

# Global transcriptional coactivators CREB-binding protein and p300 are highly essential collectively but not individually in peripheral B cells

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**CREB-binding protein (CBP) and its paralog p300 are transcriptional coactivators that physically or functionally interact with over 320 mammalian and viral proteins, including 36 that are essential for B cells in mice. CBP and p300 are generally considered limiting for transcription, yet their roles in adult cell lineages are largely unknown since homozygous null mutations in either gene or compound heterozygosity cause early embryonic lethality in mice. We tested the hypotheses that**

**CBP and p300 are limiting and that each has unique properties in B cells, by using mice with Cre/LoxP conditional knockout alleles for *CBP* (*CBP<sup>fllox</sup>*) and *p300* (*p300<sup>fllox</sup>*), which carry *CD19<sup>Cre</sup>* that initiates floxed gene recombination at the pro-B-cell stage. *CD19<sup>Cre</sup>*-mediated loss of CBP or p300 led to surprisingly modest deficits in B-cell numbers, whereas inactivation of both genes was not tolerated by peripheral B cells. There was a moderate decrease in B-cell receptor**

**(BCR)-responsive gene expression in *CBP* or *p300* homozygous null B cells, suggesting that CBP and p300 are essential for this signaling pathway that is crucial for B-cell homeostasis. These results indicate that individually CBP and p300 are partially limiting beyond the pro-B-cell stage and that other coactivators in B cells cannot replace their combined loss. (Blood. 2006;107:4407-4416)**

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## Introduction

CBP (*Crebbp*) and p300 (*Ep300*) are highly related, large (~265 kDa) nuclear proteins that function as coactivators and cofactors for an ever-increasing number of transcriptional regulators and other proteins in vitro. They enhance transcription (hence “coactivate”) predominantly through binding to the activation domains of transcription factors via specific protein-binding domains that are unique to this 2-member family of proteins. It is believed that this positions the adaptor, protein acetyltransferase, and ubiquitin ligase functions of CBP and p300 at gene promoters and enhancers in order to potentiate transcription.<sup>1</sup> The necessity of CBP and p300 for the expression of endogenous genes has mostly been unexplored, however.

Over the last decade, the number of interacting partners for CBP and p300 has grown over a hundred-fold so that these coactivators could now be considered the most heavily connected nodes in the known mammalian protein-protein interaction network.<sup>2-4</sup> Over 60 transcription factors and other proteins essential for lymphocyte development and function in mice are included among the more than 320 described CBP and p300 interaction partners.<sup>3,5</sup> Of these, 36 are required for B cells (Table 1). The “high connectivity” of CBP and p300 as nodes in transcriptional regulatory networks signifies that they may be present in limiting quantities. Several studies indicate that this may be the case.<sup>9-31</sup> The in vivo evidence is compelling, as mice that are doubly heterozygous for *CBP* and

*p300* null alleles die during early embryogenesis, and mice and humans that are haploinsufficient for *CBP* have severe developmental defects.<sup>31,32</sup>

CBP and p300 have been implicated in the activities of numerous B-cell-critical transcription factors such as NF- $\kappa$ B, PU.1, E47, EBF, and Pax5 (Table 1).<sup>33</sup> B cells originate in the bone marrow where hematopoietic stem cells (HSCs) produce multilineage progenitors that yield the first B-lineage-committed stage (pro-B), which then develop stepwise through the pre-B and immature B-cell stages before exiting the bone marrow to become transitional T1 and T2 immature B cells in the spleen, where maturation is completed.<sup>34</sup> In this regard, *CBP<sup>-/-</sup>* mouse embryos have defective hematopoiesis,<sup>35</sup> as do chimeric mice generated using *CBP* or *p300* null embryonic stem cells,<sup>36</sup> but the effects of the mutations appear to be at the level of the HSC. *CBP<sup>+/-</sup>* mice have approximately 3-fold fewer B cells as a percentage of peripheral blood cells, whereas T cells are normal, although the B-cell phenotype is statistically significant only if splenomegaly is present.<sup>37</sup> Conversely, others have reported equivalent percentages of T cells and B cells in the spleens of *CBP<sup>+/-</sup>* mice.<sup>38</sup> Additional studies have shown that CBP can have a tumor suppressor role in hematopoietic lineages, including T cells<sup>39</sup> and possibly the B-cell lineage (plasmacytomas developed in mice that received transplants of *CBP<sup>+/-</sup>* cells).<sup>37</sup> Interestingly, mice homozygous for

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**Table 1. The B-cell essential CBP and p300 interactome**

Common name and reference	Mouse gene symbol	Essential for B cells or B and T cells in mice, MGI
53BP1 <sup>6</sup>	<i>Trp53bp1</i>	B and T cells
A-Myb <sup>7</sup>	<i>Myb1</i>	B cells
BCL6 <sup>8</sup>	<i>Bcl6</i>	B and T cells
C/EBPβ <sup>9</sup>	<i>Cebpb</i>	B and T cells
CIITA <sup>9</sup>	<i>C2ta</i>	B and T cells
c-Jun <sup>10</sup>	<i>Jun</i>	B and T cells
c-Myb <sup>10</sup>	<i>Myb</i>	B and T cells
c-Myc <sup>11</sup>	<i>Myc</i>	B cells
E47 <sup>12</sup>	<i>Tcf2a</i>	B and T cells
EBF <sup>13</sup>	<i>Ebf1</i>	B cells
Ets-1 <sup>14</sup>	<i>Ets1</i>	B and T cells
GATA-2 <sup>15</sup>	<i>Gata2</i>	B and T cells
HEB <sup>16</sup>	<i>Tcf12</i>	B cells
HIF-1α <sup>10</sup>	<i>Hif1a</i>	B cells
Hoxa9 <sup>17</sup>	<i>Hoxa9</i>	B and T cells
IRF-2 <sup>18</sup>	<i>Irf2</i>	B and T cells
MDM2 <sup>9</sup>	<i>Mdm2</i>	B and T cells
Msh2 <sup>6</sup>	<i>Msh2</i>	B and T cells
Msh6 <sup>6</sup>	<i>Msh6</i>	B and T cells
NBS1 <sup>6</sup>	<i>Nbn</i>	B cells
NFAT1 <sup>19</sup>	<i>Nfatc2</i>	B and T cells
NFATc1 <sup>20</sup>	<i>Nfatc1</i>	B and T cells
NF-κB p50 <sup>21</sup>	<i>Nfkb1</i>	B and T cells
NF-κB p65 <sup>9</sup>	<i>Rela</i>	B and T cells
Pax5 <sup>22</sup>	<i>Pax5</i>	B cells
PML <sup>23</sup>	<i>Pml</i>	B and T cells
PPARγ <sup>24</sup>	<i>Pparg</i>	B cells
PU.1 <sup>15</sup>	<i>Sfp1</i>	B and T cells
Rad50 <sup>6</sup>	<i>Rad50</i>	B and T cells
Runx2 <sup>25</sup>	<i>Runx2</i>	B and T cells
Smad3 <sup>10</sup>	<i>Smad3</i>	B and T cells
Sp3 <sup>26</sup>	<i>Sp3</i>	B and T cells
Spi-B <sup>27</sup>	<i>Spib</i>	B cells
STAT1α <sup>10</sup>	<i>Stat1</i>	B and T cells
STAT6 <sup>28</sup>	<i>Stat6</i>	B and T cells
TCF-4N <sup>29</sup>	<i>Tcf4</i>	B and T cells

Thirty-six mammalian proteins that interact physically or functionally with CBP or p300 in vitro and which are encoded by genes that are essential for B cells in mice (ie, mutation results in a B-cell or combined T- and B-cell phenotype). Source for phenotype information is Mouse Genome Informatics (MGI).<sup>30</sup> More detailed and up-to-date information on the entire CBP/p300 interactome can be found at [http://www.stjude.org/faculty/0,2081,407\\_2030\\_20103,00.html](http://www.stjude.org/faculty/0,2081,407_2030_20103,00.html).<sup>5</sup>

mutations in the CREB- and c-Myb-binding domain (KIX) of p300 have a B-cell deficiency included in a multilineage defect in hematopoiesis.<sup>40</sup> It is uncertain, however, if CBP and p300 are essential for B-cell development and function after commitment of progenitor cells to the B-cell lineage. To address the roles of CBP and p300 in these processes, we induced the inactivation of *CBP<sup>flox</sup>* and *p300<sup>flox</sup>* alleles in *CD19<sup>Cre</sup>* mice that begin to express Cre recombinase at the pro-B-cell stage.

## Materials and methods

### Mice

The *p300<sup>flox</sup>* allele was constructed by flanking exon 9 of the mouse *Ep300* gene with LoxP sites.<sup>3</sup> *CBP<sup>flox</sup>* and *CD19<sup>Cre</sup>* mice were described previously.<sup>39,41</sup> The recombined null alleles are designated *p300<sup>Δflox</sup>* and *CBP<sup>Δflox</sup>*. Relevant alleles in control and mutant mice are wild type unless specified otherwise. Most experiments used mice maintained on a C57BL/6 background, except that Mx-Cre mice were maintained on BALB/c.

### Genotyping of mice

Recombination of the LoxP sites flanking *p300* exon 9 in cells expressing Cre was determined by Southern blot using an *EcoRI* digest and a cDNA probe to the region encoding amino acids (aa's) 615-681 or by semiquantitative polymerase chain reaction (PCR) using primers p4, CTCTACATC-CTAAGTGCTAGG; p5, TGGACTGGTTATCGGTTCCACC; and p6, CAG-TAGATGCTAGAGAAAGCC, producing a 540-bp wild-type band, a 720-bp *p300* flox band, and a 1.1-kb *p300* Δflox band. This semiquantitative PCR overestimates the deletion of *p300<sup>flox</sup>* that can be corrected by using a standard curve of samples of known deletion as determined by Southern blot. PCR genotyping for the *CBP<sup>flox</sup>* allele was previously reported.<sup>39</sup> Detection of the *CBP<sup>flox</sup>* and *CBP<sup>Δflox</sup>* alleles by Southern blot was performed using an *EcoRI* digest and a 5' external probe.

### B-cell purification

Magnetic beads from Miltenyi Biotec (Auburn, CA) were used for positive (B220 microbeads, 130-049-501) and negative (B-cell isolation kit, 130-090-862) selection. Fluorescence-activated cell sorter (FACS) purification of B-cells was performed as indicated.

### Indirect immunofluorescent and Western blots

Antibodies against CBP (A-22 and C-20) and p300 (N-15 and C-20) for immunofluorescence (IF) and Western blotting were from Santa Cruz Biotechnology (Santa Cruz, CA). IF was performed essentially as described using splenic B cells purified by negative selection and C-terminal-specific p300 (1:800 dilution, C-20) and CBP (1:800, C-20) antibodies.<sup>3,39</sup> After immunostaining, cells were viewed under a Nikon Eclipse E800 microscope equipped with a 40 ×/0.95 objective lens and a Nikon DMX1200 camera (Nikon, Tokyo, Japan). Images were recorded with Nikon ACT-1 software; Adobe Photoshop 5.0 software (Adobe Systems, San Jose, CA) was used to process images. Western blots using boiled SDS whole-cell lysates from purified splenic B cells were performed as previously described using cocktails of CBP (A-22 and C-20) or p300 antibodies (N-15 and C-20).<sup>40</sup>

### ELISA assays

Serum samples were collected from 3- to 6-month-old mice. Relative immunoglobulin levels (absorbance at 405 nm) were measured by enzyme-linked immunosorbent assay (ELISA; Southern Biotech, Birmingham, AL).

### Microarray

Affymetrix U74Av2 microarray chips (12 488 probe sets; Santa Clara, CA) were used to test RNA isolated from unstimulated splenic B220<sup>+</sup> B cells purified by magnetic bead-positive selection. Spotfire software version 8.1 (Cambridge, MA) was used for data analysis.

### Flow cytometry and complete blood count

Bone marrow, spleen, and lymph node cells obtained from 6- to 10-week-old control and mutant littermate mice were made into single-cell suspensions in PBS supplemented with 2% heat-inactivated FBS. Cells were stained with a combination of fluorescence-conjugated antibodies (anti-B220, anti-CD43, anti-IgM, anti-CD3, anti-IgD, anti-CD21, or anti-CD23; Becton Dickinson, San Jose, CA) and applied to a flow cytometer (FACSCalibur; Becton Dickinson). Data were collected and analyzed by using CellQuest 3.3 software (Becton Dickinson). Automated complete blood counts (CBCs) were performed using a Hemavet 3700R (CDC Technologies, Oxford, CT).

### B-cell culture and B-cell receptor (BCR) stimulation

Splenic B220<sup>+</sup> cells purified by FACS from 6- to 8-week-old littermate mice were cultured in IMDM medium containing 10% heat-inactivated FBS, 55 μM 2-mercaptoethanol, 0.25% Pluronic F-68, 2 mM L-glutamine, 100 units/mL of penicillin, and 100 μg/mL of streptomycin for 16 hours and treated with 30 μg/mL of soluble anti-IgM (Jackson ImmunoResearch, Bar

Harbor, ME) for 4 hours. Cells were harvested and total RNA was purified using Trizol (Invitrogen, Carlsbad, CA).

**RT-qPCR**

cDNA was generated from 500 ng total RNA using Superscript II reverse transcriptase (Invitrogen). Quantitative reverse transcription-PCR (RT-qPCR) was performed on an Opticon DNA Engine (MJ Research, Waltham, MA) using Qiagen (Valencia, CA) SYBR Green PCR master mix. qPCR primers (sequences available upon request) were designed using Primer Express software (Applied Biosystems, Foster City, CA) and only pairs with sense and antisense primers on different exons were chosen for RT-qPCR analysis. A single product for each primer pair was further confirmed by gel and melt-curve analysis. mRNA level for each gene was normalized to  $\beta$ -actin. Relative expression was calculated by normalizing to the lowest value for an experiment; this permits combining data from multiple experiments. BCR-dependent expression was determined by subtracting the expression for mock-treated cells from anti-IgM-treated cells.

**Results**

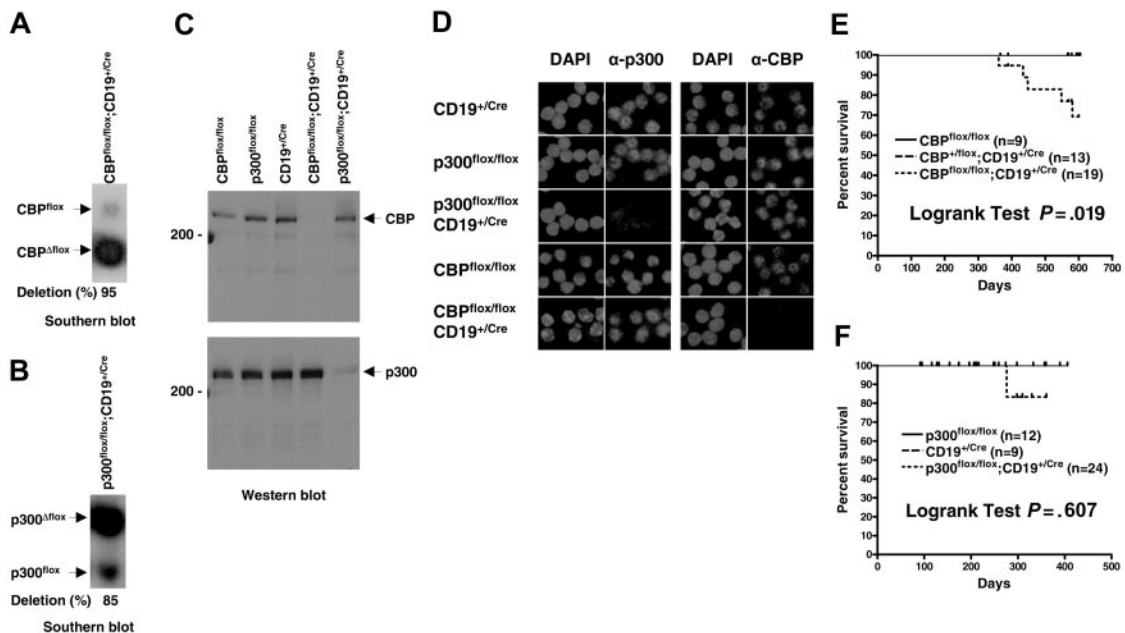
**Inactivation of *CBP<sup>flox</sup>* and *p300<sup>flox</sup>* conditional alleles in mouse B cells**

The *p300<sup>flox</sup>* allele contains LoxP sites in the 2 introns flanking the exon encoding aa's 588-626, which is part of the conserved CREB-binding domain (KIX)<sup>3</sup>; the equivalent exon is targeted in *CBP<sup>flox</sup>* and constitutes a comparable experimental system.<sup>39</sup> Cre-recombinase-mediated deletion of the intronic and exonic sequences yielding *CBP $\Delta$ flox* and *p300 $\Delta$ flox* null alleles was achieved using a *CD19<sup>Cre</sup>* gene that is expressed throughout B-cell development starting at the pro-B stage.<sup>41</sup> The *CD19<sup>Cre</sup>* mutant allele contains a Cre recombinase cDNA insertion that inactivates *CD19*, necessitating its use in the heterozygous state. *CBP<sup>flox/flox</sup>*,

*CD19<sup>+Cre</sup>* and *p300<sup>flox/flox</sup>;CD19<sup>+Cre</sup>* mice efficiently recombined the floxed genes as determined by quantitative Southern blot of splenic B-cell DNA (typically 70%-95%; Figure 1A-B). Western blot analysis using antibodies specific for the N- and C-termini of CBP or p300 confirmed the loss of both proteins in B-cell lysates (Figure 1C), as did indirect immunofluorescence using C-terminal-specific antibodies for CBP or p300 on purified B cells (Figure 1D). There was no apparent increase in the levels of wild-type CBP or p300 protein in  $\Delta$ flox mutant B cells, indicating that compensation by increased expression is unlikely (Figure 1C).

**Increased occurrence of premature death in *CBP<sup>flox/flox</sup>;CD19<sup>+Cre</sup>* mice but loss of CBP or p300 does not lead to B-cell tumors**

CBP and p300 have been identified as tumor suppressors in the myeloid and lymphoid lineages of mice.<sup>36,37,39</sup> In humans, CBP and p300 translocations occur in leukemia and somatic mutations have been found in solid tumors.<sup>42</sup> We tested whether loss of CBP specifically in lineage-committed cells results in B-cell malignancy and whether loss of p300 in B cells leads to tumors. *CBP<sup>flox/flox</sup>;CD19<sup>+Cre</sup>* mice on a mixed genetic background appeared generally healthy but had an increased incidence of death after about one year when compared with *CBP<sup>flox/flox</sup>* and *CBP<sup>+flox</sup>;CD19<sup>+Cre</sup>* littermate controls (Figure 1E;  $P = .019$ , log-rank test,  $n = 9-19$ ). The cause of death was undetermined but there were no instances of B-cell lymphoma. The *p300<sup>flox/flox</sup>;CD19<sup>+Cre</sup>* mice also appeared healthy and had lifespans that were not significantly different from control mice out to one year of age (Figure 1F;  $P = .606$ , log-rank test,  $n = 9-24$ ). It is possible that *p300<sup>flox/flox</sup>;CD19<sup>+Cre</sup>* mice will show increased rates of death after one year like *CBP<sup>flox/flox</sup>;CD19<sup>+Cre</sup>* mice, but we have not observed any incidence of lymphoma. We conclude therefore that *CBP* and *p300* do not have highly essential



**Figure 1. Efficient inactivation of *CBP<sup>flox</sup>* or *p300<sup>flox</sup>* in mouse B cells does not result in lymphoma.** Quantitative Southern blot of genomic DNA isolated from purified splenic B cells for *CBP<sup>flox/flox</sup>;CD19<sup>+Cre</sup>* (A) and *p300<sup>flox/flox</sup>;CD19<sup>+Cre</sup>* (B) mice. Nonrecombined (flox) alleles, recombined ( $\Delta$ flox) alleles, and percent deletion are indicated. (C) Western blot of cell lysates from purified splenic B cells using CBP-specific (top panel) and p300-specific (bottom panel) antisera. Genotypes, 200-kDa markers, and position of CBP and p300 are indicated. (D) Indirect immunofluorescence of control and *CBP* and *p300* null purified splenic B cells using CBP- and p300-specific antisera. Genotypes are indicated. Nuclear DNA stained with DAPI. Survival curves for *CBP<sup>flox/flox</sup>;CD19<sup>+Cre</sup>* (E) and *p300<sup>flox/flox</sup>;CD19<sup>+Cre</sup>* (F) and control mice. Age of mice (days) and percent surviving are indicated. Tick marks indicate mice that were censored (killed for experimentation) or still alive at the end of the study. Curve comparisons by 2-tailed log-rank test, number of mice of each genotype, and  $P$  values are indicated.

tumor suppressor functions in B cells after the pro-B stage of development.

### Modest reduction in peripheral B cells in $CBP^{fllox/fllox};CD19^{+/Cre}$ and $p300^{fllox/fllox};CD19^{+/Cre}$ mice

We observed an approximately 50% decrease in the number of B cells in the peripheral blood of  $CBP^{fllox/fllox};CD19^{+/Cre}$  mice compared with  $CBP^{fllox/fllox}$  and  $CD19^{+/Cre}$  controls (Figure 2A; analysis of variance [ANOVA],  $P < .001$ ,  $n = 7-12$ ). A similar decrease was observed in the spleen and lymph nodes (W.X., data not shown). The  $p300^{fllox/fllox};CD19^{+/Cre}$  mice showed only an approximately 25% decrease in peripheral B cells in the blood (Figure 2B; ANOVA,  $P = .02$ ,  $n = 14-16$ ), spleen, and lymph nodes (W.X., data not shown). T-cell numbers were not disturbed in either mutant, as expected (Figure 2A-B). Flow cytometric analysis of splenic B cells revealed similarly modest decreases in the proportions of all 3 major  $IgM^{+}$  and  $IgD^{+}$  B-cell classes in  $CBP^{fllox/fllox};CD19^{+/Cre}$  mice:  $IgM^{hi} IgD^{lo}$  cells include mature marginal zone (MZ) and B-1 B cells as well as T1 immature B cells;  $IgM^{hi} IgD^{hi}$  includes T2 immature B cells and proliferating B cells; and  $IgM^{lo} IgD^{hi}$  includes follicular mature (FM) B cells (Figure 2C-D).  $IgM^{hi} IgD^{lo}$  B cells were the most affected proportionally (2- to 3-fold lower,  $P = .004$ , ANOVA), but examination by IgM and CD21 staining did not reveal a disproportionate loss of  $IgM^{hi} CD21^{lo}$  T1

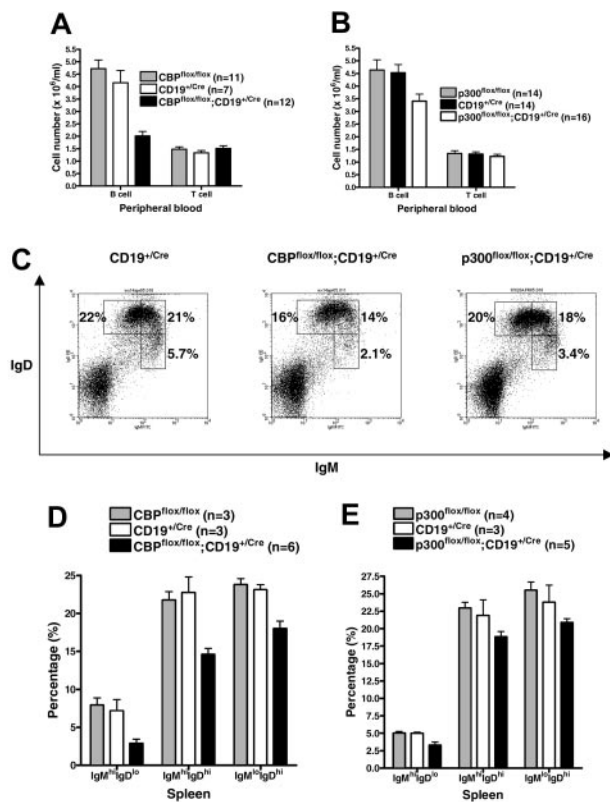
cells (W.X., data not shown). IgM and IgD staining of  $p300^{fllox/fllox};CD19^{+/Cre}$  splenic B cells showed a similar, albeit more modest, phenotype (Figure 2C,E). Together, these results demonstrate that B cells lacking CBP or p300 can still efficiently populate the lymphoid organs, with only a modest reduction in total cell numbers.

### B-cell development in the bone marrow proceeds without obvious blockage in $CBP^{fllox/fllox};CD19^{+/Cre}$ and $p300^{fllox/fllox};CD19^{+/Cre}$ mice

We did not detect significant alterations in the proportions of the pro-B, pre-B, and immature stages of B-cell development in the bone marrow of  $CBP^{fllox/fllox};CD19^{+/Cre}$  and  $p300^{fllox/fllox};CD19^{+/Cre}$  mice. B220 and IgM staining revealed proportions of  $B220^{lo} IgM^{-}$  pro- and pre-B cells and  $B220^{int} IgM^{+}$  immature B cells that were not significantly different between mutant and control mice (Figure 3A-C). The fraction of  $B220^{hi} IgM^{+}$  mature recirculating B cells was approximately 2-fold lower in  $CBP^{fllox/fllox};CD19^{+/Cre}$  (Figure 3B;  $P < .001$ , ANOVA) but not  $p300^{fllox/fllox};CD19^{+/Cre}$  mice (Figure 3C;  $P = .322$ , ANOVA). Staining for B220 and CD43 also revealed that the proportions of  $B220^{lo} CD43^{+}$  (pro-B) and  $B220^{lo-int} CD43^{-}$  (pre-B and immature B cells) were unaffected in  $CBP^{fllox/fllox};CD19^{+/Cre}$  and  $p300^{fllox/fllox};CD19^{+/Cre}$  mice (W.X., data not shown). Interestingly, there was a decrease in the mean fluorescence intensity for surface B220 in pre-B and immature B cells, but not mature B cells, from  $CBP^{fllox/fllox};CD19^{+/Cre}$  but not  $p300^{fllox/fllox};CD19^{+/Cre}$  mice (Figure S1, available at the Blood website; see the Supplemental Materials link at the top of the online article). This correlated with a reduction in the level of B220 mRNA, indicating that the expression of this isoform produced from the CD45 protein tyrosine phosphatase receptor gene is dependent on CBP in B-cell progenitors (Figure S1).  $CD45$  null mice have a decreased percentage of mature B cells in the bone marrow,<sup>43</sup> as do  $CBP^{fllox/fllox};CD19^{+/Cre}$  mice (Figure 3B), suggesting that reduced expression of B220 may contribute to the phenotype of the latter.

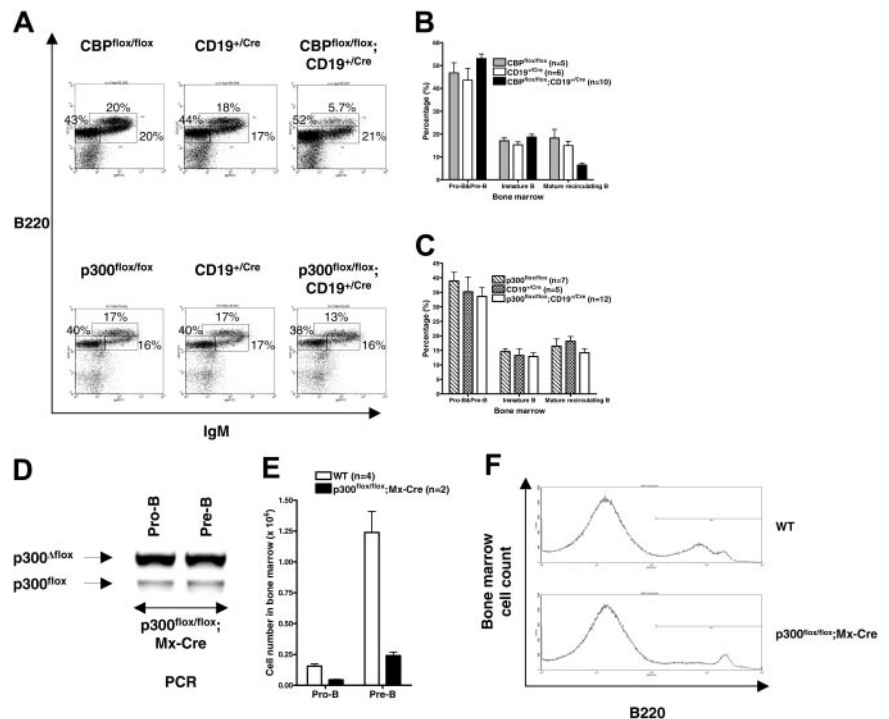
### Loss of p300 before B-lineage commitment compromises B-cell development

We previously showed that a mutation in the CREB- and c-Myb-binding KIX domain of p300 causes multilineage defects in hematopoiesis that include B-cell deficiency in mice.<sup>40</sup> We were therefore surprised that the loss of p300 did not cause a similar B-cell deficiency in  $p300^{fllox/fllox};CD19^{+/Cre}$  mice. This inconsistency may indicate that p300 (ie, the p300 KIX domain) is necessary for B cells before, or at, the pro-B stage of development when  $CD19^{Cre}$  begins expression. We tested this prediction by using mice with the interferon-inducible  $Mx-Cre$  transgene that express Cre efficiently in hematopoietic stem cells (HSCs) following treatment of the mice with double-stranded RNA (dsRNA). The number of pro-B- and pre-B-cell progenitors in the bone marrow was examined in wild-type and  $p300^{fllox/fllox};Mx-Cre$  mice 6 months after injection of dsRNA. The 6-month delay ensured that any effects of interferon on B cells had subsided and that B-cell progenitors have been replaced by new cells originating from HSCs (mature peripheral B cells may not have turned over, however<sup>44</sup>). The  $p300^{fllox/fllox};Mx-Cre$  mice with efficient deletion of  $p300^{fllox}$  in pro-B and pre-B cells had 3.5-fold fewer pro-B cells and 5-fold fewer pre-B cells in the bone marrow compared with wild-type mice ( $n = 2-4$ ; Figure 3D-E). B220 flow cytometric analysis also emphasized the deficiency of B cells in the bone marrow of  $p300^{fllox/fllox};Mx-Cre$  mice (Figure 3F). Overall, these findings support the model that p300 is more



**Figure 2. Modest deficit in peripheral B cells of  $CBP^{fllox/fllox};CD19^{+/Cre}$  and  $p300^{fllox/fllox};CD19^{+/Cre}$  mice.** The number of B cells and T cells per mL of peripheral blood for  $CBP^{fllox/fllox};CD19^{+/Cre}$  (A) and  $p300^{fllox/fllox};CD19^{+/Cre}$  (B) mice and controls. Number of mice of each genotype is indicated. Mean  $\pm$  SEM. (C) Representative flow cytometric analysis using anti-IgM and anti-IgD antibodies and splenic lymphocytes from  $CD19^{+/Cre}$ ,  $CBP^{fllox/fllox};CD19^{+/Cre}$ , and  $p300^{fllox/fllox};CD19^{+/Cre}$  mice. Percentage of total splenic lymphocytes representing the major B-cell subtypes ( $IgM^{hi} IgD^{lo}$ ,  $IgM^{hi} IgD^{hi}$ ,  $IgM^{lo} IgD^{hi}$ ). (D-E) Quantification of IgM and IgD flow cytometry data as in panel C for the indicated number of mice of each genotype (mean  $\pm$  SEM). Flow cytometric analysis and complete blood count analyses for the  $CBP^{fllox}$  and  $p300^{fllox}$  groups were performed at different times and may not be directly comparable to each other.

**Figure 3. Proportions of B-cell progenitors in *CBP<sup>flox/flox</sup>;CD19<sup>+/-Cre</sup>* and *p300<sup>flox/flox</sup>;CD19<sup>+/-Cre</sup>* mice are normal but p300 is essential before or during the pro-B stage.** (A) Representative B220 and IgM flow cytometric analyses of lymphocyte gated bone marrow cells from the indicated mouse genotypes. Percentage of each major subtype is indicated. (B-C) Quantification of bone marrow B-cell progenitors by B220 and IgM flow cytometry as in panel A. Pro-B and pre-B cells (B220<sup>lo</sup> IgM<sup>-</sup>), immature B cells (B220<sup>hi</sup> IgM<sup>+</sup>), and mature recirculating B cells (B220<sup>hi</sup> IgM<sup>+</sup>). Number of mice of each genotype is indicated. Mean  $\pm$  SEM. (D) Recombination of the *p300<sup>flox</sup>* allele in FACS-purified pro-B (B220<sup>+</sup> CD43<sup>+</sup> IgM<sup>-</sup>) and pre-B (B220<sup>+</sup> CD43<sup>-</sup> IgM<sup>-</sup>) cells from a representative *p300<sup>flox/flox</sup>;Mx-Cre* mouse with efficient deletion 6 months after inducing Cre expression with dsRNA. (E) Number of pro-B and pre-B cells in dsRNA-treated wild-type (WT; n = 4) and *p300<sup>flox/flox</sup>;Mx-Cre* mice (n = 2) as in panel D (mean  $\pm$  SEM). (F) Representative histogram showing B220<sup>+</sup> bone marrow cells as in panels D-E.



important for B-cell development before, or at, the pro-B stage, rather than later.

#### Slightly increased serum IgM and IgG3 levels in *CBP<sup>flox/flox</sup>;CD19<sup>+/-Cre</sup>* mice

We next tested a crucial parameter of B-cell function by measuring serum immunoglobulin levels in *CBP<sup>flox/flox</sup>;CD19<sup>+/-Cre</sup>* and *p300<sup>flox/flox</sup>;CD19<sup>+/-Cre</sup>* mice. When compared with *CBP<sup>flox/flox</sup>* and *CBP<sup>+/-flox</sup>;CD19<sup>+/-Cre</sup>* controls, the relative levels of IgG3 ( $P = .007$ , ANOVA, n = 14-16) and IgM ( $P = .003$ , ANOVA, n = 14-16) were increased approximately 2-fold in *CBP<sup>flox/flox</sup>;CD19<sup>+/-Cre</sup>* mice. The *p300<sup>flox/flox</sup>;CD19<sup>+/-Cre</sup>* mice had normal amounts of IgG3 and IgM, and levels of IgG1, IgG2a, IgG2b, IgA, Igk, and Igλ were comparable between controls and both mutants (W.X., data not shown). We next inquired if marginal zone B cells were increased in the spleens of *CBP<sup>flox/flox</sup>;CD19<sup>+/-Cre</sup>* mice, as they tend to secrete greater amounts of IgM and IgG3, but there were no significant differences in proportions of marginal zone (B220<sup>+</sup> CD21<sup>hi</sup> CD23<sup>lo</sup>), follicular (B220<sup>+</sup> CD21<sup>int</sup> CD23<sup>hi</sup>), and newly formed (B220<sup>+</sup> CD21<sup>lo</sup> CD23<sup>lo</sup>) B cells between control and mutant mice (D.W. and W.X., data not shown). Although untested here, Notch signaling may be defective in *CBP* null B cells, as loss of the Notch-responsive factor RBP-J reportedly leads to approximately 3-fold higher levels of IgG3.<sup>45</sup>

#### Resting *CBP* null B cells have nominal changes in global gene expression

We next tested if widespread alterations in gene expression would occur in B cells lacking a global coactivator such as CBP. Affymetrix microarrays were used to test RNA isolated from unstimulated purified splenic B cells. Unexpectedly, we found that global gene expression in B cells from *CBP<sup>flox/flox</sup>;CD19<sup>+/-Cre</sup>* mice (deletion frequency ~80%-85% confirmed by semiquantitative PCR, not shown, n = 2) was largely unperturbed compared with *CBP<sup>flox/flox</sup>* (n = 2) and *CBP<sup>+/-flox</sup>;CD19<sup>+/-Cre</sup>* controls (n = 2). Only

59 probe sets were downregulated more than 1.7-fold in *CBP* null B cells compared with controls in a statistically significant manner (Table 2;  $2.6 \pm 1.9$ -fold, mean  $\pm$  SD,  $P < .05$ ,  $t$  test). Another 30 probe sets were upregulated 1.7-fold or more ( $2.2 \pm 0.6$ -fold,  $P < .05$ ; Table S1). Thus, only 89 (1.3%) of 6651 probe sets (omitting probe sets with unreliable signals in all 6 samples) were more than 1.7-fold different in the mutants and statistically significant. As these criteria are not highly stringent, some of the differences observed in the mutants could have occurred by chance. Therefore, most genes were not measurably disturbed in resting *CBP* null B cells, although a few potential outliers are worth noting. Genes with reduced expression (~2- to 3-fold) in the mutant arrays include effectors (eg, Ig kappa and heavy chains, Ccr7) and transcriptional regulators with roles in B cells (eg, *C21a*, *Ets1*, *Relb*). Transcriptional cofactors (eg, *Mcm2* and *Smarca3*) that are upregulated may hint at compensatory mechanisms for loss of CBP.

#### Attenuated BCR-responsive gene expression in *CBP* and *p300* null B cells

BCR-signaling-responsive gene transcription has a central role in maintaining B-cell homeostasis and is thought to principally involve several CBP/p300 interacting transcription factors that include NF- $\kappa$ B and NF-AT. We tested if CBP or p300 was limiting for BCR-inducible target genes using real-time quantitative RT-PCR (RT-qPCR) to measure mRNA from FACS-purified splenic B cells following 4-hour stimulation with anti-IgM ex vivo. Test gene expression was normalized to  $\beta$ -actin mRNA. Efficient recombination (typically ~80%-90%) of the *CBP<sup>flox</sup>* and *p300<sup>flox</sup>* alleles was confirmed by PCR (W.X., data not shown). We tested 5 BCR-inducible genes that represent putative CBP/p300 target genes and play roles in B-cell biology: tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; *Tnf*), c-Myc (*Myc*), Bcl2-related protein A1a (A1, Bfl-1; *Bcl2a1a*), cyclin D2 (*Ccnd2*), and cyclin-dependent kinase 4 (*Cdk4*). TNF- $\alpha$  is regulated in lymphocytes by the CBP/p300 interacting factors

**Table 2. Fifty-nine probe sets downregulated at least 1.7-fold in *CBP* null B cells**

Probe set ID	Gene title	Gene symbol	Controls, average	Mutants, average	Mutant-control ratio
100368_at	—	—	161	34	0.21
100682_f_at	Kappa	—	8 657	4532	0.52
101319_f_at	Similar to kappa light chain	—	2 629	1453	0.55
103700_r_at	—	—	140	75	0.53
161423_r_at	—	—	135	18	0.14
161899_f_at	—	—	402	218	0.54
162035_f_at	—	—	97	18	0.18
93506_at	—	—	266	147	0.55
97563_f_at	Similar to Ig H-chain	—	10 986	5338	0.49
98765_f_at	Similar to immunoglobulin heavy chain	—	6 232	2863	0.46
98896_at	RIKEN cDNA 5830457O10 gene	<i>5830457O10Rik</i>	1 743	1012	0.58
99466_at	RIKEN cDNA 6230401O10 gene	<i>6230401O10Rik</i>	348	180	0.52
104444_at	RIKEN cDNA 9430098E02 gene	<i>9430098E02Rik</i>	559	282	0.50
96188_at	Adenosine deaminase, RNA specific	<i>Adar</i>	681	388	0.57
97574_f_at	Expressed sequence AI324046	<i>AI324046</i>	5 750	2619	0.46
98372_at	Aldehyde dehydrogenase family 1, subfamily A3	<i>Aldh1a3</i>	65	19	0.30
102118_at	Ankyrin repeat domain 17	<i>Ankrd17</i>	124	68	0.55
93372_at	Acidic (leucine-rich) nuclear phosphoprotein 32 family	<i>Anp32a</i>	1 024	202	0.20
100462_at	ADP-ribosylation factor 6	<i>Arf6</i>	265	117	0.44
93631_at	rho/rac guanine nucleotide exchange factor (GEF) 2	<i>Arhgef2</i>	140	80	0.57
93797_g_at	ATPase, Na <sup>+</sup> /K <sup>+</sup> transporting, alpha 1 polypeptide	<i>Atp1a1</i>	1 174	526	0.45
102917_at	Class II transactivator	<i>C2ta</i>	1 176	671	0.57
99899_at	Chemokine (C-C motif) receptor 6	<i>Ccr6</i>	2 791	1238	0.44
104443_at	Chemokine (C-C motif) receptor 7	<i>Ccr7</i>	1 063	583	0.55
97832_at	CD97 antigen	<i>Cd97</i>	732	286	0.39
95466_at	Coactosin-like 1 (Dictyostelium)	<i>Cotl1</i>	725	156	0.21
104681_at	Diaphanous homolog 1 (Drosophila)	<i>Diap1</i>	822	462	0.56
104673_at	Eph receptor A4	<i>Epha4</i>	143	62	0.43
99397_at	Excision repair cross-complementing repair deficiency	<i>Ercc2</i>	454	260	0.57
94720_at	E26 avian leukemia oncogene 1, 5' domain	<i>Ets1</i>	919	292	0.32
94141_at	Fc receptor, IgE, low-affinity 11, alpha polypeptide	<i>Fcer2a</i>	4 414	1627	0.37
100693_at	GABA-A receptor, subunit gamma 3	<i>Gabrg3</i>	138	76	0.55
93750_at	Gelsolin	<i>Gsn</i>	657	297	0.45
97867_at	Hydroxysteroid 11-beta dehydrogenase 1	<i>Hsd11b1</i>	331	181	0.55
92614_at	Inhibitor of DNA binding 3	<i>Idb3</i>	1 161	506	0.44
100360_f_at	Immunoglobulin heavy chain 1a (serum IgG2a)	<i>Igh-1a</i>	7 765	3612	0.47
101743_f_at	Immunoglobulin heavy chain (J558 family)	<i>Igh-VJ558</i>	2 042	914	0.45
101747_f_at	Immunoglobulin heavy chain (J558 family)	<i>Igh-VJ558</i>	2 195	903	0.41
97577_f_at	Similar to Ig H-chain V-JH3-region	<i>Igh-VJ558</i>	2 165	1052	0.49
100376_f_at	Ig heavy chain	<i>Igh-VJ558///J558</i>	4 370	2321	0.53
97576_f_at	Ig heavy chain	<i>J558</i>	2 746	1375	0.50
102793_at	Keratin-associated protein 8-1	<i>Krtap8-1</i>	78	5	0.07
100721_f_at	Single-chain Ig VH and VL domains	<i>LOC56304</i>	13 928	8156	0.59
102630_s_at	Lymphotoxin A	<i>Lta</i>	818	346	0.42
100362_f_at	Unknown (protein for MGC:60843)	<i>MGC60843</i>	7 778	4004	0.51
98373_at	Membrane-spanning 4 domains, subfamily A, member 4C	<i>Ms4a4c</i>	725	400	0.55
101981_at	Nuclear receptor subfamily 1, group H, member 2	<i>Nr1h2</i>	417	191	0.46
102197_at	Nucleobindin 2	<i>Nucb2</i>	107	43	0.40
93694_at	Period homolog 2 (Drosophila)	<i>Per2</i>	77	44	0.57
95325_at	Pleckstrin homology, Sec7 and coiled-coil domains 1	<i>Pscd1</i>	359	150	0.42
104442_at	RAB14, member RAS oncogene family	<i>Rab14</i>	583	291	0.50
103091_at	v-rel oncogene-related B	<i>Relb</i>	1 038	597	0.58
101967_at	Stromal cell-derived factor 2	<i>Sdf2</i>	300	142	0.47
96131_at	TBC1 domain family, member 10	<i>Tbc1d10</i>	463	240	0.52
103541_at	t-complex-associated testis expressed 2	<i>Tcte2</i>	137	69	0.50
101918_at	Transforming growth factor, beta 1	<i>Tgfb1</i>	2 410	1334	0.55
104192_at	Translocated promoter region	<i>Tpr</i>	77	37	0.48
101167_at	Ubiquitin specific protease 53	<i>Usp53</i>	424	221	0.52
92432_at	Zinc finger protein 574	<i>Zfp574</i>	297	166	0.56

Microarray analysis was performed using Affymetrix U74Av2 chips and RNA isolated from unstimulated splenic B cells purified by positive selection. Average signal intensity for *CBP<sup>flax/lox</sup>;CD19<sup>+/-Cre</sup>* (mutants, n = 2) and combined control mice (*CBP<sup>flax/lox</sup>* [n = 2] and *CBP<sup>+/lox</sup>; CD19<sup>+/-Cre</sup>* [n = 2]). Probe sets shown had at least 1.7-fold lower average signal intensity in the mutants compared with combined controls. *P* < .05, 2-tailed *t* test. Probe sets scored as absent in all 6 samples by the Affymetrix analysis software were excluded. Ratio of mutant average signal to control average signal is indicated in the right-hand column.

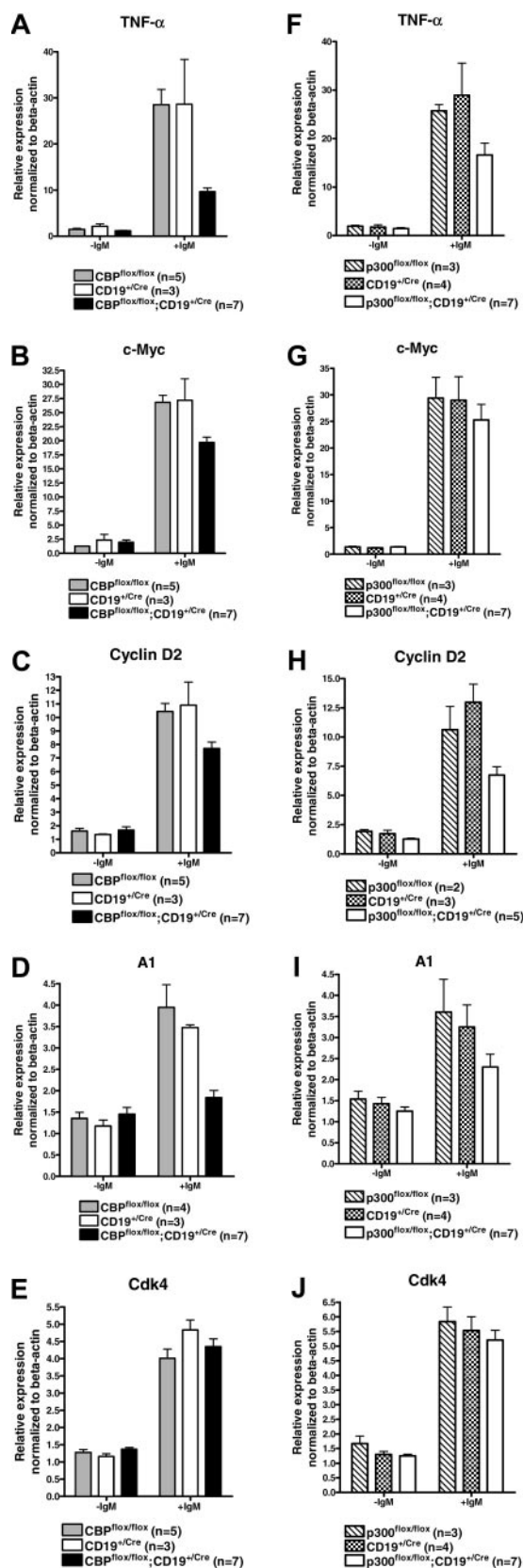
— indicates not available.

ATF2, Jun, and NF-AT and has reduced expression in T cells with *CBP* haploinsufficiency.<sup>38,46</sup> BCR-dependent TNF- $\alpha$  expression was reduced approximately 68% in *CBP* null B cells ( $P = .002$ , one-tailed ANOVA) and approximately 40% in *p300* null B cells compared with controls ( $P = .044$ ; Figure 4A,F). A comparable deficit in TNF- $\alpha$  expression was observed in *CBP* null follicular and marginal zone splenic B cells, indicating that the defect is not specific for a particular B-cell subtype (W.X., data not shown). The role of TNF- $\alpha$  in B cells is unclear but it may act as an autocrine growth factor, suggesting that reduced expression may affect B-cell homeostasis.<sup>47,48</sup> *c-Myc* expression in B cells requires the CBP/p300 interacting factor NF- $\kappa$ B,<sup>49</sup> and BCR-dependent expression was reduced approximately 30% in *CBP* null cells ( $P = .004$ ) but only approximately 14% in *p300* null B cells ( $P = 0.33$ ; Figure 4B,G). *c-Myc* is highly essential in B cells, suggesting that modestly reduced levels may be detrimental in conjunction with reductions in other essential factors.<sup>50</sup> Cyclin D2 is an NF- $\kappa$ B target that is limiting for BCR-dependent B-cell proliferation<sup>51,52</sup> and its BCR-dependent expression was reduced approximately 34% in *CBP* null cells ( $P = .01$ ) and approximately 44% in *p300* null B cells ( $P = .075$ ; Figure 4D,I). Not all tested genes were affected by the loss of CBP or p300, however, as the E2F target *Cdk4* showed normal BCR-inducible expression in both mutants<sup>53</sup> (Figure 4E,J). Together, these results indicate that CBP and p300 are generally limiting for BCR-dependent transcription.

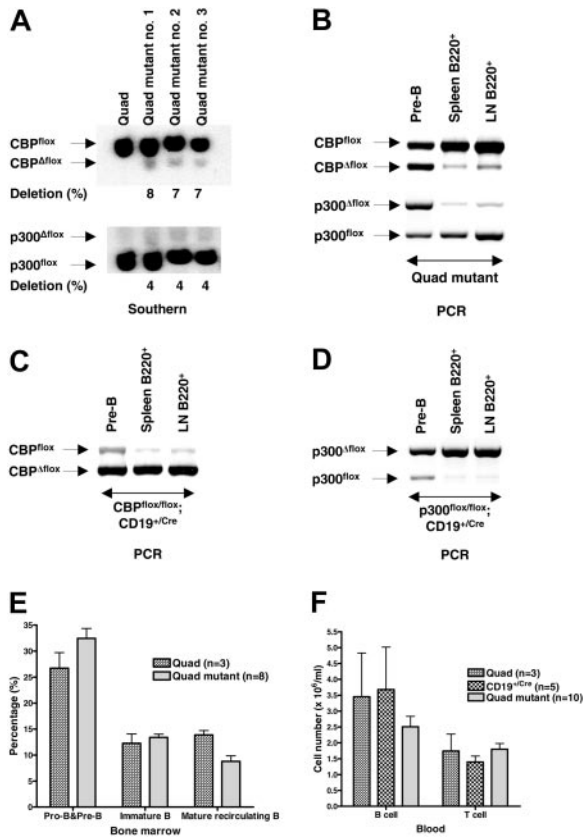
#### The combined dosage of CBP and p300 is critical for peripheral B cells

The ability of B cells to develop and survive with only CBP or p300 raised the possibility that cells lacking both coactivators could be generated. We tested this by creating *CBP<sup>fllox/fllox</sup>;p300<sup>fllox/fllox</sup>;CD19<sup>+Cre</sup>* mice (“quad mutants”), which were compared with *CBP<sup>fllox/fllox</sup>;p300<sup>fllox/fllox</sup>* “quad” control mice. Quantitative Southern blot analysis of FACS-purified quad mutant splenic B cells detected only about 4% to 8% recombination of *CBP<sup>fllox</sup>* and *p300<sup>fllox</sup>* alleles, suggestive that cells lacking both CBP and p300 are selected against (Figure 5A). The low levels of  $\Delta$ fllox alleles detected could be from B cells with incomplete recombination or perhaps viable double-null B cells. We next tested if quad mutant B cells were at a selective disadvantage by determining the frequency of gene deletion in purified pre-B cells from the bone marrow and B cells from the spleen and lymph nodes. Semiquantitative PCR analysis of DNA isolated from quad mutants showed a marked reduction in the percentage of recombined alleles in peripheral B cells when compared with pre-B cells (Figure 5B), the former of which was quantitatively similar ( $\sim 6\%$  *CBP<sup>\Delta</sup>fllox*) to the Southern blot result (Figure 5A). In contrast, *CBP<sup>fllox/fllox</sup>;CD19<sup>+Cre</sup>* and *p300<sup>fllox/fllox</sup>;CD19<sup>+Cre</sup>* B cells from the spleen and lymph node had a greater percentage of the recombined alleles compared with bone marrow pre-B cells (Figure 5C-D; PCR quantitation is fairly linear for *CBP<sup>\Delta</sup>fllox* but tends to overestimate the relative abundance of the *p300<sup>\Delta</sup>fllox* allele). These results strongly indicate that B cells require CBP or p300 in order to populate the periphery.

We unexpectedly observed marginal effects on B-cell development in the quad mutants as determined by flow cytometric analysis of bone marrow cells for B220 and IgM (Figure 5E) and B220 and CD43 (W.X., data not shown). The quad mutant looked



**Figure 4. Loss of CBP or p300 attenuates BCR-inducible gene expression.** Real-time RT-qPCR of RNA from FACS-purified splenic B cells of the indicated genotypes following 4 hours treatment with or without soluble anti-IgM. Test gene expression was normalized to signal derived from  $\beta$ -actin mRNA and then normalized to the lowest signal for each gene in a given experiment. Gene tested and the number of mice of each genotype are indicated (mean  $\pm$  SEM).



**Figure 5. Loss of both CBP and p300 is highly detrimental for peripheral B cells.** (A) Quantitative Southern blot of DNA of FACS-purified splenic B cells from control  $CBP^{flox/flox}; p300^{flox/flox}$  (Quad) and 3 different quadruple mutant  $CBP^{flox/flox}; p300^{flox/flox}; CD19^{+/Cre}$  (Quad mutant) mice. Position of each allele and percent deletion are indicated. (B) Semiquantitative PCR of DNA isolated from quad mutant bone marrow pre-B cells ( $B220^+ CD43^- IgM^-$  purified by FACS) and B cells ( $B220^+$  purified by FACS) from the spleen and lymph node (LN). Positions of PCR products representing each allele are indicated. PCR tends to overestimate the abundance of the  $p300^{\Delta/flox}$  allele. Semiquantitative PCR of DNA isolated from  $CBP^{flox/flox}; CD19^{+/Cre}$  (C) and  $p300^{flox/flox}; CD19^{+/Cre}$  (D) B cells as in panel B. (E) Quantification of B-cell progenitors as determined by B220 and IgM flow cytometry (lymphocyte gate) of bone marrow derived from mice of the indicated genotypes. Number of mice is indicated (mean  $\pm$  SEM). (F) The number of B and T cells per mL of peripheral blood from mice of the indicated genotypes. Number of mice is indicated (mean  $\pm$  SEM).

similar to the *CBP* mutant in this respect. Quad mutant mice also had only an approximately 30% decrease in the number of B cells in the blood compared with controls (Figure 5F), indicating that there is compensation by homeostatic proliferation of peripheral B cells that did not recombine the conditional alleles. These results suggest 3 possibilities. First, loss of CBP and p300 does not strongly affect B-cell development but is highly detrimental to peripheral B cells. Second, loss of CBP and p300 is detrimental to B-cell progenitors, but cells having nonrecombined alleles can expand to fill the bone marrow niche. Third, quad mutant progenitors retain sufficient CBP and p300 protein to enable development because of insufficient protein turnover/dilution but die as CBP and p300 become limiting.

## Discussion

Our results demonstrate that individually CBP and p300 are not highly essential for major aspects of B-cell development and function beyond the pro-B stage. This was unexpected, as the combined levels of CBP and p300 are generally presumed to be

limiting; their described interactions with 36 different B-cell-critical transcriptional regulators support this view. CBP was moderately more important than p300 in peripheral B cells when it was lost after the pro-B stage. This may indicate that CBP protein is relatively more abundant compared with p300 in B cells or that it has unique B-cell-critical biochemical properties.

Although individual loss of CBP or p300 was fairly well tolerated, B cells lacking both coactivators were nonexistent or very rare in the periphery. This indicates that this small family of coactivators is indispensable and that other transcriptional cofactors like GCN5 and P/CAF that share functionalities with CBP and p300 (ie, protein acetyltransferase activity and a bromodomain for binding acetylated lysines) cannot supply completely redundant functions, at least in lymphocytes. CBP and p300 have a number of unique domains that function predominately to bind other proteins; our results suggest that there are no functional substitutes for all these domains outside the CBP/p300 family.

It is uncertain if loss of both CBP and p300 starting at the pro-B stage was detrimental to quad mutant B-cell development in bone marrow, although the effect on peripheral B cells was clear (Figure 5). *CD19<sup>Cre</sup>*-mediated recombination of both *CBP<sup>flox</sup>* and *p300<sup>flox</sup>* in quad mutant pre-B cells was considerably less than in cells deleting either floxed gene alone. This would suggest that progenitors lacking both CBP and p300 are selected against. The percentages, however, of pro-B, pre-B, and immature B cells were comparable between quad mutant and quad control mice, implying that cells lacking both CBP and p300 develop normally, although it is possible that cells retaining CBP and p300 protein expand to fill the niche. Comparison of the floxed gene recombination frequencies in pro-B, pre-B, and immature B cells in quad mutant mice would help resolve this question.

Cell survival in the bone marrow and periphery, or migration from bone marrow, may be aberrant in the quad mutants. We believe that survival is likely to be affected because the deletion frequency of both *CBP<sup>flox</sup>* and *p300<sup>flox</sup>* in quad mutant pre-B cells is less than if only one of the conditional alleles is present (ie, possible selection against double-null cells). There is also no obvious accumulation or blockage at any precursor stage in quad mutant bone marrow (Figure 5). The mechanism causing the loss of quad mutant B cells is unknown but may involve a deficit in BCR-responsive transcription (discussed in the following paragraph). In this regard, attenuated NF- $\kappa$ B-dependent transcription in quad mutant B cells may contribute to the phenotype; *CD19<sup>Cre</sup>*-mediated inactivation of the NF- $\kappa$ B regulators IKK2 and NEMO has little effect on B-cell development in the bone marrow but reduces mature B-cell numbers.<sup>54</sup>

Global gene expression was not greatly altered in resting *CBP* null B cells as measured by microarray, which is consistent with the modest B-cell phenotype. It is worth noting, however, that the effects of CBP and p300 loss on gene expression are underestimated because the frequency of deletion in bulk populations was always less than 100%. In contrast, the moderately reduced numbers of peripheral B cells in *CBP<sup>flox/flox</sup>; CD19<sup>+/Cre</sup>*, and to a lesser degree *p300<sup>flox/flox</sup>; CD19<sup>+/Cre</sup>*, mice correlated with attenuated BCR-responsive gene expression. BCR signaling is essential for the maintenance of resting mature B cells,<sup>55</sup> although the crucial downstream target genes are unclear. It is believed that NF- $\kappa$ B is one of the key transcription factors mediating BCR survival and proliferation signals, and we observed attenuated BCR-inducible expression in *CBP* and *p300* null B cells of the NF- $\kappa$ B targets A1 and cyclin D2, which are thought to have roles in these processes. Expression measured by microarray of the NF- $\kappa$ B family member



genes *Nfkb1*, *Nfkb2*, *Rel*, and *Rela* was not significantly different in *CBP* null B cells, although *Relb* was reduced approximately 1.7-fold (data not shown; Table 2).

TNF- $\alpha$  expression in response to BCR signaling was sensitive to the loss of CBP or p300, in general agreement with the findings by Falvo et al<sup>38</sup> who used TCR-activated *CBP*<sup>+/-</sup> T cells. LPS-dependent TNF- $\alpha$  expression in macrophages that were deficient for *CBP* or *p300* was not strongly affected, however, indicative that transcriptional regulators for this gene differ between cell lineages or signaling pathways.<sup>3</sup>

The mild B-cell phenotype seen in *p300*<sup>fllox/fllox</sup>; *CD19*<sup>+/-Cre</sup> mice was initially perplexing because we previously showed that *p300*<sup>KIX/KIX</sup> knock-in mutant mice have a marked deficiency in peripheral B cells.<sup>40</sup> The *p300*<sup>KIX</sup> mutation abrogates the interaction of the p300 KIX domain with the principal activation domains of c-Myb and CREB. The disparity between the *p300*<sup>KIX/KIX</sup> and *p300*<sup>fllox/fllox</sup>; *CD19*<sup>+/-Cre</sup> phenotypes could be explained if the p300 KIX domain is essential prior to, or during, the pro-B stage of development. Alternatively, there may be a delay in p300 protein and mRNA turnover following *p300*<sup>fllox</sup> recombination, which initiates at the pro-B stage in mice with the *CD19*<sup>Cre</sup> allele. This would prevent a deficiency of p300 KIX domain function at the pro-B and possibly later stages. We addressed these 2 models by inactivation of *p300*<sup>fllox</sup> in HSCs using *p300*<sup>fllox/fllox</sup>; *Mx-Cre* mice, which led to a substantial deficit in pro-B and pre-B progenitors, indicating that p300 has a crucial role in B lymphopoiesis prior to, or during, the pro-B stage. There is precedence for CBP/p300 interacting transcription factors being required very early in B-cell development but not at later stages. For example, the conventional knockout of PU.1 shows that it is essential for B cells but the conditional knockout reveals that it is not required beyond the pre-B-cell stage.<sup>56</sup>

It is somewhat counterintuitive that loss of both CBP and p300 did not lead to a significant decrease in peripheral B cells, even though loss of CBP alone causes a modest deficit. There are several possible explanations for this observation. First, B cells lacking

CBP alone may function in a cell-nonautonomous manner to suppress homeostatic mechanisms; since B cells lacking both CBP and p300 are very rare or nonexistent outside of the bone marrow where B-cell development occurs, homeostatic compensation of peripheral B-cell numbers would function normally in *CBP*<sup>fllox/fllox</sup>; *p300*<sup>fllox/fllox</sup>; *CD19*<sup>Cre</sup> mice. Second, homeostatic "cell counting" mechanisms may respond more efficiently to a profound deficit in peripheral B cells (ie, the lack of double-null B cells) than if cells are modestly affected in the periphery as with *CBP*<sup>fllox/fllox</sup>; *CD19*<sup>Cre</sup> mice. Third, *CBP* null peripheral B cells themselves may be defective for homeostatic proliferation.

In summary, we have discovered that the 2 most heavily connected transcriptional coactivators known are individually only marginally limiting in peripheral B cells. Even though they do not appear to separately have very unique roles in mature B cells, CBP and p300 function to maintain B-cell homeostasis, and p300 is particularly important during very early B lymphopoiesis. The loss of both CBP and p300, however, shows that the CBP/p300 family is extremely important for B lymphocytes. Finally, the absence of a robust phenotype in singly homozygous null mice suggests that there is an unexpected degree of functional redundancy supplied by coactivators unrelated to CBP and p300. This may indicate that coactivator networks are decidedly more robust and interconnected than typically appreciated,<sup>2</sup> although the identities of the CBP/p300-compensating factors are unknown.

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