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ZBTB32 is an early repressor of the class II transactivator and MHC class II gene expression during B cell differentiation to plasma cells[1]

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Abstract

The MHC class II transactivator (CIITA) and MHC class II expression is silenced during the differentiation of B cells to plasma cells. When B cell differentiation is carried out ex vivo, CIITA silencing occurs rapidly but the factors contributing to this event are not known. ZBTB32, also known as repressor of GATA3, was identified as an early repressor of *CIITA* in an ex vivo plasma cell differentiation model. ZBTB32 activity occurred at a time when Blimp-1, the regulator of plasma cell fate and suppressor of *CIITA,* was minimally induced. Ectopic expression of ZBTB32 suppressed *CIITA* and *I-A* gene expression in B cells. ShRNA depletion of ZBTB32 in a plasma cell line resulted in reexpression of *CIITA* and *I-A*. Compared to conditional *Blimp-1* knock out and wild-type B cells, B cells from *ZBTB32/ROG*-knock out mice displayed delayed kinetics in silencing *CIITA* during ex vivo plasma cell differentiation. ZBTB32 was found to bind to the *CIITA* gene, suggesting that ZBTB32 directly regulates *CIITA*. Lastly, ZBTB32 and Blimp-1 coimmunoprecipitated, suggesting that the two repressors may ultimately function together to silence *CIITA* expression. These results introduce *ZBTB32* as a novel regulator of *MHC-II* gene expression and a potential regulatory partner of Blimp-1 in repressing gene expression.

Introduction

To initiate and regulate the adaptive and humoral immune responses, major histocompatibility complex class II (MHC-II) genes encode heterodimeric proteins that present antigenic peptides to CD4 T lymphocytes (1, 2). *MHC-II* genes are transcriptionally regulated by the MHC class II transactivator (CIITA). CIITA is expressed in a limited number of cell types, including dendritic cells, macrophages, and B lymphocytes (3, 4). CIITA functions as a transcriptional coactivator, connecting the DNA-binding transcription factors at *MHC-II* promoters with chromatin-modifying complexes and the RNA polymerase machinery (5–7). Depending on the species, the expression of *CIITA* is controlled by three or four tissue specific promoters, with promoter III principally used by B cells (8, 9). Following immune stimulation, activated B lymphocytes differentiate/mature into antibody-secreting plasma cells (10). As B cells transition to plasma cells, the

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expression of *CIITA* is silenced, resulting in the subsequent loss of MHC-II expression (11, 12).

Plasma cell differentiation requires extensive epigenetic reprogramming and morphological changes that allow them to secrete high levels of serum antibodies (13, 14). This process represents a critical event in the development and control of the humoral immune response. The B lymphocyte induced maturation protein-1 (Blimp-1) encoded by the *Pdrm1* gene is an important regulator of this transition. Ectopic expression of Blimp-1 in some B cell lines induces plasma cell differentiation (15, 16) and the silencing of *CIITA* and *MHC-II* gene expression (17). Moreover, B cells carrying a B cell specific, conditional knockout allele of Blimp-1 fail to form plasma cells, leading to a decrease of serum immunoglobulin levels even though B cell development to this point was normal (18, 19). Biochemically, Blimp-1 functions as a transcriptional repressor of gene activity (18, 20). However, Blimp-1 is only part of this cell fate decision. A complex network of activators and repressors controls B cell fate and development. For example, while B cell lymphoma-6 (BCL6) maintains the B cell program and represses *Blimp-1* expression, Blimp-1 represses the B cell program by silencing *BCL6*, *Pax5*, and *SpiB*, critical modulators of B cell fate (20, 21). Blimp-1 also serves to induce the plasma cell program by contributing to the regulation of *XBP-1* and *syndecan*-1 (22). While this seems complete, additional data suggest that other factors may act independent of Blimp-1 to initiate this differentiation program (23). With respect to *CIITA* silencing in plasma cells, Blimp-1 was found to bind directly to sequences upstream of three of the tissue specific promoters of the *CIITA* gene (promoters I, III, and IV), suggesting that it directly repressed *CIITA* expression (17, 24, 25). The molecular mechanism(s) by which Blimp-1 mediates repression is still not well defined. Blimp-1 was found to interact with histone deacetylases-1 (HDAC1) and -2 (HDAC2) in repressing the *c-Myc* gene during plasma cell differentiation (26). During dendritic cell maturation, Blimp-1 bound *CIITA* promoter I and recruited G9a and HDAC2, resulting in the acquisition of repressive histone marks (25). In other systems, Blimp-1 formed a complex with histone methyltransferases G9a (27) and PRMT5 (28), suggesting a role in modulating the remodeling of chromatin. However, the participating factors and the mechanism of Blimp-1 repression at *CIITA* promoter III in plasma cells are largely unknown.

ZBTB32 encodes a transcriptional repressor and member of the zinc finger, broad complex, tram track, bric-a-brac (BTB) protein family of proteins (29). ZBTB32, which is also known as repressor of GATA3 (ROG), PLZF-like zinc finger protein (PLZP), testis zinc finger protein (TZFP), and Fanconi anemia zinc finger (FAZF), was identified in multiple screens involving either immune cell tumorigenesis or development (30–32). In T cells, ZBTB32 activity inhibits the development of Th2 cells by interfering with the activity of GATA3, a factor required for Th2 development and *IL-4* expression (33, 34). ZBTB32 repressed *IL-13* expression through direct binding and recruitment of HDAC1 and 2 to deacetylate the locus (34). ZBTB32 also plays a role in T lymphocyte homeostasis (31, 34), but a function for ZBTB32 in terminal differentiation events, such as B cell to plasma cell has not been previously described.

While Blimp-1 is highly expressed in plasma cells, it is not fully expressed at early time points in vivo or in ex vivo differentiation models when *CIITA* is repressed and the *CIITA* locus has lost the binding of positive regulators of transcription and its active chromatin marks (23, 35). This suggests that other factors may initiate or contribute to *CIITA* silencing. To examine early events, cDNA microarrays were used to analyze the gene expression profiles of ex vivo differentiated murine B cells; a set of transcriptional and chromatin regulators during this transition was identified. Among these, *ZBTB32* was highly induced and further analysis demonstrated that ZBTB32 could directly suppress *CIITA* gene expression. In comparison to wild-type and Blimp-1-deficient B cells, B cells

from *ZBTB32* knock out animals displayed delayed down regulation of *CIITA* gene expression, suggesting a role for ZBTB32 early in the differentiation process. The results suggest that ZBTB32 may function by direct binding of ZBTB32 to the proximal promoter region of *CIITA* and by formation of a complex with Blimp-1. Thus, these data describe ZBTB32 as a novel repressor of CIITA gene expression and suggest a role for *ZBTB32* in the regulation of *CIITA* and the silencing of *MHC-II* gene expression during the formation of plasma cells.

Materials and Methods

Mice, cells, and cell culture

C57BL/6 and Balb/c mice were purchased from the Jackson Laboratory. *ZBTB32/ROG* mice were described previously (36) and were generously provided by Dr. I. Ho-Cheng (Harvard University) through Dr. Jianlin Gang (Boston University). *Blimp-1fl/fl, CD19*-Cre transgenic mice (19) were generously provided by Dr. Kathryn Calame (Columbia University) through Dr. S. Speck (Emory University). Double knock out mice were generated by crossing and backcrossing the ZBTB32 and Blimp-1/CD19-Cre mice and monitoring for the respective alleles. Mice were housed in the Emory University School of Medicine Facilities. All animal protocols used were approved by the Emory University Institutional Animal Care and Use Committee.

CW13.20-3B3 (CRL-1669) referred to here as BCL1 cells, Raji (CCL-86), P3X63Ag8 (CRL-1580), and NCI-H929 (CRL-9068) cell lines were purchased from the American Type Tissue Collection. Murine BCL1 cells, P3X63Ag8 plasma cells, and murine splenic B cells were grown in RPMI 1640 medium (Mediatech, Inc.) supplemented with 10% heat inactivated FBS (HyClone Laboratories), 10 mM HEPES (HyClone Laboratories), 1 mM sodium pyruvate (HyClone Laboratories), 1X non-essential amino acids (HyClone Laboratories), and 0.05 mM β-mercaptoethanol (Sigma-Aldrich). Primary murine splenic B cells were purified from five-eight week old C57BL/6 mice using magnetic separation columns (MACS; Miltenyi Biotech, Inc.) as previously described (35). Ex vivo differentiation of B cells to plasma-like cells (35) was performed by incubating murine primary splenic B cells for the indicated time in the above culture media supplemented with LPS (20 μg/ml, Sigma-Aldrich), IL-2 (20 ng/ml, Sigma-Aldrich), and IL-5 (5 ng/ml, Sigma-Aldrich). Additionally, each 24 hours the culture media was supplemented with LPS (10 μg/ml), IL-2 (10 ng/ml), and IL-5 (2.5 ng/ml) for the duration of the time course. The Burkitt's lymphoma B cell line Raji was grown in RPMI 1640 medium supplemented with 5% FBS (HyClone Laboratories), 5% bovine calf serum (HyClone Laboratories). The plasmacytoma cell line H929 was cultured in RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate, and 0.05 mM β-mercaptoethanol. HEK293 and HEK293FT cells were cultured in DMEM with 10% heat inactivated FBS.

Primary plasmablast purification

To obtain primary plasmablasts from the spleen, Balb/c mice (6 per experiment) were infected by i.p. injection with 2×10^5 plaque forming units of Lymphocytic Choriomeningitis Virus (LCMV) Armstrong strain (37). Eight days after infection, mice were euthanized and their spleens harvested. After homogenization and RBC lysis with ACK buffer (Gibco, Inc.), splenocytes were suspended in Dulbecco's PBS (DPBS) with 2% FBS and 2 mM EDTA and stained with PE- or APC-labeled anti-mouse CD138, PerCP-labeled anti-mouse B220, and APC-or PE-labeled anti-mouse CD44. For flow cytometry, antibodies were purchased from BD Pharmingen, Inc. CD138-positive cells were enriched by magnetic bead positive selection using LS columns and anti-PE or anti APC-microbeads

(Miltenyi Biotec, Inc). $CD138^{hi}$ $CD44^{hi}$ cells were then sorted by flow cytometry. Post sort purity was ~90–95% and approximately 5–10 million sorted cells were obtained per experiment. To assess the antibody secreting plasmablasts, ELISPOT assays, performed essentially as described (37) were used. Multiscreen HA plates (Millipore Corporation) were coated with goat anti-mouse IgG/IgA/IgM capture antibody (0.25 μg/well) (Caltag) and blocked with 10% FCS in PBS. Three-fold serial dilutions of sorted plasma cells were seeded into each well in growth medium (RPMI 1640 with 10% FCS, 10 mM HEPES, 5.5 μM β-mercaptoethanol, and 100 U/ml penicillin-streptomycin). Plates were incubated 6–8 hours at 37°C in a 5% CO_2 atmosphere. After washing, foci of antibody secretion were detected with anti IgG-biotin or anti IgM-biotin (Southern Biotech) and Avidin-HRP (Vector Laboratories), using chromogen 3-amino-9-ethylcarbazole (AEC) substrate (Sigma Aldrich). Spots were counted manually and divided by the number of cells seeded to determine the proportion of antibody-secreting cells in the sorted population.

Flow Cytometry

Flow cytometry was performed following standard protocols using a FACSCalibur. Briefly, 1×10^6 control or LPS, IL-2, IL-5 stimulated primary B cells were stained with either FITC or PE conjugated I-A/E and PE conjugated CD138 (syndecan-1) antibodies (BD Pharmingen, Inc.). For assessing splenic B cell populations 1×10^6 total splenocytes were stained with the following fluorophore-antibody conjugations: FITC-CD19, PE-I-A, APC-B220, Cy5.5PerCP-IgM, and V450-CD5 (BD Pharmingen, Inc.). Cells were washed with PBS containing 0.1% BSA, incubated for 30 min on ice and washed again in PBS containing 0.1% BSA. Flow cytometry data was analyzed using CellQuest software. All experiments were performed at least three times from independently isolated and purified cells.

cDNA microarray analysis

RNA was prepared from purified murine primary B cells that were either untreated or treated as described above with IL-2, IL-5, and LPS for 24 hours. RNA was submitted to the Emory Winship Cancer Institute Cancer Genomics core facility for whole-genome expression profiling using Applied Biosystems Mouse Genome Survey Microarray chips. Single color microarray technology was used to measure the absolute level of RNA. Two biological replicate experiments were performed. The data were analyzed using SAM (significant analysis of microarray) with a 2-fold cutoff and false discovery rate (FDR) of <0.05 (38). Genes with 2-fold or more change in expression are presented in Supplemental Table 2. The full dataset has been deposited into NCBI GEO database (http://www.ncbi.nlm.nih.gov/gds), accession number GSE17999.

RNA isolation and RT-real time PCR

Total RNA was isolated using RNeasy mini prep kit (Qiagen, Inc.). 2 μg RNA was used to generate cDNA with reverse transcriptase (Invitrogen, Inc.) and random hexamers and oligo dT (Applied Biosystem, Inc.). Approximately $1/100^{th}$ of the reverse transcription generated cDNA was analyzed by real time PCR using an iCycler (Bio-Rad, Inc.) to measure SYBR green incorporation of the PCR product. Primers used in RT-real time PCR are provided in Supplemental Table 1. 18S rRNA measurements by real-time PCR were used to normalize between samples. Normalized data from at least three independent cell preparations were averaged and plotted as fold over control treated samples. Student's t-tests were used to determine if significant differences between control and experimental samples were observed. Standard error of the mean was used to represent experimental variation.

Plasmids and transfection

Expression plasmids used in transient transfection experiments purchased from OriGENE Technologies, Inc., included those for *CBX2* (MC204062), *HMGN3* (SC110044), *Rad54l* (MC205395), *SATB1* (MC200989), and *TfDP1* (SC127935). These vectors were transfected transiently into 2×10^6 log phase growing Raji cells using Amaxa Nucleofection Kit V following the manufacturer's protocol. Myc-tagged *ZBTB32* and Flag-tagged *Blimp-1* cDNAs were cloned into the pcDNA 3.1(−) expression vector directly from PCR amplified cDNA from P3X63Ag8 cells. Flag-tagged GFP was cloned from the pEGFP expression construct. 1×10^7 log phase growing Raji cells were electroporated as described previously (39) with 50 μg of the indicated expression vector. HEK293 cells were transfected with Lipofectamine-2000 (Invitrogen Corporation) in 10 cm dishes with 10 μg of plasmid DNA encoding Myc-*ZBTB32* or Flag-*Blimp-1*.

Lentivirus generation and infection

Flag-tagged *ZBTB32* cDNA was cloned into the YFP expressing lentivirus vector (pHR-UBQ-IRES-eYFP). These lentivirus constructs were co-transfected with the pseudo envelope protein VSV-G gene into HEK293FT packaging cells using FuGENE 6 (Roche), according to the manufacturer's protocols. 48 and 72 hours post transfection, virus was harvested, and infected into purified primary murine B cells in the presence of 8 μg/ml polybrene (Sigma Aldrich, Inc.). Infection efficiency was determined by YFP expression and western blot using Flag antibody. Lentivirus constructs (pLKO.1) harboring shRNA to specific *ZBTB32* (cat. RMM4534-NM_021397; clone number, TRCN0000096484, TRCN0000096485, TRCN0000096486, TRCN0000096487, TRCN0000096488) were purchased from Open Biosystems (now, Thermo Scientific Inc.). For primary B cell experiments, five different lentivirus that express shRNA specific to *ZBTB32* were infected into purified murine primary B cells. Cells were then treated as above with LPS, IL-2, IL-5 to induce plasma cell differentiation for five days. To deplete *ZBTB32* in the plasma cell line P3X63Ag8, lentivirus expressing the indicated shRNA to *ZBTB32* was used, and cells were selected on puromycin (encoded in pLKO.1) for 6 days, prior to analysis.

Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously (35, 40). 1×10^7 P3X63Ag8 or A20 were crosslinked with 1% formaldehyde for 15 min in culture media. Chromatin was isolated and sonicated to generate average 600 bp size of DNA. Chromatin (30 μg) was pre-cleared, and immunoprecipitated with the indicated antibodies overnight at 4°C. DNA was purified from the reverse crosslinked chromatin and 1/10 of each sample was analyzed by quantitative real time PCR using SYBR green incorporation for the *CIITA* DNA sequence of interest. Primers for *CIITA* promoter III, and a control region are provided in Supplemental Table 1. Quantitation for each real-time PCR assay was carried out by comparison to a standard curve generated with genomic DNA. The average of at least three experiments was normalized to an irrelevant antibody control for the locus and plotted with respect to the amount of input chromatin. Anti-ZBTB32 (sc-25358) and anti-PU.1 (sc-352) antibodies were purchased from Santa Cruz Biotech. The anti-Blimp-1 antiserum was developed in collaboration with Rockland Immunochemicals, Inc. This antiserum recognizes both murine and human Blimp-1 (data not shown). Non-immune control IgG antiserum was purchased from Millipore, Inc.

Coimmunoprecipitation and immunoblotting

Myc-*ZBTB32* and Flag-*Blimp-1* expression vectors were transiently transfected into HEK293 cells for 48 hours. Nuclear lysates were prepared as described previously (41). Antibodies were pre-incubated with magnetic beads (Invitrogen Corporation) overnight at

4°C. 500 μg nuclear extract was immunoprecipitated with antibody pre-bound beads overnight at 4°C. After incubation, the beads were washed in 50 mM Tris (pH 8.0), 100 mM NaCl, and 0.05% NP-40, and eluted with SDS-PAGE loading buffer. Immunoblotting using the indicated antisera was carried out under standard conditions. For H929 cell immunoprecipitation experiments, 250 μg of nuclear extracts (250 μg) were incubated with specific antibodies for 1 hour on ice followed by the addition of 40 μl protein A sepharose and an additional 3 hour incubation at 4°C. Following incubation, the beads were washed in 50 mM Tris (pH. 8), 150 mM NaCl, and 1% NP-40. Precipitates were separated by SDS-PAGE and immunoblotting was conducted with the indicated antibodies. Antibodies used in these experiments include: monoclonal anti-GST (sc-138, Santa Cruz Biotech), anti-Myc (16-213, Millipore, Inc.), anti-Flag (F4042, Sigma-Aldrich, Inc.), anti-ZBTB32 and anti-Blimp-1 as described above, anti-RFX5 (42), secondary anti-mouse IgG (A6782, Sigma-Aldrich, Inc.), and anti-rabbit IgG (A0545, Sigma-Aldrich, Inc.).

Listeria monocytogenes and influenza infection

Listeria monocytogenes strain 10403S, grown in Difco Brain Heart Infusion (BHI, BD Biosciences) to an OD of about 0.2 was concentrated and 9.8×10^6 colony forming units were used to infect wild-type C57Bl/6 and ZBTB32ko mice. Cohorts of wild type and ZBTB32ko mice were also infected with $0.1xLD_{50}$ A/PR/8/34 mouse adapted virus. Sera were individually collected on the indicated days, and anti-influenza specific antibody levels were determined quantitatively by hemagglutination inhibition (HAI) assays as described previously (43). Briefly, maxi-sorb Nunc 96-well plates (Southern Biotechnology Associates) were coated with 4 μg/ml inactivated A/PR/8/34 egg grown inactivated virus and sera incubated with 4 HA units/50 μl of A/PR/8/34 virus for 30 min. Infected mice were monitored daily for signs of morbidity (body weight changes, fever and hunched posture) and mortality.

LPS injection

Mice were injected retro-orbitally with 50 μg LPS (Enzo Life Sciences) diluted into 100 μl PBS. Three days post-injection splenocytes were harvested, red blood cells lysed in ACK lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1M EDTA, pH 7.2), and total splenocytes stained with PE conjugated anti-CD138 and APC conjugated anti-B220 (BD Biosciences) for 30 minutes on ice. Samples were washed once in cold PBS and resuspended in cold PBS containing 0.1% BSA. Flow cytometry data were analyzed with CellQuest software.

Results

A large number of transcription factors and chromatin remodeling proteins are differentially regulated during ex vivo B cell differentiation

Ex vivo differentiation of murine B cells to plasma cells using LPS, IL-2, and IL-5 (35) was used as a model system to dissect the mechanisms that silence *CIITA* and ultimately MHC-II gene expression. This model results in the silencing of *CIITA* and MHC-II, as well as the acquisition of canonical plasma cell markers, such as syndecan-I (CD138) (Figure 1A and (35)). While plasma cell phenotypic changes develop over at least a five day period using this protocol, a significant reduction in the steady state levels of *CIITA* mRNA was observed within the first 24 hours of culture (35). At this early time point, the expression of Blimp-1, a transcriptional repressor of *CIITA* (17) and master regulator of plasma cell fate (20), is not fully expressed (Figure 1B), suggesting that other factors may also play a role in *CIITA* silencing. To identify potential candidate genes that were differentially expressed between B cells and plasma cells at this 24-hour time point the above ex vivo differentiation model was employed. Freshly isolated and purified $CD43^-$ splenic B cells were cultured in media or with LPS, IL-2, and IL-5 for 24 hours, and RNA transcripts were compared using

cDNA microarrays. Of the 32,970 murine targets queried, 2,370 genes were differentially expressed by more than 2 fold (FDR<0.05) with 1,169 up regulated and 1,201 down modulated in the 24 h treated cells (Figure 1C, Supplemental Table 2). A heat map of the 2,370 modulated genes, sorted by fold change, from two biological replicates showed clear segregation of the modulated genes (Figure 1C). In addition to the *MHC-II* genes, some of the many highly differentially expressed genes were transcription factors or modifiers of chromatin structure (Table 1). For example, key B cell transcription factors, such as *BCL6, EBF1, CD19,* and *SpiC* were significantly repressed during the differentiation process. Enhancer of zest 2 (*EZH2*) and chromobox homology 2 (*CBX2*), genes that encode members of the chromatin remodeling polycomb group (PcG) repressor complexes PRC2 and PRC1, respectively, were induced, whereas *EZH1*, a component of the PRC2 complex was repressed. The expression of other chromatin assembly/remodeling factor genes, *Chalf, TfDP1, HMGN3,* and *Smyd2* were also increased. Additionally, *ZBTB32*, which encodes a zinc finger repressor protein previously characterized in the regulation of T helper cell differentiation as a repressor of GATA3, was increased 102-fold during the differentiation process and was the most significantly induced transcript. These results raised the possibility that the above genes may function to regulate *CIITA* expression and/or B cell differentiation.

B cell specific genes show diverse kinetics of regulation

To verify the cDNA microarray data and to provide a kinetic view of the expression of some of the differentially regulated genes, real-time RT-PCR was carried out on 6 repressed genes and 6 induced genes over a 5 day period following treatment with IL-2, IL-5, and LPS. For the repressed genes, two overall kinetic profiles were observed. Profile one showed a near continual decline in gene expression over the time course (Figure 1B). *BCL6*, *SpiC,* and *CIITA* fell into this group. Profile two transcripts (*EBF1, PAX5,* and *SpiB*) were initially induced up to a few fold before being repressed around day 2. Differences in these initial expression profiles from the 24 h microarray results may reflect the overall sensitivity of the cDNA microarrays. Two profiles were also observed for the induced genes. The profile exhibited by *Blimp-1,* and *XBP-1* showed an increase and stabilization of mRNA expression over the time course (Figure 1B). The other induced gene profile, which included *ZBTB32*, *Tfdp1*, *Smyd2*, and *EZH2* displayed a transient, high burst of induction over the first 2 days and a slow decline in expression over the rest of the time course with the final levels still significantly above the starting steady state levels in resting B cells.

To determine whether a similar set of genes were differentially regulated in primary plasma cells, plasmablasts ($CD138^+CD44^+$) were purified from the spleens of mice eight days following LCMV infection (Figure 2A), and the RNA profiles of 18 genes were analyzed. Enrichment of plasmablasts led to a population of predominantly IgG secreting cells that were 94% $CD138^+CD44^+$ (Figure 2A and 2B). Although there were differences in magnitude, the induction/repression of genes was similar to that observed in the ex vivo system (Figures 1B and 2C). Induction of *Blimp-1*, *XBP-1*, and *syndecan-1* was more pronounced. The steady-state level of *ZBTB32* mRNA, at 13-fold over naive B cells, was less than the peak but similar to the day five levels observed in the ex vivo system. As expected (44), *CIITA*, *I-Ab*, *CD19,* and *c-Myc* genes were repressed in the plasmablasts (Figure 2C). Overall, these results verify the ex vivo cDNA microarray data obtained in the in vivo model and provide a series of candidates that may regulate *CIITA* and potentially other genes involved at the early stages of B cell differentiation.

ZBTB32 regulates *CIITA* and *MHC-II* gene expression

To determine if some of the induced plasma cell transcription factors could repress *CIITA* expression, cDNA expression vectors encoding seven of the genes were obtained and

transiently transfected into the *MHC-II* and *CIITA* expressing human B lymphoblast cell-line Raji (Figure 3A). Selection of this set of candidates was based on the overall induction strength and known function such that transcriptional repressors and chromatin assembly/remodeling proteins were chosen. The DNA binding protein *Satb1* was repressed following B cell differentiation and was chosen as a control gene that was not expected to repress *CIITA* expression. The transient transfection efficiency by nucleofection of Raji cells ranged between 35 and 60% (unpublished data). Of the seven genes transfected, only three had a statistically significant effect on the repression of *CIITA*. Ectopic expression of CBX2 and EZH2 in Raji cells, which are members of the polycomb PRC1 and PRC2 complexes, respectively, resulted in small but statistically significant decreases in *CIITA* expression. By contrast, ectopic expression of ZBTB32 resulted in a 65% reduction in the steady state mRNA levels of *CIITA*. Thus, ZBTB32 could potentially represent a novel regulator of *CIITA* expression, and as described below was further characterized. Because the available vectors did not contain an epitope tag, the expression of the transfected genes in this system could not be easily examined. Thus, this assay could not rule out a potential role for candidate genes that displayed no effect on *CIITA* expression.

The repressive ability of ZBTB32 was compared to the known regulator of *CIITA*, Blimp-1. Epitope tagged vectors expressing Flag-GFP, Myc-ZBTB32, or Flag-Blimp-1 were electroporated into Raji cells and ability of each construct to regulate *CIITA*, *HLA-DRA*, *BCL6*, and a control gene *MTA3* were measured 72 hours later (Figure 3B). As expected, due to a reduction in *CIITA*, *HLA-DRA* mRNA expression was also significantly reduced by ZBTB32 compared to a control GFP electroporation. GFP vector control transfection did not affect the level of *HLA-DRA* mRNA. Importantly, *MTA3* and *BCL6* mRNA expression levels were not affected, suggesting that ZBTB32's role as a repressor was specific to *CIITA*. On its own, Blimp-1 significantly repressed *CIITA* levels but this repression was very modest. As such, *HLA-DRA* mRNA levels were not significantly affected. Consistent with previous data (20), Blimp-1 repressed *BCL6* levels but not a control gene *MTA3*. Western blotting confirmed that each construct was expressed to significant levels (Figure 3C).

To determine if ZBTB32 could repress *CIITA* in primary murine B cells, a YFP lentiviral expression vector co-expressing *ZBTB32* was used to infect freshly purified murine B cells. The levels of *CIITA* mRNA were assessed after 4 days. In these cultures a low level of LPS (5 μg/ml) was provided to maintain cell viability over the course of the experiment. The level of *CIITA* mRNA in the YFP-lentiviral control cultures was similar to uninfected cultures (data not shown); whereas, the ectopic expression of ZBTB32 in primary murine B cells repressed *CIITA* expression (Figure 3D). These results indicate that ZBTB32 can repress *CIITA* expression.

ZBTB32-depleted plasma cells reactivate expression of *CIITA* and *I-A*

The murine plasmacytoma cell line P3X63Ag8 represent a fully differentiated murine plasma cell and express *ZBTB32.* To determine the effects of depletion of ZBTB32 in plasma cells, P3X63Ag8 was infected with lentiviral vectors expressing a series of ZBTB32 shRNAs and the effect on expression of *CIITA*, *I-A*, and other B and plasma cell-specific genes were assessed. Three of the five-lentiviral shRNA vectors were able to reduce the ZBTB32 protein levels to varying degrees (Figure 4A). The levels of actin protein did not change with any of the shRNA vectors (Figure 4A). ZBTB32 shRNA vector 3 demonstrated the greatest effect on *CIITA* derepression, resulting in a ~2,000 fold increase (Figure 4B). The other vectors (2 and 4) showed a ~250 fold increase (Figure 4B). Consistent with *CIITA* expression, *I-Ab* expression was derepressed (70–300 fold) using the same vectors. The levels of *BCL6* mRNA were slightly induced at levels between 1.5 and 3-fold depending on the shRNA. While no significant difference was observed for *syndecan-1* or *XBP-1*

mRNAs, a 20–50% reduction in *Blimp-1* mRNA levels was observed, suggesting that ZBTB32 may affect the expression of other genes in this cell line required to maintain the plasma cell phenotype.

Because *ZBTB32* depletion in P3X63Ag8 cells resulted in the derepression of *CIITA* and *I-Ab* mRNA, it was of interest to determine if *ZBTB32* depletion would also result in an increase in MHC-II surface expression. Flow cytometry of the P3X63Ag8 cells infected with a ZBTB32 shRNA-lentivirus were compared to that of control vector infected cells. Here, ~15% of the cells exhibited strong I-A surface expression (Figure 4C), indicating that this subpopulation of cells had been derepressed for *CIITA* and *MHC-II* expression. This small proportion of cells may reflect an epigenetic pathway that must be overcome to allow derepression of a terminally differentiated cell, such as that described with dedifferentiation of plasma cells transfected with vectors expressing *MTA3* and *Bcl6* (41).

ZBTB32-deficient B cells fail to efficiently repress *CIITA*

The ZBTB32/ROG knockout mouse (referred to here as ZBTB32ko) was created previously and contains a deletion spanning exons 2–6 of the *ZBTB32* gene (36). The ZBTB32ko mouse displays minor defects in Th2 cell development and homoeostasis (32, 33) but B cells appear to develop normally as they express wild-type levels of typical B cell phenotypic markers, such as B220, CD5, CD19, MHC-II, and IgM (Supplemental Figure 1A). B cell differentiation to plasma cells or the ability of these mice to repress *CIITA* transcription was not investigated in these mice previously. To determine if CIITA expression was altered in ZBTB32ko mice, splenic B cells from ZBTB32ko and wild-type mice were isolated and differentiated ex vivo in LPS, IL-2, IL-5 and mRNA levels of *CIITA* were examined by real-time RT-PCR. Here, a three-day time course was used that corresponded to the peak and early induction of *ZBTB32* mRNA. In wild-type cells, *ZBTB32* mRNA levels were increased ~50 fold and *CIITA* mRNA levels were reduced to ~30% of their B cell levels at 4 hours, the earliest time point examined (Figure 5a). As a point of reference, *Blimp-1* mRNA has not been induced at this time point in the wild-type differentiated cells (Figure 5g). *CIITA* mRNA levels continued to decrease during the time course, and this occurred in a manner that was concordant with the increase in *Blimp-1* mRNA levels after 48 hours. Comparison of the kinetics of ZBTB32ko and wild-type B cells in this differentiation protocol showed that the ZBTB32ko cells were significantly delayed in their ability to silence *CIITA* (Figure 5a), indicating that ZBTB32 plays a role early in the process.

To examine and compare the role of *Blimp-1* to *ZBTB32*, the CD19-Cre, Blimp-1 conditional knock out mouse (Blimp-1cko), which deletes *Blimp-1* from 85–90% of B cells (19), was investigated in this ex vivo system. Additionally, the influence of both genes in this system was assessed using a ZBTB32ko/Blimp-1cko double knock out (dko) mouse line. Ex vivo differentiation of Blimp-1cko B cells resulted in the repression of *CIITA* mRNA through the first 24 hours that mirrored wild-type kinetics (Figure 5b), suggesting that Blimp-1 does not play a role in the early suppression of *CIITA*. At 48 hours, *CIITA* transcript levels increased slightly, suggesting that repression was not fully maintained in the absence of Blimp-1. Ex vivo differentiation of B cells from the dko mouse displayed a delayed decrease in *CIITA* mRNA that paralleled ZBTB32ko cells for the first 24 hours; and similar to the Blimp-1cko, *CIITA* mRNA levels increased after 48 hours (Figure 5c). These data suggest that ZBTB32 initially functions to repress *CIITA* and that Blimp-1 is required to maintain the repression. Analysis of *ZBTB32* and *Blimp-1* mRNAs over the same time course verified each genetic deletion (Figures 5d-i). In the Blimp-1cko, slight increases in *Blimp-1* mRNA were observed at the 48 and 72 hour time points (Figures 5h and i), confirming previous observations about the efficiency of the CD19-Cre system (19). *Blimp-1* deficient B cells were found to express less *ZBTB*32 mRNA than wild-type cells

(Figure 5e); however, this level was ~25-fold above resting B cells and was likely sufficient to repress *CIITA* at early time points.

ZBTB32 directly binds *CIITA* promoter III

The above data suggest that ZBTB32 plays an important role in *CIITA* silencing during plasma cell differentiation, but do not distinguish between a direct or indirect role, nor do the experiments provide mechanistic insight into how ZBTB32 may function. ZBTB32 contains a *Krüppel*-type zinc finger DNA binding domain and was previously reported to bind to the *Aurora kinase C* (*Aie1*) promoter (45) and *IL-13* gene (34), suggesting that it may also bind to the *CIITA* gene and directly silence expression. *CIITA* is expressed from three promoters in mouse cells, with promoter III (pIII) being principally used in B cells (4, 8, 46). A sequence similar to the ZBTB32 binding site in the *Aie1* gene was identified and lies within 50 bp of a known Blimp-1 binding site (17) upstream of *CIITA* pIII. Chromatin immunoprecipitation (ChIP) assays were performed in P3X63Ag8 plasma cells, which express high levels of ZBTB32 and amplicons were designed around the predicted binding site at pIII. A20 B cells which do not express ZBTB32 and express high levels of *CIITA* were used as a control cell line. ZBTB32 was found to bind specifically to *CIITA* pIII but not a control amplicon from the *CIITA* locus or in A20 cells (Figure 6A). Blimp-1 binding was also tested and found to bind to the same amplicon, in agreement with previous reports (17, 47). The known B cell activator PU.1 was found to bind a site upstream of pIII in A20 but not P3X cells in agreement with previous reports (48), providing a positive control for these chromatin lyates. Control IgG antisera did not precipitate either of the specific or non-specific DNAs. These data support the hypothesis that ZBTB32 directly binds and represses the *CIITA* gene.

ZBTB32 associates with Blimp-1

The binding of both ZBTB32 and Blimp-1 to the same pIII upstream promoter region in plasma cells suggested the possibility that the two proteins may interact. To determine if that may be the case, Flag-tagged Blimp-1 was co-expressed with Myc-tagged ZBTB32 in HEK293 cells and the ability of the proteins to associate was tested by immunoprecipitation. Anti-Flag and anti-Myc antibodies were each able to coimmunoprecipitate both Flag-Blimp-1 and Myc-ZBTB32 proteins (Figure 6B). A control antibody to GST failed to immunoprecipitate either protein (Figure 6B). A similar set of immunoprecipitation assays was carried out on the endogenous proteins using human H929 plasma cells. The results showed that ZBTB32 immunoprecipitated with Blimp-1 but not a control protein RFX5 (Figure 6C). Control antisera did not pull down either protein. These results therefore suggest that ZBTB32 can interact with Blimp-1 and that these two proteins may function together or in a complex to silence gene expression.

Discussion

The transition from B cell to plasma cell represents a critical fate decision leading to effective humoral immune responses. The silencing of *CIITA* and *MHC-II* gene expression effectively removes the ability of these cells to receive TCR-MHC-II mediated signals. During this transition, transcriptionally active chromatin marks are removed and replaced by repressive chromatin modifications (35). Blimp-1 was identified previously as a key regulator of this transition and as a repressor of *CIITA* expression (15, 17, 49). However, using an ex vivo differentiation model to mimic early events, it was observed that silencing of *CIITA* begins at a time before Blimp-1 reached a high level of expression. While it could be that the *CIITA* gene is sensitive to very low levels of Blimp-1 expression, this inconsistency prompted a search for factors expressed early in this transition that could

mediate *CIITA* silencing. As shown, ZBTB32 was identified as such a factor and had the highest level of induction in this system.

An ex vivo system to globally stimulate and differentiate naïve B cells was chosen such that gene expression and biochemical analyses could be achieved during early time points in the process. Intriguingly, the cDNA microarrays identified 2,370 genes whose expression had already changed at 24 hours, with several genes showing log differences in their expression patterns. Verification of the microarray results using primary plasmablasts isolated 8 days after virus infection, verified that the ex vivo system mimicked a plasma cell fate differentiation process. Previously, a number of gene expression profile comparisons between B cells and in vivo generated plasma cells were performed to identify genes associated with this genetic program (50, 51). Although the data generated here were at an earlier time point during the differentiation process, a number of hallmark genes were differentially regulated in both studies, including the up regulation of cell cycle/proliferation associated genes and *IL6, IRF4*, *IRF6*, *TfDP1.* Likewise, as in this study *Pax5*, *CD19*, *BCL6*, *SpiB* and others were among some of the key down modulated genes observed previously. Many of these genes were found previously to be direct targets of Blimp-1, including *Pax5*, *CIITA*, and *SpiB* (20). Surprisingly, *ZBTB32* was not identified in either of the two previous B cell/plasma cell microarray screens, perhaps because those studies were performed at later time points in the differentiation of plasma cells (>6 days), a significant time after the peak of ZBTB32 expression.

ZBTB32 is a member of the BTB containing zinc finger protein family that recruits co-repressors, such as N-CoR and HDACs to its target genes (29, 34). ZBTB32 over expression and shRNA analysis revealed that *ZBTB32* expression was able to repress *CIITA* and suggested that it could play a role in this differentiation process. Using homology searches from the *Aie1* (45) gene, a potential BTB-ZF family member binding site upstream of promoter III of the *CIITA* gene was identified that was in close proximity to the Blimp-1 binding site (17). A positive ChIP assay result for ZBTB32 at pIII provided evidence for direct binding of ZBTB32 to the upstream promoter region of the *CIITA* gene.

Early kinetic analysis from wild-type mice suggest a model for the regulation of *CIITA* during plasma cell differentiation in which *ZBTB32* is induced and functions during the very early stages of the ex vivo plasma cell differentiation system, when *CIITA* silencing occurred within four hours. At this time point, *Blimp-1* mRNA was not detectable. Cells from ZBTB32ko animals showed delayed kinetics of *CIITA* silencing and were consistent with a model in which only Blimp-1 was controlling *CIITA* silencing. Likewise, at early time points, the cells from a Blimp-1cko mouse displayed kinetics more similar to the wild-type mouse as *ZBTB32* (although reduced in this background) was fully functional at this stage. At later time points, the levels of *CIITA* were derepressed in the Blimp-1cko, suggesting a role for Blimp-1 in maintenance or establishing a fully repressed state. These data are consistent with Blimp-1 being required for establishment of the plasma cell fate. *CIITA* silencing in the dko followed a combined pattern of the two single knockouts with delayed silencing kinetics and a late derepression of *CIITA*. The combined pattern was likely due to the loss of function of ZBTB32 and Blimp-1, respectively. It should be emphasized that in the absence of Blimp-1, plasma cells do not form either in vivo or through the ex vivo differentiation protocol used here. Thus, at extended time points (after 24 h; i.e., when Blimp-1 is expressed), it is likely that the cell differentiation program in Blimp-1cko is altered and does not follow a normal path. As such, it was not possible to separate Blimp-1 and ZBTB32 using the genetic models. The silencing of CIITA by ZBTB32 early in the differentiation time course suggested that there could be a reduction in surface MHC-II expression. This was not observed (data not shown) and is likely due to the

stability of CIITA bound to MHC-II promoters (52), as well as the long half-life of MHC-II proteins on the cell surfaces (53).

At the *CIITA* locus, activation associated histone modifications are replaced with repressive modifications as cells transition from B cells to plasma cells (35). One set of repressive marks, H3K27me3, is produced by the polycomb complexes. Consistent with the appearance of H3K27me3 in plasma cells was the fact that components of the polycomb repressor complex EZH2 and CBX2 were identified in the microarray screen. In addition, when over expressed, CBX2 and EZH2 had small but significant repressive effects on *CIITA* expression. Consistent with this finding is that EZH2 has been shown to play a role in regulating *CIITA* in other systems (54, 55). Although not investigated here, the role of these proteins is likely pertinent to many of the genes that are repressed during this transition.

Kallies et al. (23) provided evidence that the plasma cell program was initiated through the loss of *Pax5*, and that this occurred at a time prior to Blimp-1 expression. They suggested that an unknown factor could be the mediator of this process. Whether ZBTB32 is this factor is not clear but raised the issue of the breadth of the role that *ZBTB32* may play in plasma cell differentiation and B cell mediated immune responses. To examine the role that ZBTB32 may have in ultimately augmenting humoral immune responses, ZBTB32ko mice were compared to wild-type mice in two infection models. In the first, ZBTB32ko were infected with a mouse adapted influenza strain, which elicits a strong humoral immune response in wild-type mice. No differences in mortality or morbidity were observed, indicating that these mice had a functional immune system (data not shown). ZBTB32ko mice developed wild-type levels of anti-influenza antibodies during a primary infection, indicating the presence of a T-dependent plasma cell response (Supplemental Figure 1B). ZBTB32ko mice were also infected with *Listeria monocytogenes* and also demonstrated no defect in mounting an effective immune response (Supplemental Figure 1C). In contrast, and as previously noted (19, 56, 57), Blimp-1 is required for T-independent plasmablast formation as ZBTB32ko but not Blimp-1cko mice injected with LPS formed wild-type numbers of $CD138^+$ splenic plasmablasts (Supplemental Figure 1D). These data argue that *ZBTB32* was not essential for in vivo B cell to plasma cell differentiation in either the T-dependent or independent model models tested.

Experiments in which shRNAs to ZBTB32 were used to reduce ZBTB32 in a plasmacytoma cell line had a pronounced effect on the mRNAs levels of CIITA and Ia, even though Blimp-1 was expressed. In addition, two of the three shRNAs to ZBTB32 resulted in reduced Blimp-1 expression and small increases in BCL-6 mRNA. One interpretation to these findings is that in these cells, which represent a fully differentiated plasma cell, ZBTB32 plays a role in maintaining the cell fate program. Alternations in that program would lead to increased BCL-6 and reduced Blimp-1.

It is intriguing that ZBTB32 was identified in at least three other systems (32, 33, 45). The complete role that ZBTB32 played in those systems was not elucidated fully. The infection model data presented here were consistent with the analyses of ZBTB32 in T cells, where the *ZBTB32/ROG* knockout was found to be associated with small changes in lymphocyte homeostasis (32) but no severe phenotype or essential function of ZBTB32 was uncovered (32, 33, 36, 45, 58). Thus, in the system queried, the function of ZBTB32 may be redundant in part to the major role that Blimp-1 plays in terminal differentiation of various lymphocytes (59–62) and perhaps other cell types. The finding of ZBTB32 as a gene that is induced early upon the plasma cell differentiation pathway may suggest that in some cases, this induction may be important but not necessary to begin plasma cell differentiation transition. Considering the stability of the CIITA enhanceosome complex (52) a rapid loss

of CIITA expression through ZBTB32 may allow for a more efficient loss of MHC-II during this process. Decreases and ultimately loss of MHC-II would prevent these cells from receiving further stimulation or help from antigen specific T cells and lock the cell fate pathway that these cells undertake. Irrespective of its other potential functions during terminal differentiation events in lymphocytes, the data reported here introduce ZBTB32 as a novel regulator of *CIITA* gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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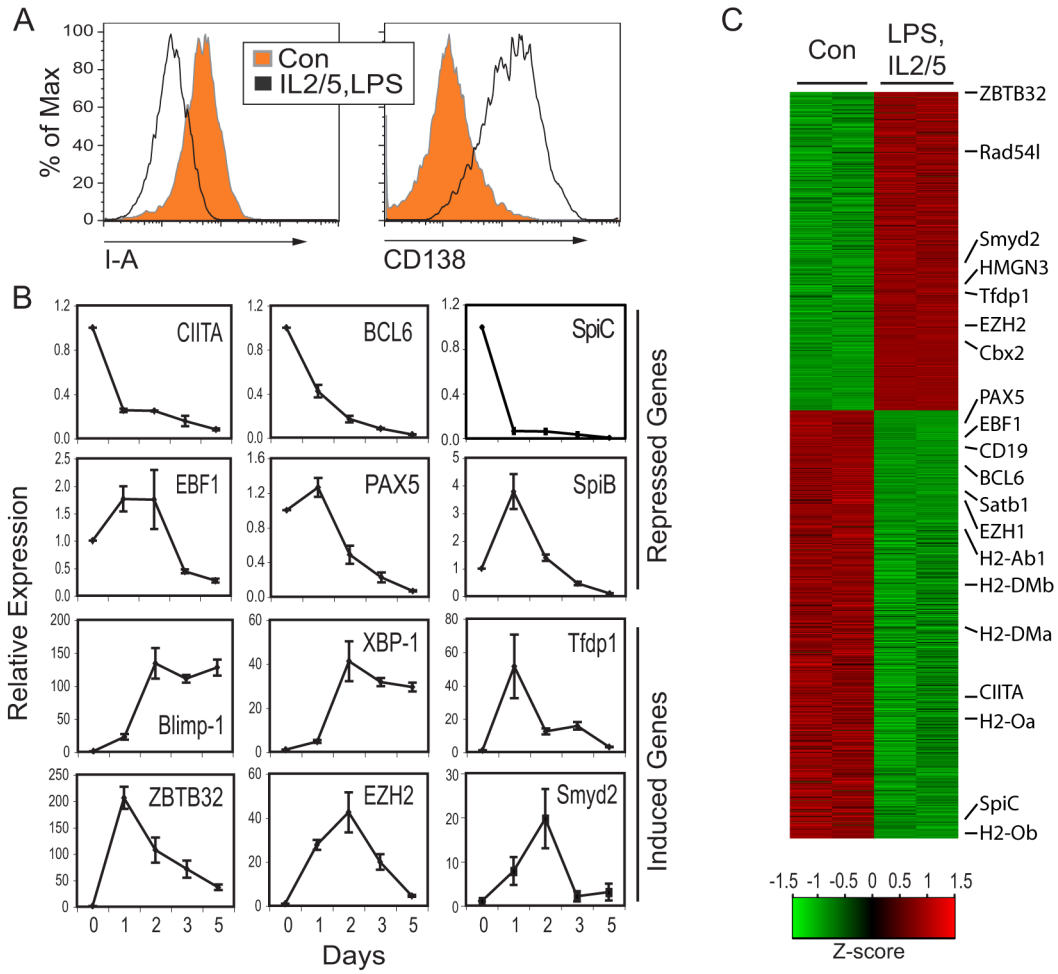

Figure 1. Ex vivo differentiation of murine B cells to plasma-like cells results in the induction and repression of 2,370 genes

Primary mouse B cells purified from splenocytes of C57BL/6 mice were either untreated (Con) or differentiated ex vivo using LPS, IL-2, and IL-5 for the various times as indicated. (A) Flow cytometry using FITC conjugated I-A and PE conjugated CD138 (syndecan-1) antibodies was carried out after 5 days of the above treatment to assess the loss of MHC-II surface expression and acquisition of a key plasma cell marker. Histograms are representative of three experiments with similar results. (B) Quantitative RT-PCR was used to determine the steady state mRNA levels of representative genes encoding transcription factors and chromatin remodeling proteins identified from the cDNA microarrays over a five-day time course of the ex vivo differentiation protocol. The data from three independent biological replicates were analyzed and normalized to the expression level of 18S rRNA and plotted with respect to the day 0 time point. Standard error of the mean was used to represent experimental variability. (C) To assess events that occurred early in the differentiation process, control and 24 hour treated cultures were examined by RNA profiling using cDNA microarrays (See Materials and Methods). A heat map, representing 2,370 z-score normalized genes displaying a change in expression greater than two-fold is presented was plotted with respect to fold change. The relative locations of significantly changed genes discussed in this manuscript are indicated. Two biological replicates are shown.

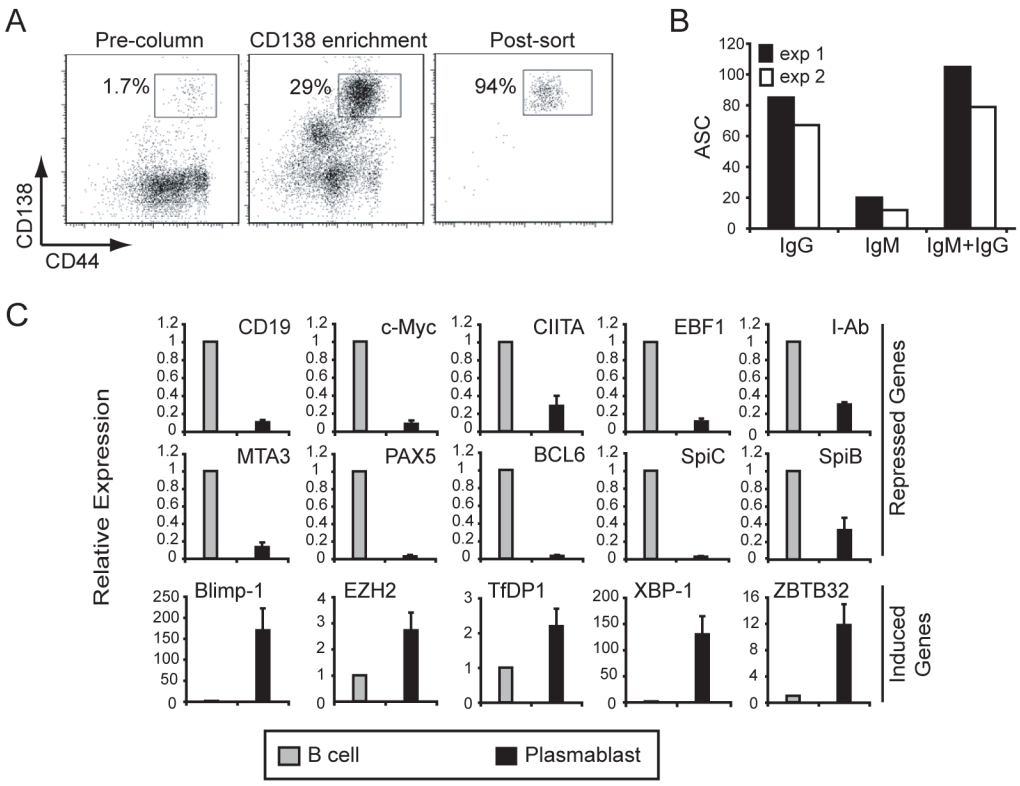

Figure 2. Primary murine plasmablasts repress B cell genes while inducing plasma cell genes

Primary murine splenic plasmablasts were collected and analyzed for expression of select transcription factors identified in the microarray screen above. (A) Eight days following infection with LCMV, splenocytes were isolated and plasmablasts were sorted based on their expression of high levels of CD138 and CD44. (B) Purified cells from two independent isolations were tested for antibody secretion by ELISPOT. Between 70% and 100% of the sorted cells represented antibody-secreting cells (ASC). (C) Steady state mRNA levels of representative genes identified in the microarray screen were analyzed by qRT-PCR. Samples were analyzed as above using three independent RNA samples and plotted relative to fold change over B cells. Standard error of the mean was used to represent experimental variability.

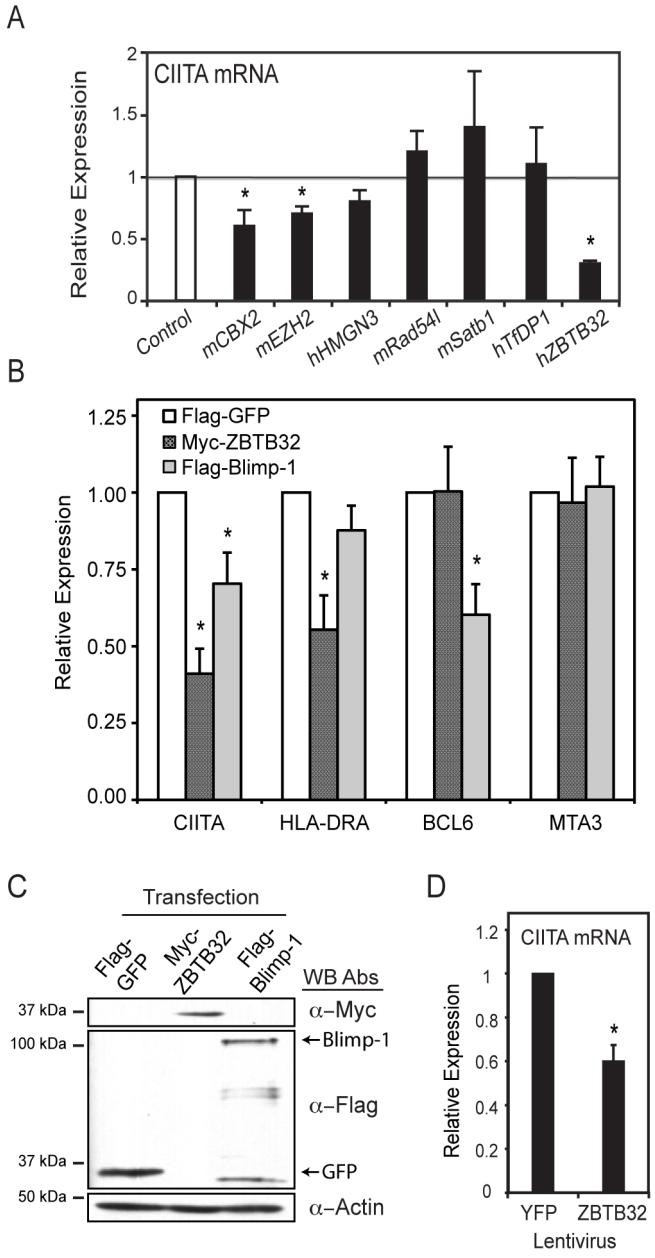

Figure 3. ZBTB32 and the polycomb complex proteins EZH2 and CBX2 repress *CIITA* expression

(A) cDNA expression vectors encoding seven transcription factors/chromatin assembly proteins identified from the cDNA microarrays were transiently expressed in the human CIITA positive B-cell line Raji. At 3 days post transfection, RNA was extracted and the level of *CIITA* mRNA was evaluated by qRT-PCR. The origin of the cDNAs is indicated by the prefix (m for mouse and h for human). The control lane represents a GFP expression vector. (B) The expression of *CIITA*, *HLA-DRA*, *BCL6,* and *MTA3* mRNA steady state levels was evaluated by qRT-PCR following ectopic expression of Flag-GFP, Myc-ZBTB32, or Flag-Blimp-1 in Raji cells. (C) Expression vectors containing Flag-GFP, Myc-ZBTB32, or Flag-Blimp-1 were electroporated into the human Raji B cell line. Expression levels of each tagged protein were determined by western blotting with anti-Flag and anti-

Myc antibodies. The actin western blot is provided as a loading control. The positions (arrows) of Blimp-1 and GFP are indicated in the middle anti-Flag epitope blot. The positions of relevant molecular weight standards are shown. (D) Purified primary murine B cell cultures were infected with a lentivirus vector expressing either YFP or ZBTB32. RNA was prepared and analyzed for *CIITA* mRNA levels as above. Data from three independent experiments were averaged, normalized to the expression of 18S rRNA, and presented with respect to the levels of a control gene, which was arbitrarily set at 1. Asterisks indicate differences between control and experiments that show significant differences with p values < 0.05 as determined by the Student's t-test. Standard error of the mean was used to represent experimental variability.

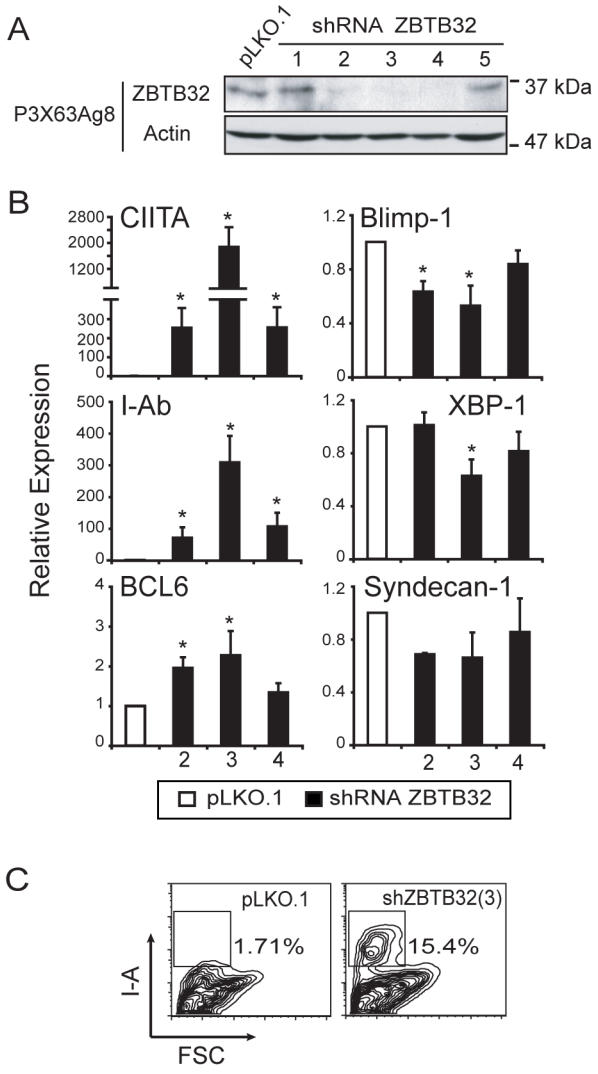

Figure 4. ZBTB32 is required for *CIITA* silencing in a plasma cell line

(A) Lentiviruses containing five different shRNAs specific to *ZBTB32* were produced and used to infect the murine plasma cell line P3X63Ag8. The efficiency of ZBTB32 knock down compared to the control empty vector pLKO.1 was evaluated by western blot. The actin loading control showed that none of the shRNAs affect expression of the actin gene. Lentivirus with shRNAs 2, 3, and 4 reduced ZBTB32 expression; whereas constructs 1 and 5 did not. (B) qRT-PCR was performed on RNA isolated 6 days after infection of P3X63Ag8 cells with lentivirus expressing shRNAs 2, 3, or 4. The RNA levels of *CIITA* and other B cell specific genes, *I-Ab* and *BCL6*, as well as plasma cell specific genes, *Blimp-1*, *XBP-1*, and *syndecan-1* were examined and compared to the control (pLKO.1) samples. The results were plotted with respect to the control infection for each of the genes. (C) P3X63Ag8 cells, infected for 6 days with control pLKO.1 or *ZBTB32* shRNA number 3, were evaluated for I-A surface expression by flow cytometry and are shown with forward side scatter (FSC). All experiments were performed at least three times from independent cultures. Single asterisks indicate Student's t-test values of $p<0.05$. Standard error of the mean was used to represent experimental variability.

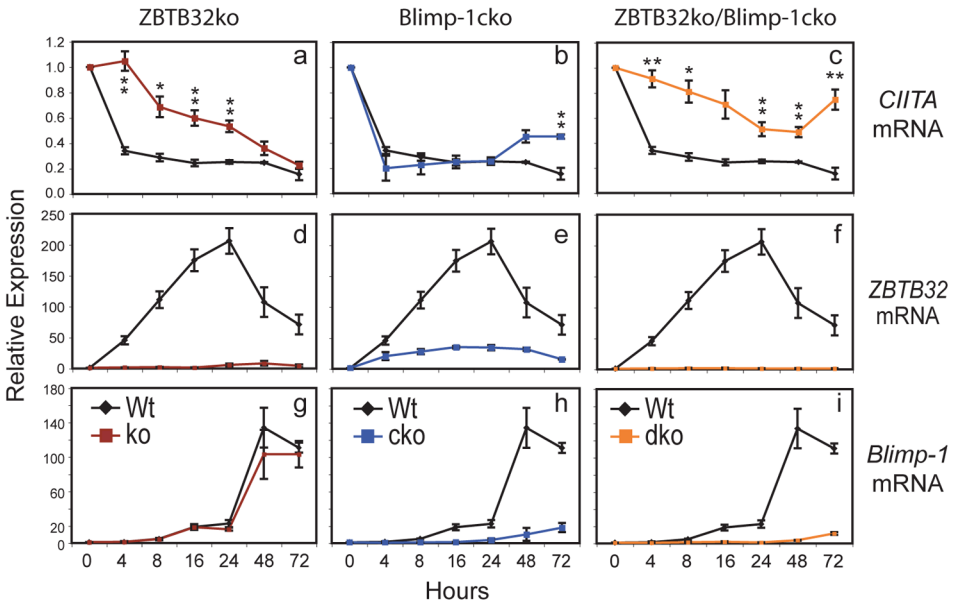

Figure 5. ZBTB32-deficient B cells fail to acutely repress *CIITA* during ex vivo differentiation

Splenic B cells from wild-type (n=6), ZBTB32ko (n=10), Blimp-1cko (n=3), and ZBTB32/Blimp-1dko (n=9) mice were ex vivo differentiated for 3 days. RNA was isolated at indicated time points and *CIITA* (top row), *ZBTB32* (middle row), and *Blimp-1* (bottom row) mRNA levels were quantitated by qRT-PCR, normalized to 18S rRNA, and plotted with respect to the day 0 time point for the gene analyzed. The data for the wild-type animals is replotted in each row so that a visual comparison to each knock out can be made. Student's t-test differences from wild type are indicated by * ($p < 0.05$) and ** ($p < 0.01$). Standard error of the mean was used to represent experimental variability.

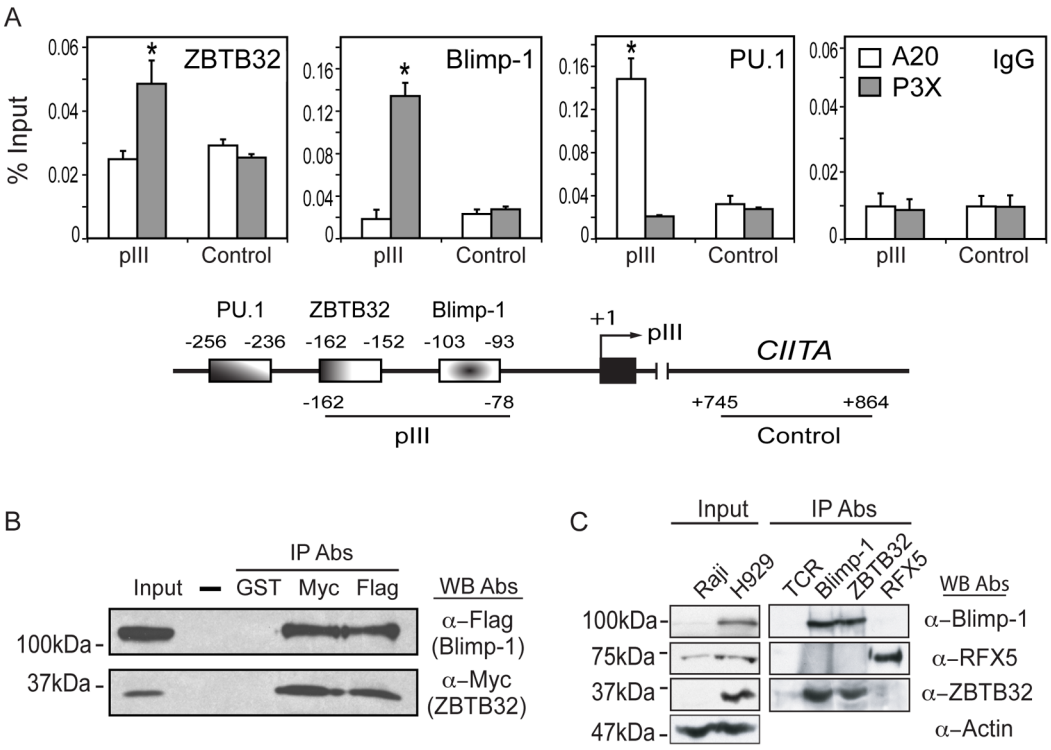

Figure 6. ZBTB32 binds *CIITA* promoter III in differentiated plasma cells and can interact with Blimp-1

(A) ChIP assays from murine A20 B cells or P3X63Ag8 plasma cells were performed using antibodies specific to ZBTB32, Blimp-1, PU.1, or a non-immune IgG control antisera. The occupancy of these factors at *CIITA* promoter III and a negative control site was measured by quantitative PCR compared to a standard curve generated for these regions. The positions of these ChIP PCR amplicons are indicated in the schematic, as are the positions of the putative binding sites for ZBTB32, Blimp-1 and PU.1. The data from three independent chromatin preparations were expressed with respect to the input chromatin. Student's t-test differences from both the control site and control cell line are indicated by * ($p < 0.05$). Standard error of the mean was used to represent experimental variability. (B) Flag-Blimp-1 and Myc-ZBTB32 were co-expressed in HEK293 cells. Nuclear extracts were prepared and subjected to immunoprecipitation using anti-GST, anti-Myc and anti-Flag antibodies. The precipitates were analyzed by western blotting with the indicated antisera. Input lane represents 10% of the extract. The data are representative of three independent experiments. (C) Coimmunoprecipitation of Blimp-1 and ZBTB32 were performed with nuclear extracts from H929 human plasma cells using either control (anti-T cell receptor (TCR)), anti-Blimp-1, anti-ZBTB32, or anti-RFX5 antisera. The input represents 16% of extract. The data are representative of two independent experiments.

Table 1

Differential expression of selected genes during *ex vivo* plasma cell differentiation

Induced genes				Repressed genes			
Symbol[1]	Fold[2]	Symbol	Fold	Symbol	Fold	Symbol	Fold
ZBTB32	102	*Nek2*	15	***Pax5***	0.479	*Fyb*	0.163
Ptgir	68	*Kif2c*	15	*Elf2*	0.479	*Emr1*	0.162
Cdc6	30	*Pycr1*	14	*Pou2af1*	0.460	*Acas2l*	0.154
Il2ra	29	*Cdc25c*	14	*Klf7*	0.419	*Metrnl*	0.154
Il6	27	*Bcat1*	14	*Jarid2*	0.399	*Zfp318*	0.153
Bub1	24	*Ckap2*	13	*Arid1a*	0.385	*Btg1*	0.072
Melk	24	*Mybl2*	13	*Vezf1*	0.384	*Zfp36*	0.071
Ccna2	23	*Sytl3*	12	***EBF1***	0.373	*C1qb*	0.016
Rad51	23	*Idi1*	12	***CD19***	0.300	*Slpi*	0.013
Plk1	22	*Pole2*	11	*Zfp353*	0.292	*Tgfbi*	0.012
Pbk	19	*Sgol2*	11	*Foxo1*	0.287	***SpiC***	0.012
Rrm2	19	*Cenph*	11	*Hhex*	0.254	*Sgk*	0.009
Tpx2	19	*Aurka*	11	*Nfatc1*	0.243	*Axl*	0.009
Chek1	18	*Nusap1*	11	*Ifngr1*	0.228	*Fcna*	0.009
Shcbp1	18	*Prc1*	11	*Il17r*	0.222	*C1qg*	0.004
Gcat	18	*Bcl2l1*	11	*Btla*	0.222	*C1qa*	0.002
Pask	17	*Cdc20*	10	***BCL6***	0.218	**MHC Genes**	
Clspn	17	***Smyd2***	9.3	*Pink1*	0.213	*Symbol*	Fold
Birc5	16	***HMGN3***	7.8	***Satb1***	0.182	***H2-Ab1***	0.20
Rad54l	16	***TfDP1***	7.5	***EZH1***	0.170	*H2-DMb*	0.18
Batf	16	***EZH2***	6.5	*Add3*	0.170	*H2-DMa*	0.16
Chaf1a	16	*Bach*	6.4	*Apc*	0.166	***CIITA***	0.14
Fignl1	15	***CBX2***	6.0	*Hbp1*	0.166	*H2-Oa*	0.14
Kif11	15	*Acat2*	6.0	*Sectm1*	0.163	*H2-Ob*	0.07

[1] Genes in bold are referred to in the manuscript

[2] Fold reflects the level in stimulated cells divided by control untreated cells.