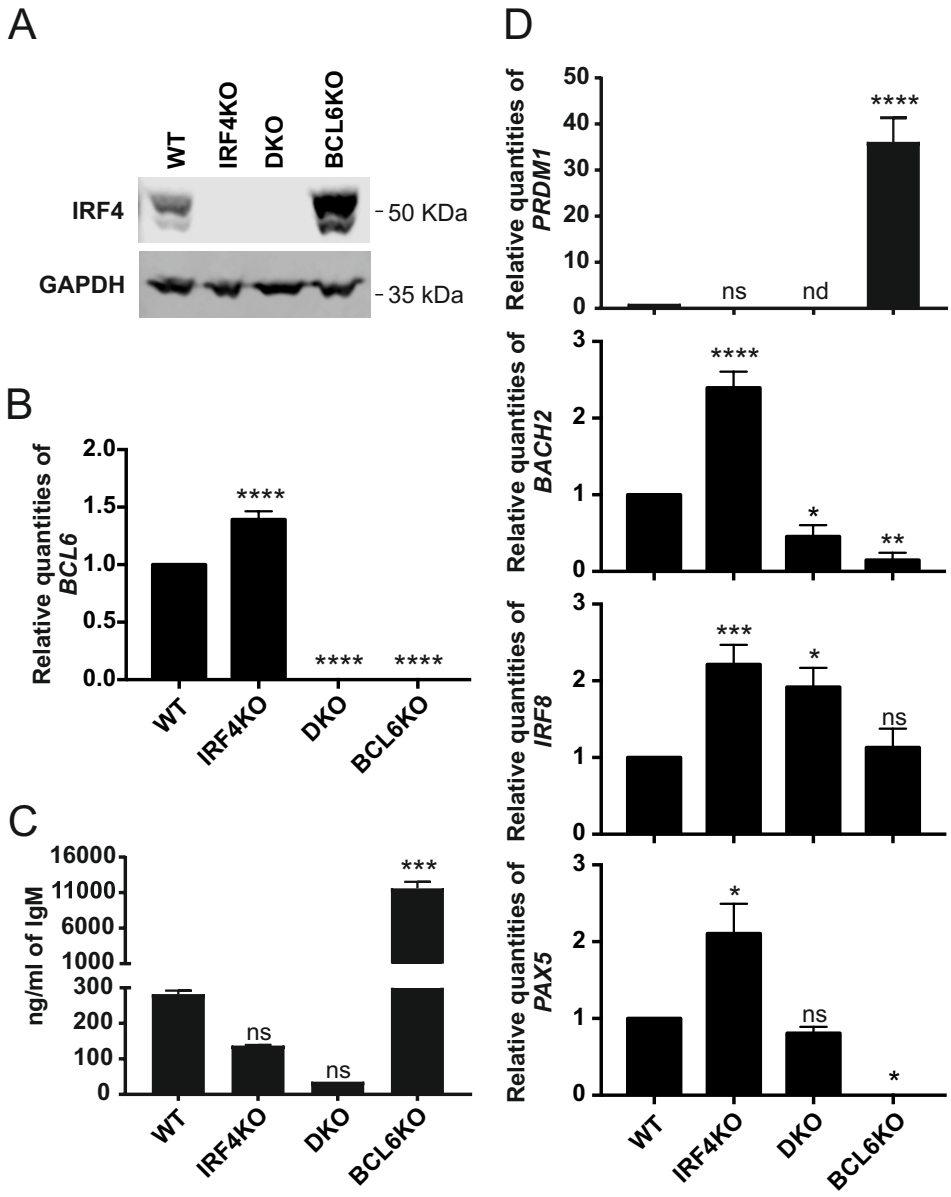
Supporting Information Figure 6. The mRNA levels of *ID3* in BLIMP-1 transduced cell lines; quantified by RT-qPCR and normalized to *GAPDH*, shown in comparison to WT/GFP Mock, set as 1. Mean +SEM of at least 2 independent RNA preparations. Statistical significance was analyzed by unpaired two-tailed Student's t-test; *** $p<0.001$; ns, not statistically significant.

Supporting Information Table 1. Differentially expressed genes in BCL6KO, IRF4KO and DKO cell lines relative to WT DT40 cell line.

Supporting Information Table 2. A list of genes from Fig. 2B with gene expression changes relative to WT cell line.

Supporting Information Table 3. A list of 138 genes from Fig. 2D with gene expression changes relative to WT cell line.

Figure 1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DKO
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1.0
0.5
0.0
****
****
****
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IRF4KO
DKO
BCL6KO
C
ng/ml of IgM
16000
11000
6000
1000
300
200
100
0
***
ns
ns
WT
IRF4KO
DKO
BCL6KO
D
Relative quantities of PRDM1
50
40
30
20
10
0
****
ns
nd
Relative quantities of BACH2
3
2
1
0
****
*
**
Relative quantities of IRF8
3
2
1
0
***
*
ns
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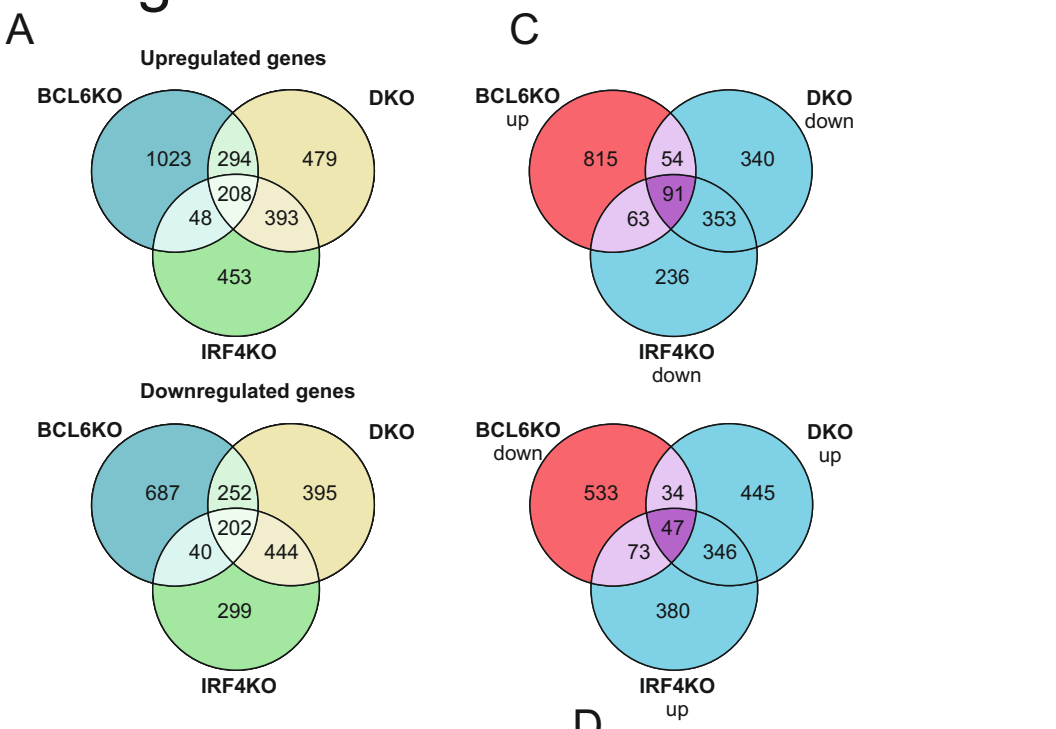
ns
*
WT
IRF4KO
DKO
BCL6KO

Figure 2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DKO
IRF4KO
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Downregulated genes
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IRF4KO
687
252
395
202
40
444
299
C
BCL6KO up
DKO down
IRF4KO down
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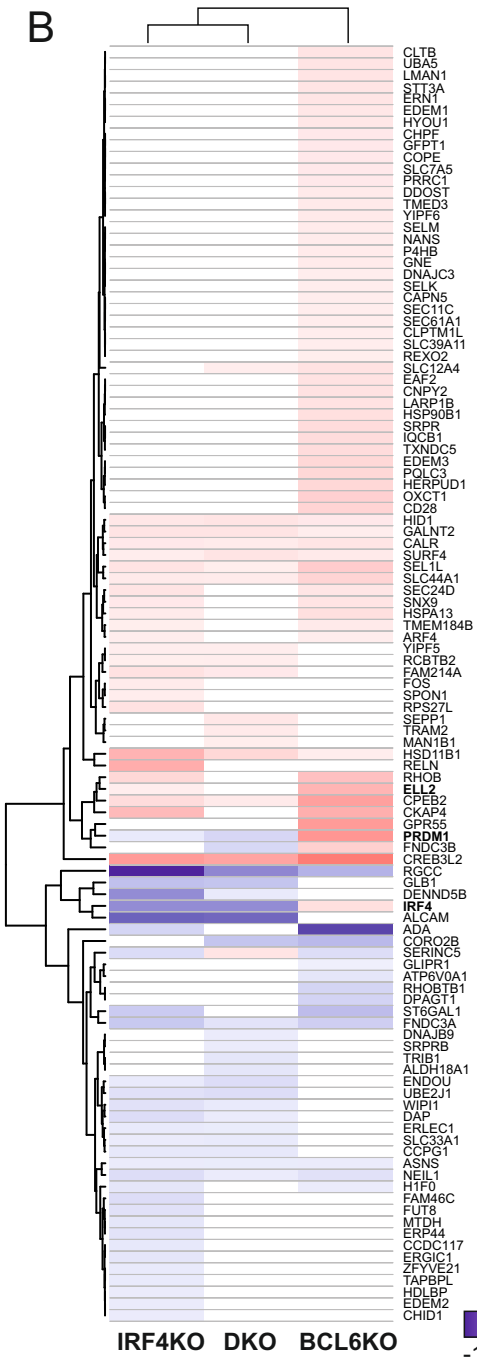
47
73
346
380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SELM
NANS
P4HB
GNE
DNAJC3
SELK
CAPN5
SEC11C
SEC61A1
CLPTM1L
SLC39A11
REXO2
SLC12A4
EAF2
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HSP90B1
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GALNT2
CALR
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SEL1L
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RELN
RHOB
ELL2
CPEB2
CKAP4
GPR55
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GLB1
DENND5B
IRF4
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ADA
CORO2B
SERINC5
GLIPR1
ATP6V0A1
RHOBTB1
DPAGT1
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FNDC3A
DNAJB9
SRPRB
TRIB1
ALDH18A1
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UBE2J1
WIPI1
DAP
ERLEC1
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COPG1
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NEIL1
H1F0
FAM46C
FUT8
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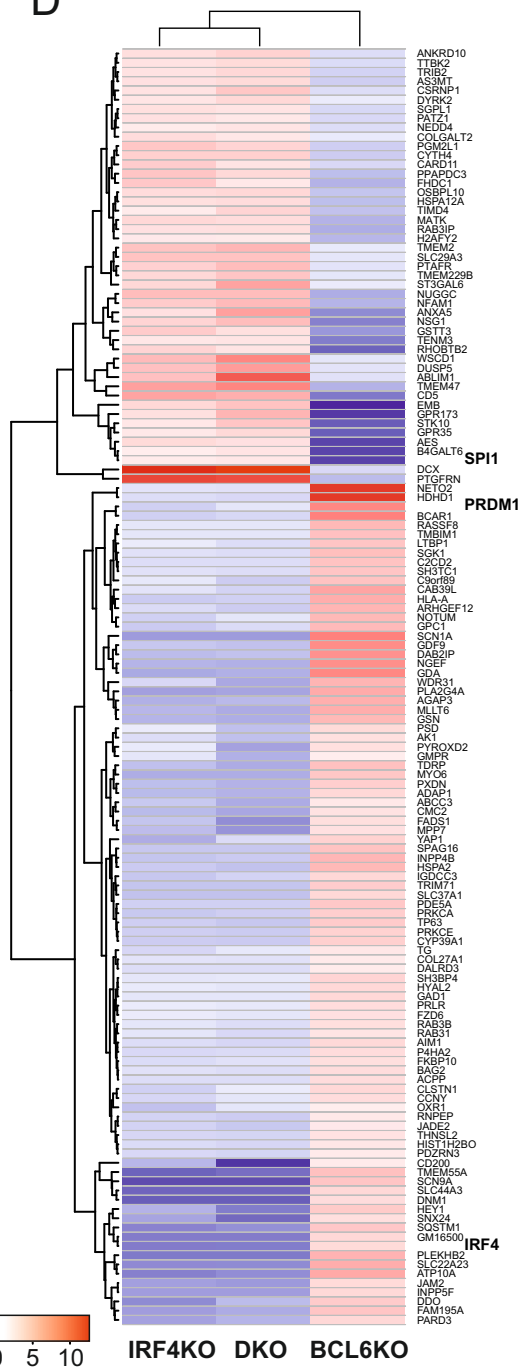
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CARD11
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FHDC1
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HDHD1
PRDM1
BCAR1
RASSF8
TMBIM1
LTBP1
SGK1
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SH3TC1
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CMC2
FADS1
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SPAG16
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ACPP
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CCNY
OXR1
RNPEP
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THNSL2
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HEY1
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GM16500
IRF4
PLEKHB2
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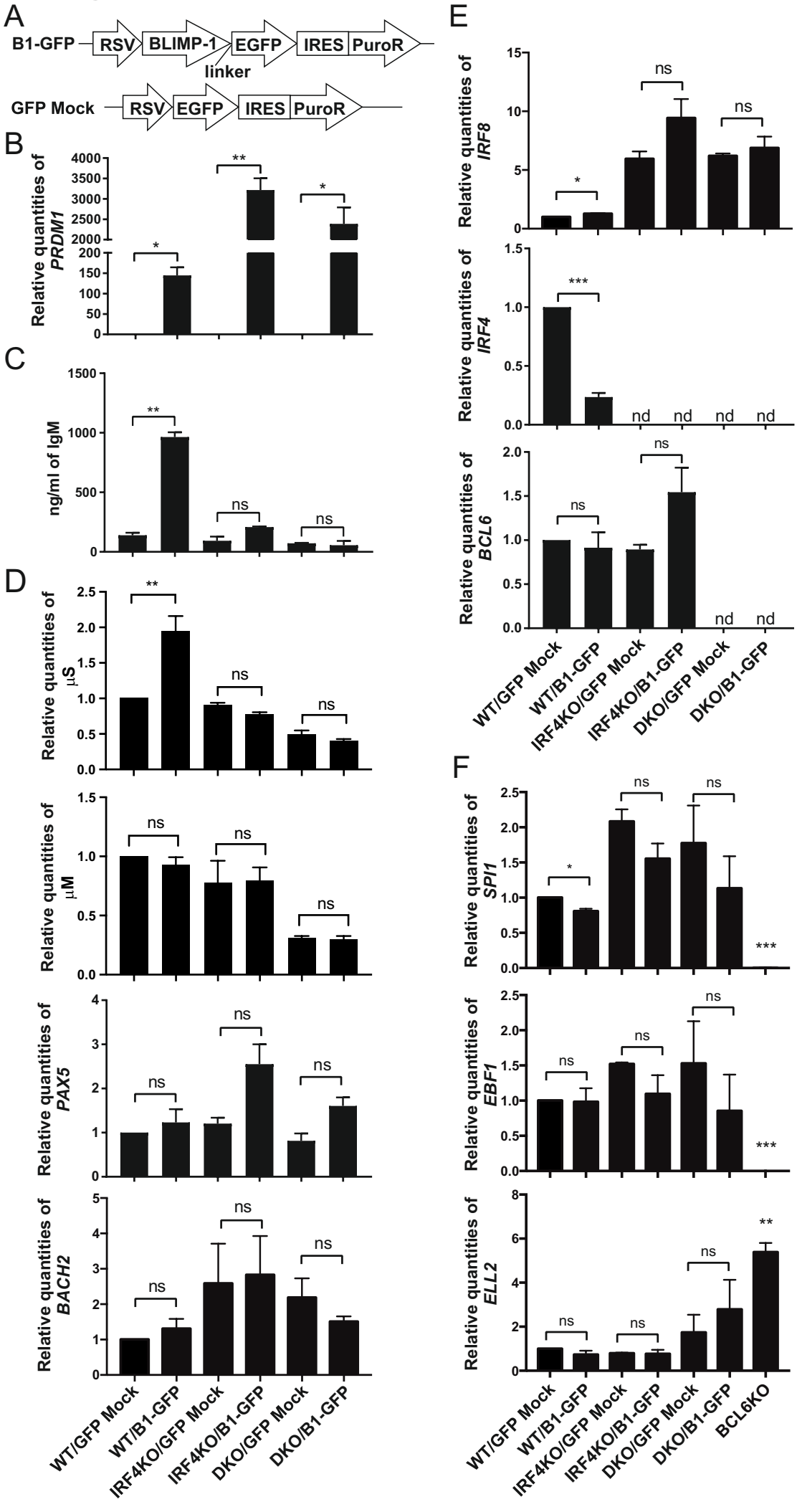
BCL6KO

Figure 3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B1-GFP
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BLIMP-1
EGFP
IRES
PuroR
linker
GFP Mock
RSV
EGFP
IRES
PuroR
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Relative quantities of PRDM1
C
ng/ml of IgM
D
Relative quantities of μS
Relative quantities of μM
Relative quantities of PAX5
Relative quantities of BACH2
E
Relative quantities of IRF8
Relative quantities of IRF4
Relative quantities of BCL6
F
Relative quantities of SPI1
Relative quantities of EBF1
Relative quantities of ELL2
WT/GFP Mock
WT/B1-GFP
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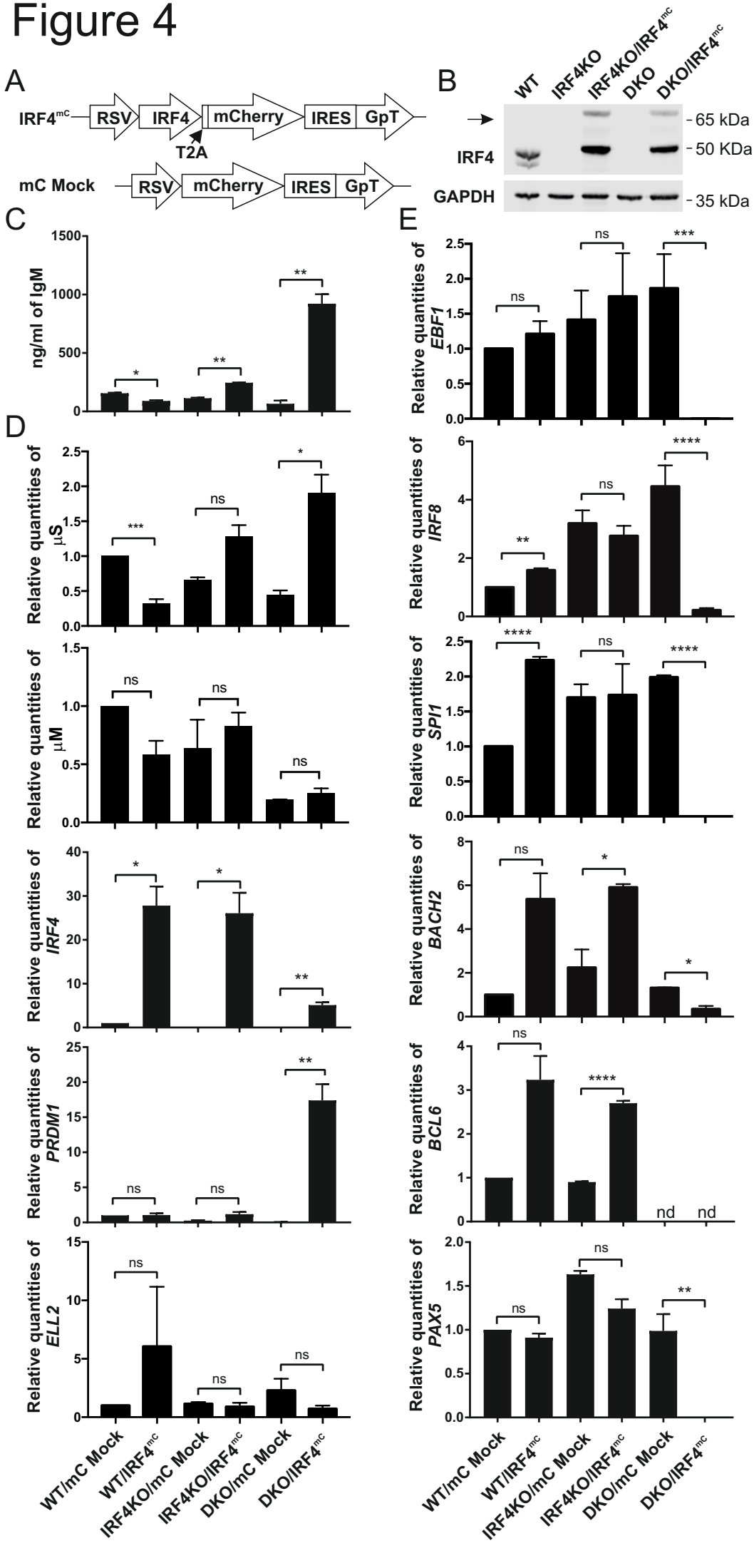
DKO/B1-GFP
BCL6KO
ns
nd

Figure 4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IRF4
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IRES
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mC Mock
RSV
mCherry
IRES
GpT
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IRF4KO/IRF4mC
DKO
DKO/IRF4mC
IRF4
GAPDH
65 kDa
50 KDa
35 kDa
C
ng/ml of IgM
D
Relative quantities of μS
Relative quantities of μM
Relative quantities of IRF4
Relative quantities of PRDM1
Relative quantities of ELL2
E
Relative quantities of EBF1
Relative quantities of IRF8
Relative quantities of SPI1
Relative quantities of BACH2
Relative quantities of BCL6
Relative quantities of PAX5
ns
nd
WT/mC Mock
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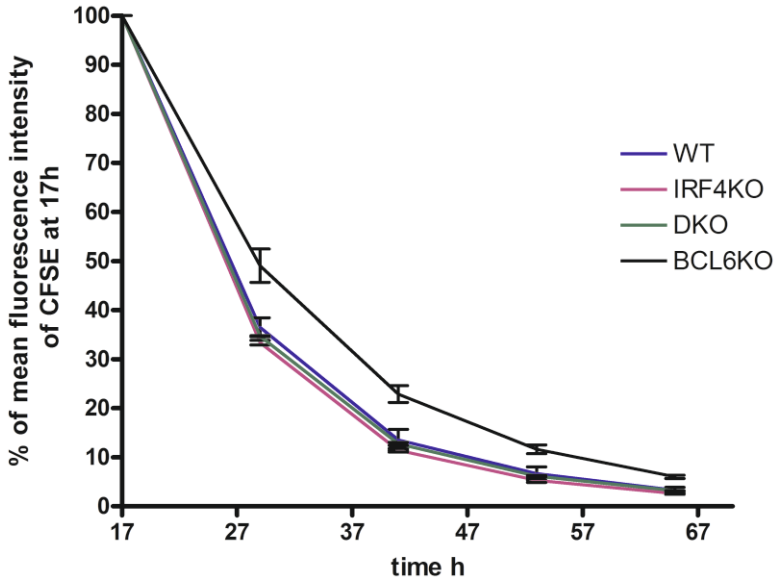
DKO/IRF4mC

Supporting Information Figure 1



Supporting Information Figure 2

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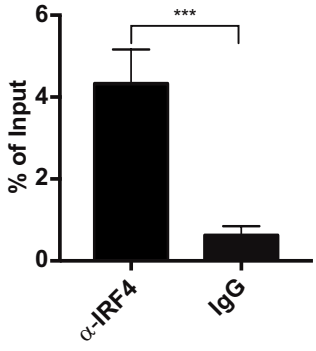

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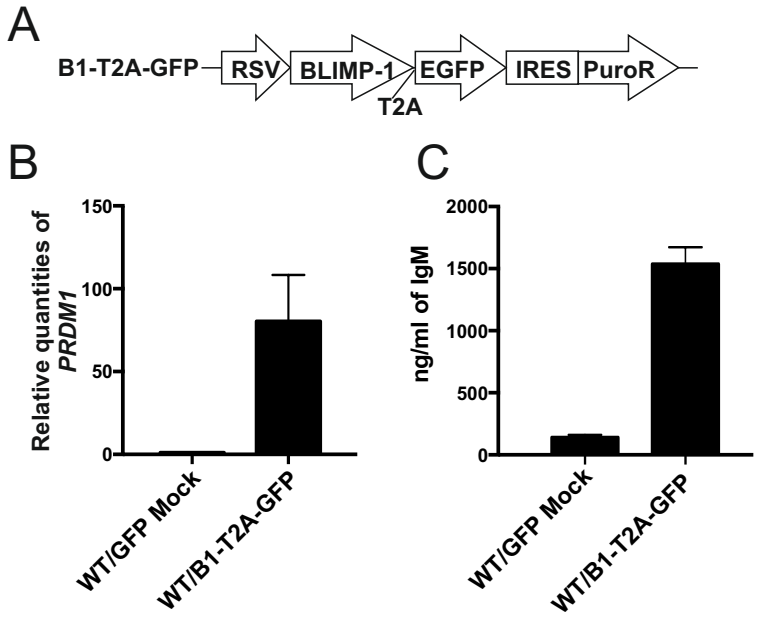

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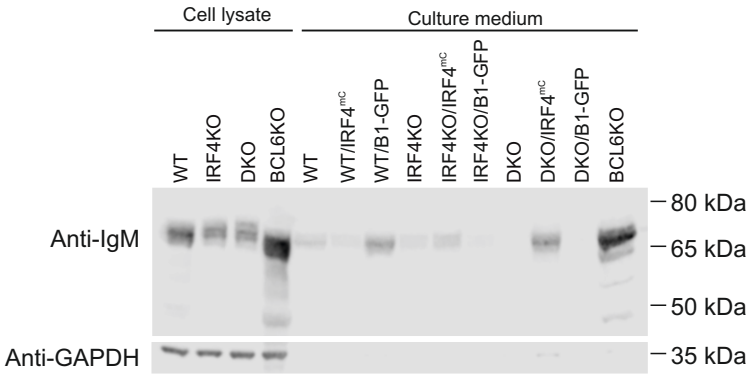
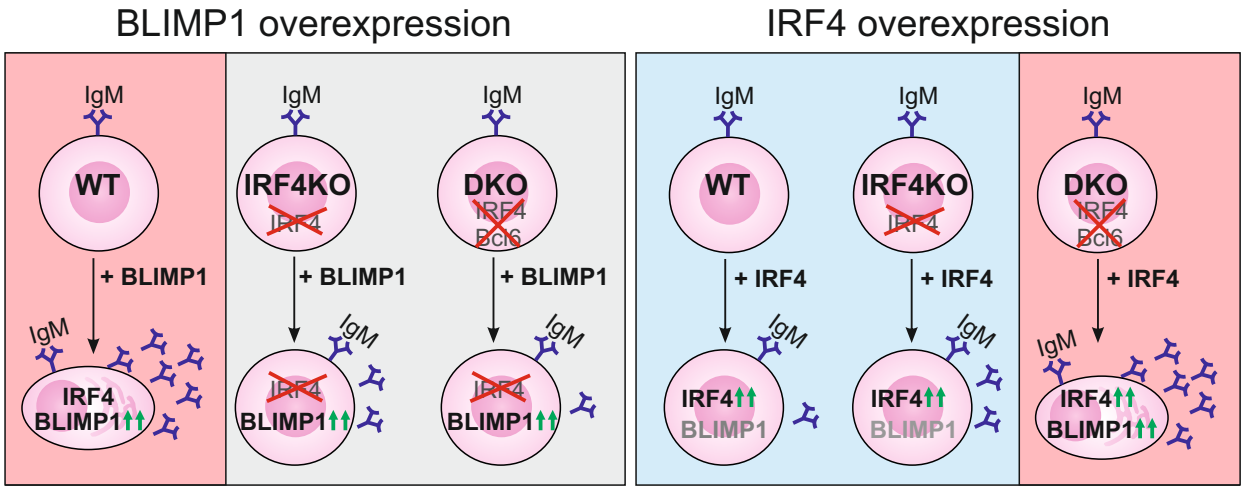


Supporting Information Figure 5



Supporting Information Figure 6