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Transgenic Mice Expressing Dominant-Negative Bright Exhibit Defects in B1 B Cells

Jamee C. Nixon,* Scott Ferrell,*† Cathrine Miner,* Athenia L. Oldham,**‡
Ute Hochgeschwender,*‡ and Carol F. Webb2*‡

The transcription factor Bright up-regulates Ig H chain production from select V region promoters and requires Bright dimerization, Bruton’s tyrosine kinase (Btk), and the Btk substrate, TFII-I, for this activity. Defects in Btk cause X-linked immunodeficiency disease in mice and humans. Btk-deficient mice exhibit decreased serum IgM production, B cell developmental blocks, absence of peritoneal B1 cells, and subnormal immune responses against Ags, including phosphorylcholine, which confer protection against Streptococcus pneumoniae. Transgenic mice expressing dominant-negative Bright share similarities with Btk-deficient mice, including decreased serum IgM, poor anti-phosphorylcholine responses, and slightly reduced numbers of mature B cells. Although dominant-negative Bright mice developed B1 B cells, these were functionally deficient in Ig secretion. These data suggest a mechanistic explanation for the abnormal responses to phosphorylcholine observed in Btk-deficient mice, and indicate that Bright functions in a subset of Btk-dependent pathways in vivo, particularly those responses dominated by B1 B cells. The Journal of Immunology, 2008, 181: 6913–6922.

The B cell regulator of Ig H chain transcription (Bright) was the first eukaryotic member of the A + T-rich interacting domain (ARID) family of DNA-binding proteins described (1, 2), but its function in vivo has not been elucidated. ARID-containing proteins are expressed in a wide range of organisms, including Drosophila, Caenorhabditis elegans, and Xenopus (3), where they have diverse functions, including roles in gene expression, physical development, and cell growth (4). Although all contain a similar ARID sequence, only a few of the ARID3 subfamily members (including Bright/ARID3a) bind specific DNA motifs, and gene targets have been identified for only a handful of those (3, 4). Bright binds A + T regions flanking the intronic Ig μ H chain enhancer as well as to regions 5′ of select VH promoters, where it up-regulates Ig H chain transcription 5- to 7-fold (5, 6). The transcriptionally active complex is comprised of a Bright dimer, Bruton’s tyrosine kinase (Btk), and BAP135/TFII-I (7, 8), and transcription activation by this complex in vitro depends upon phosphorylation of TFII-I by Btk (6, 9). These data implied that Bright functions in a subset of Btk-dependent pathways.

Btk, a Tec family tyrosine kinase, was first identified as the defective gene in X-linked immunodeficient, or xid mice (10, 11). Xid and Btk-deficient mice are characterized by blocks in B cell development that result in reduced levels of serum IgM and IgG3 (11, 12), increased numbers of immature B cells in the periphery (13), deficient calcium and cell cycle responses in activated B lymphocytes (14, 15), absence of peritoneal B1 cells, and failure to respond to immunizations with type II pneumococcal polysaccharide or infection with Streptococcus pneumoniae (16). Although the contributions of Btk to B cell signaling pathways have been clarified (reviewed in Refs. 17 and 18), the mechanisms by which Btk deficiency blocks early B cell development, particularly in Btk-deficient humans who typically exhibit earlier and more pronounced blocks in B cell development than occur in mouse models, are unknown (19). Recently, we and other labs (6, 20, 21) have obtained data suggesting a role for Btk in transcription-mediated processes, including pathways that require Bright.

B cells from xid and Btk-deficient mice express Bright protein after stimulation with either LPS or CD40L, but do not form stable Bright transcription complexes (22), implying that Bright-mediated transcription is defective in these mice. Indeed, early studies indicated that canonical T15 Id responses to phosphorylcholine (PC) were deficient in xid male mice, but were unchanged in female littermates having a functional copy of Btk (16). Anti-PC Abs result almost exclusively from use of the S107 VH family V1 gene in normal mice. Data from our laboratory demonstrated that both Bright and Btk are required for up-regulation of V1 transcription in vitro (6). We therefore hypothesized that impairment of Bright function should lead to defects in V1 gene expression in mice and might explain the defective anti-PC responses observed in xid mice. Furthermore, because Btk also affects B cell development, we hypothesized that inhibition of Bright function could impair B cell development.

Bright is expressed in multiple tissues in the mouse embryo, becoming B cell restricted after birth (23). Similarly, the Drosophila homologue, dri, is expressed throughout embryonic development, but is gut associated in the adult (24). Therefore, we speculated that a Bright knockout might have an embryonic lethal phenotype, as was the case for Drosophila and Xenopus homologues (25, 26). Therefore, to address the role of Bright in Ig H chain expression and B cell development in vivo, dominant-negative (DN) Bright transgenic (TG) mice were generated that express a double point mutation in the DNA binding domain of

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2 Address correspondence and reprint requests to Dr. Carol F. Webb, 825 N. E. 13th St., Oklahoma City, OK 73104. E-mail address: webbc@omrf.org
3 Abbreviations used in this paper: ARID, A + T-rich interacting domain; Btk, Bruton’s tyrosine kinase; DN, dominant negative; FOL, follicular mature; KLH, keyhole limpet hemocyanin; MZ, marginal zone; NF, nitrophenyl; PC, phosphorylcholine; TG, transgenic; WT, wild type.

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Bright. These mutations do not affect interactions of Bright with Btk and TFII-I, but inhibit endogenous Bright activity by allowing formation of inactive Bright dimers that cannot bind DNA (9, 27). To eliminate effects due to expression in multiple tissues, the transgene was expressed from the B lineage-specific CD19 promoter. Phenotypic analyses of these mice indicate that Bright contributes directly to the function of peritoneal B1 B cells and normal immune responses against PC. Results indicate that Bright is particularly important for B1 B cell function.

Materials and Methods

Generation of TG mice

DN Bright was created, as previously described (27). The C terminus of DN Bright was tagged with the myc-Has sequence of pcDNA/TO/myc-HisB (Invitrogen). SV40 poly(A) (amplified from pSP55) and LoxP (ATAACTCTGGATAAATGCATGACGATATTAT) (28) sequences were subcloned onto the 3′ end of the DN-myc-Has construct, and the resulting fragment was ligated downstream of the B cell-specific 6.3-kb human Bcl6 promoter yeast extract R. HindIII, Erasmus Medical Center, Rotterdam, The Netherlands) (29). TGs were generated in FVB/N mice by the Oklahoma Medical Research Foundation Transgenic Core Facility. Founder mice were identified using primers within Bright (CAGATCCTCTTCTGAGTATGAG) and the myc-Has tag (CAGATCTTTCTGAGTAAAG) with PCR conditions of 93°C for 1 min, and 40 cycles of 93°C for 30 s, 57°C for 30 s, and 72°C for 45 s. Homozygous TGs were bred from heterozygous mice and confirmed by production of 100% TG positive progeny (at least 20 pups) with a non-Tg control. Male and female homozygous mice were analyzed at ages 6–13 wk. In some cases, mice overexpressing wild-type (WT) Bright from the CD19 promoter (WT-TG) were used (30). C57BL/6 and C57BL/6–2 Epi TG mice were obtained from Jackson ImmunoResearch Laboratories. All studies were performed under institutional guidelines for animal use.

Tissue collection, immunization, and infection

Sera, spleen, thymus, and bone marrow were harvested, as described (30). Peritoneal cells were obtained by lavage with PBS-3% FCS. In some cases, mice were immunized i.p. with keyhole limpet hemocyanin (KLH) or LPS, or with CD40L-expressing Sf9 or WT Sf9 control cells, as described (22). Cells were pulsed with 1 μCi of [3H]thymidine for 6 h. Peritoneal cells and sorted subpopulations were resuspended (2.5×106 cells/ml) and cultured with or without 10 μg/ml LPS for 3 days. Supernatants were collected for ELISA and RNA was isolated. For activation experiments, B cells were isolated using the B220 enrichment B cell isolation kit (Miltenyi Biotec), plated in 6-well plates at 1.0×106 cells/ml, and stimulated with LPS (25 μg/ml) for 18 h. EMSAs were performed for Bright and octamer-binding activity, as previously described (22).

ELISAs and ELISPOTs

The clonotype system-AP kit (Southern Biotechnology Associates) was used, according to the manufacturer’s directions, to test for serum isotypes. Standard curves were generated with isotopes of known concentration, and Ig levels were quantified using Excel software. Ag-specific Abs were detected using PC-BSA- or NP-BSA-coated plates, as described (30). Samples were assayed in duplicate at four or more dilutions, and samples were run with an MRX microtiter reader (Dynatech Laboratories). The BD ELISPOT assay (BD Biosciences) was used. Peritoneal cells were serially diluted onto MultiScreen-IP plates (Millipore) precoated with goat anti-mouse Ig and were incubated for 3 h at 37°C, followed by goat anti-mouse IgG or IgM directly or indirectly conjugated to HRP. Spots were detected with a 3-aminophenylcarbazole substrate and were counted by the ImmunoSpot Series 1 analyzer with ImmunoSpot 4.0 software (Cellular Technology).

Results

Generation of DN Bright TG mice

DN Bright (27) expressed from the B cell-specific human CD19 promoter (Fig. 1A) was used to generate TG mice. The CD19 promoter was chosen because CD19 expression begins at the early pro-B cell stage in mice and continues throughout B cell differentiation with down-regulation occurring in terminally differentiated plasma cells (34). Bright mRNA is first expressed in mouse B lineage cells at the pro-B to pre-B cell stage (23) and is therefore

were as follows: IgM exon 4, 5′-GTGAGCAACTGGAACCTGGAGGAA GTC-3′; IgM Sec, 5′-CAATAGAAGGTGGGCGCTTGTACG-3′; and IgM Mem, 5′-GGTACGCTCAGAGTGCTGAGAAGG-3′.

In vitro stimulation

Non-B cells were depleted from splenic cell suspensions with anti-Thy-1 and complement and incubated at 2×10^6 cells/ml, alone, with 25 μg/ml LPS, or with CD40L-expressing S9 or WT S9 control cells, as described (22). Cells were pulsed with 1 μCi of [3H]thymidine for 6 h. Peritoneal cells and sorted subpopulations were resuspended (2.5×10^6 cells/ml) and cultured with or without 20 μg/ml LPS for 3 days. Supernatants were collected for ELISA and RNA was isolated. For activation experiments, B cells were isolated using the B220 enrichment B cell isolation kit (Miltenyi Biotec), plated in 6-well plates at 1.0×10^6 cells/ml, and stimulated with LPS (25 μg/ml) for 18 h. EMSAs were performed for Bright and octamer-binding activity, as previously described (22).

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founder mice, four generated transgene-positive progeny (Fig. 1d). Endogenous protein, allowing us to monitor protein expression. Of 17 slightly larger product that distinguished DN Bright from the endogenous protein, allowing us to monitor protein expression. Of 17 founder mice, four generated transgene-positive progeny (Fig. 1B). Transgene expression levels varied among these lines from very low to moderate, as measured by RT-PCR of splenic B cell RNA (Fig. 1C). Because we expected robust transgene expression to more effectively inhibit endogenous Bright function, the B and D DN Bright TG lines exhibiting moderate DN Bright expression were bred to homozygosity, and the resulting homozygous D and B lines were interbred to produce double-TG (D/B) heterozygous mice. Both homozygous lines, the double-TG line, and heterozygous lines were analyzed. All animal studies were reviewed and approved by the appropriate institutional review board.

**DN Bright TG mice produce decreased levels of serum IgM**

To determine whether DN Bright affects Ig production in vivo, as we observed previously in vitro (6), serum IgM, IgG2a, IgG2b, IgG3, and IgA Ab levels were measured in age-matched male mice from three of the TG lines (Fig. 2). IgM levels were decreased by an average of 50% in the TG mice. IgM levels were decreased in each of the strains and in both heterozygous and homozygous mice. This contrasted with serum IgG2b, IgG3, and IgA levels that were not statistically different between controls and DN Bright mice, irrespective of the level of transgene expression. Unexpectedly, IgG2a levels increased ~2-fold in homozygous DN Bright mice relative to controls for reasons that remain unclear. Because these mice were on an FVB background, we have been unable to measure IgG1, as previously documented for TG mice overexpressing Bright (30). These data clearly indicate that DN Bright expression decreases normal IgM production in vivo.

**Peripheral lymphocytes develop normally in DN Bright TG mice**

DN Bright spleens appeared to be slightly smaller than non-TG age-matched controls upon visual examination, particularly in one of the three lines; however, total numbers of CD19+ B cells and CD3+ T cells were similar to control mice (Fig. 3A). Likewise, splenic architecture appeared normal in tissue sections stained for metallophyllic macrophages and IgM-positive B cells (Fig. 3B). Because preliminary examination of the DN Bright TG mice suggested that they were slightly smaller than WT littermates, thymic analyses were routinely performed on each mouse to ensure that any effects we observed were not due to generalized stress. Total numbers of CD4- and CD8-positive T cells were similar in all TG and control mice (Fig. 3C). Therefore, expression of DN Bright did not noticeably perturb peripheral lymphopoiesis.

**DN Bright transgene expression occurs early in B cell development, but is not maintained in mature B cell subpopulations**

Western blots of proteins from DN Bright TG tissues showed no DN Bright protein in any tissue of the three heterozygous and two homozygous lines examined, except in the spleen, where it was barely detectable even in homozygous mice (Fig. 4A and data not shown). In contrast, robust expression of WT-TG protein relative to endogenous Bright was evident in whole spleen cells from TG mice overexpressing WT Bright (WT-TG) (Fig. 4A, lane 1). The identical CD19 promoter was used for both DN and WT Bright TG mice. CD19 expression begins in early B cell development; therefore, bone marrow B cell subpopulations served as an initial starting point for our analysis of DN Bright expression. Total bone
marrow was isolated, and Abs against B220, IgM, and CD43 were used to distinguish individual B cell subpopulations by flow cytometry. Fig. 4B shows a typical analysis of pro-B cells (IgM⁺B220⁺CD43⁺), pre-B (CD43⁻B220lowIgM⁻), immature (CD43⁻B220lowIgM⁻), and recirculating (CD43⁻B220highIgM⁻) B cell compartments. No significant differences were observed in
relative percentages of bone marrow B lymphocytes between control and DN TG mice.

To confirm that transgene expression occurred in early B cells, equal numbers of sorted bone marrow B cell subpopulations were analyzed for Bright expression by Western blotting (Fig. 4C). DN Bright was expressed at low levels in pro-B cells, and levels increased (from 2- to 7-fold) in pre-B and immature B cells. Although endogenous Bright was detected at low levels in each of the four subpopulations analyzed (Fig. 4C), DN Bright protein expression was not detectable in the mature, recirculating subpopulation of B cells that has migrated back to the bone marrow from the periphery. In contrast, TG Bright protein was clearly evident in all bone marrow B cell subsets from WT-TG mice, including the mature, recirculating population (30). These data indicate that DN Bright protein expression begins early in B cell development, but is not maintained at detectable levels at all stages of B cell differentiation.

Because robust levels of DN Bright were detected in immature B cells from bone marrow, but not in the more mature recirculating cells, we determined which splenic B cell subpopulations expressed DN Bright. Four-color analyses were used to identify the transitional T1, T2, T3, marginal zone (MZ), and follicular mature (FOL) B cell subpopulations (35). Fig. 4D shows immature Bright (B220<sup>+</sup>CD93<sup>+</sup>) analyzed for IgM and CD23 expression to reveal T1, T2, and T3 transitional subpopulations, and the mature MZ and FOL cells (B220<sup>−</sup>CD93<sup>−</sup>). Western blots using equivalent numbers of sorted cells indicated that only the T1 subpopulation most recently emigrated from the bone marrow exhibited appreciable levels of DN Bright protein. In some cases, very low levels of protein were detected in T2 transitional cells (Fig. 4E and data not shown). However, none of the heterozygous, homozygous, or double-TG lines examined expressed detectable DN Bright protein in MZ or FOL B cells. In contrast, sorted subpopulations from WT-TG mice showed robust TG protein expression in multiple lines in all splenic B cell subpopulations assessed (30) (Fig. 4E). These data indicate that the absence of detectable DN Bright protein in FOL B cells could not be overcome by increasing transgene copy number.

Our earlier findings showed that endogenous Bright was not normally transcribed in most mature splenic B cells, but was abundant in germinal center cells (23). Therefore, we asked whether DN Bright transcript levels differed in immature cells that expressed TG protein vs mature cells that did not exhibit detectable Bright protein. We reasoned that cells expressing high levels of DN Bright might be eliminated at the T1 to T2 transition such that only cells that had eliminated TG protein expression could survive. Therefore, DN TG mice crossed 10 generations onto the C57BL/6 background were bred with mice of the same background expressing a BCL-2 transgene, which acts in an anti-apoptotic fashion to prolong survival of B cells (36). Although total B cell numbers were expanded in the double-TG mice produced, DN Bright was not detectable in the follicular B cells from these mice by Western analyses (data not shown). DN Bright mRNA measured by semi-quantitative RT-PCR was present at similar levels in both T1 and FOL B cells in the original lines and in the BCL-2 TGs (Fig. 4F and data not shown), suggesting that the mature B cells from the DN Bright TG mice should be capable of producing DN Bright protein. The reasons for down-regulation of DN Bright protein in the mature B cells are unknown, but are likely to occur via a posttranscriptional mechanism.

### DN Bright expression decreases total B cell numbers only slightly

Flow cytometric analyses of B cell subpopulations in DN TG mice (Fig. 4) did not show statistically significant alterations in relative proportions of B cell subpopulations; however, total numbers of B cells were also determined in the DN Bright TG vs littermate control mice. Neither total cells nor numbers of B cells within individual bone marrow subpopulations were statistically different among control mice and heterozygous (Tg<sup>D<sup>+/−</sup></sup>) or homozygous (D<sup>++/−</sup>) DN TG mice (Table I). However, total splenic B cells in Tg<sup>D<sup>++/−</sup></sup> mice 7–9 wk old were decreased slightly compared with littermate controls, and both FOL and MZ B cell subpopulations were significantly reduced compared with controls (Table I). Homozygous mice (10–13 wk old) did not exhibit differences in total B cell or individual B cell subpopulation numbers compared with age-matched controls (Table I). To discern whether younger mice exhibited greater decreases in peripheral B cells due to DN bright expression, 3-wk-old mice were examined. Although WT-TG mice of 3 wk of age already exhibited increased B cell subpopulations (30), the younger DN TG splenic B cell numbers did not show age-dependent decreases due to DN Bright expression (data not shown). Data were supported by analyses from two additional heterozygous founder lines, another homozygous line (n = 13), and the double TG (n = 12). Thus, whereas DN Bright expression did not block maturation of immature splenic B cells into mature B cells, as occurs in sid mice, DN Bright expression reduced total mature B cell numbers in spleens of young heterozygous TGs.

<table>
<thead>
<tr>
<th>Bone marrow cells (per femur)</th>
<th>CON&lt;sup&gt;b&lt;/sup&gt; (7–9 wk)</th>
<th>TG D&lt;sup&gt;++/−&lt;/sup&gt; (7–9 wk)</th>
<th>CON (10–13 wk)</th>
<th>TG D&lt;sup&gt;++/−&lt;/sup&gt; (10–13 wk)</th>
</tr>
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<tbody>
<tr>
<td>Total (&lt;×10&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>2.10 ± 0.34 (8)</td>
<td>2.05 ± 0.31 (9)</td>
<td>1.96 ± 0.22 (13)</td>
<td>1.79 ± 0.13 (9)</td>
</tr>
<tr>
<td>B220&lt;sup&gt;+&lt;/sup&gt; (&lt;×10&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>1.7 ± 0.3</td>
<td>2.2 ± 0.2</td>
<td>2.8 ± 0.4</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>Pro B (&lt;×10&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>4.7 ± 0.8</td>
<td>5.3 ± 0.6</td>
<td>4.4 ± 0.4</td>
<td>4.4 ± 0.7</td>
</tr>
<tr>
<td>Pre B (&lt;×10&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>8.8 ± 2.0</td>
<td>11.6 ± 1.9</td>
<td>10.3 ± 2.0</td>
<td>8.9 ± 1.7</td>
</tr>
<tr>
<td>Immature (&lt;×10&lt;sup&gt;4&lt;/sup&gt;)</td>
<td>3.1 ± 0.9</td>
<td>3.4 ± 0.6</td>
<td>3.8 ± 0.7</td>
<td>3.3 ± 0.6</td>
</tr>
<tr>
<td>Recirculating (&lt;×10&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>8.0 ± 2.2</td>
<td>6.1 ± 1.3</td>
<td>6.7 ± 1.0</td>
<td>5.4 ± 1.2</td>
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Table I. Lymphocyte subpopulations in heterozygous and homozygous DN transgenics<sup>a</sup>

<sup>a</sup>Male or female mice of ages indicated were analyzed. Numbers represent the mean ± SEM. Sample size is shown in parentheses after average total cell number for each tissue. Student’s t test; *, p = 0.0325; **, p = 0.0285.

<sup>b</sup> CON, Control.
CD40L and anti-IgM induce normal responses in DN Bright splenic lymphocytes

To determine whether DN Bright TG B cells exhibited defects in B cell activation, B cells were purified from TG and control spleens and were stimulated with F(ab')2 anti-IgM (data not shown) or LPS. Relative levels of surface MHC II, CD69, CD86, CD80, and CD40 were assessed by flow cytometry. DN Bright splenic B cells expressed and up-regulated each of these activation markers as effectively as non-TG control cells with either stimulus (data not shown). In addition, proliferative responses to CD40L were tested. DN Bright TG and control lymphocytes responded similarly to CD40L, as measured by [3H]thymidine incorporation (data not shown); however, in all cases, cultured DN Bright cells that were not stimulated with CD40L incorporated more [3H]thymidine than unstimulated non-TG cells. These data suggest that DN Bright splenic B cells can be activated similarly to non-TG control cells with either stimulus.

Peritoneal B cells develop in DN Bright TG mice, but are functionally deficient

Half of the naturally protective serum IgM produced in mice is secreted by peritoneal B1 cells (reviewed in Ref. 37). It was therefore possible that reduced serum IgM in the DN Bright TG mice was due to defects in peritoneal B cells. Because Btk-deficient mice fail to develop B1a peritoneal B cells, but can produce B1b and B2 peritoneal B cells (38, 39), we therefore hypothesized that DN Bright mice would be deficient in B1a cells. The DN Bright mice, however, not only produced both B1a and B1b cells (Fig. 5A), but total numbers of peritoneal cavity B cells were significantly increased (p = 0.00486, two-tailed Student’s t test) by ~30% in 11 TG mice relative to controls (2.6 × 10^6 cells vs 2.0 × 10^6 cells, respectively). Relative proportions of B1a, B1b, and B2 B cells did not differ between TGs and control mice (Fig. 5A).

Peritoneal B cells develop in DN Bright TG mice, but are functionally deficient

To determine whether DN Bright peritoneal B cells were functionally normal, these cells were stimulated in vitro with LPS, and IgM production was measured by ELISA (Fig. 6A). Although basal IgM production from unstimulated cells was similar in all mice, IgM production was consistently decreased from DN Bright
TG peritoneal B cells relative to controls. WT-TG peritoneal B cells showed no statistical difference in secretion from normal non-TG controls (Fig. 6A, right panel), suggesting the decrease in secretion in the DN TGs is due to the DN protein. However, ELISPOT analyses of total peritoneal B cells indicated that total numbers of DN Bright IgM-producing cells were not decreased, but were slightly increased relative to controls, as were the numbers of WT-Bright IgM-producing cells (Fig. 6B). Therefore, the decreased secretion of IgM from DN Bright peritoneal cells in Fig. 6A cannot be explained by fewer IgM-secreting cells. Semiquantitative RT-PCR showed no differences in Blimp-1 or XBP mRNAs in the DN Bright TG B1 cells compared with control B1 cell RNA (data not shown), suggesting that decreased IgM production was not the result of defects in regulation of these transcripts. However, examination of membrane vs secretory IgM by PCR demonstrated that whereas control B cells expressed 60-fold more secretory IgM than membrane IgM, DN TG B cells consistently expressed less secretory vs membrane message (μsec/μmem = 6.1) (Fig. 6C). Peritoneal B cells from WT-TG mice produced approximately equivalent amounts of secretory and membrane IgM mRNA, but this may be consistent with a general increase in Ig transcription due to increased levels of Bright protein, particularly because IgM secretion was not inhibited in these cells (Fig. 6A). Together, these data suggest that reduced serum IgM in the DN Bright mice is due to decreased IgM production from each cell, rather than decreased numbers of IgM-producing cells. Therefore, we conclude that Bright function in peritoneal B cells is important for maintaining normal IgM production.

FIGURE 6. DN Bright expression inhibits B1 cell function. A, Peritoneal B cells from CON and TG-D mice (left panel) or WT-Bright (WT-Br) mice (right panel) were cultured with 20 μg/ml LPS for 72 h, and secreted IgM was measured by ELISA. Symbols represent individual cultures. Data shown are representative of two to three experiments performed. Means and SE bars are indicated. p = 0.0183; Student’s t test for TG-D compared with CON. B, ELISPOT analyses of LPS-stimulated peritoneal B cells from CON, TG-D, and WT-Br mice show numbers of IgM+ cells obtained per input cell. SE bars and means are shown. Data are representative of three experiments. C, Peritoneal B cell mRNAs from control (CON), DN, and WT-TG mice were stimulated with LPS, as in A, and assessed by PCR for relative levels of μ membrane (mem) vs secretory (sec) IgM. Ratios were quantified and are listed below. Actin levels for each sample are shown and quantified. Data are representative of samples from four to seven mice each.

FIGURE 7. DN Bright expression causes deficient anti-PC responses and increased susceptibility to S. pneumoniae. Sera from five control (CON) and four TG (TG-D) preimmune and postimmunized 8- to 12-wk-old male mice were analyzed by ELISA for PC-specific IgM (A). The last positive dilution from triplicate samples was the defined endpoint. At day 7, p = 0.0268 by two-tailed Student’s t test. At day 25, p = 0.0483 by one-tailed Student’s t test. B, Mice immunized with NP-KLH were analyzed similarly for anti-NP-specific Abs. SE bars are indicated. C, RNA from CON and TG-D DN Bright follicular B cells was amplified for S107 V1 IgM and actin by RT-PCR. D, CON and TG-D mice were infected with S. pneumoniae and monitored for survival. Symbols represent the hours survived by one mouse. All samples fall within 2 SDs from the mean. Means and SE bars are indicated. p = 0.019, Mann-Whitney analysis, and 0.0079 by Student’s t test.

Anti-PC and responses to S. pneumoniae infection are attenuated in DN Bright TG mice

Ab responses to PC have been shown to be predominantly of the IgM isotype and are primarily generated by B cells using the S107 V1 H chain gene (16). We showed that enhanced expression of V1 in vitro depends on Bright function and is inhibited by DN Bright (6). Furthermore, immune responses against PC were shown to be defective in xid mice, whereas responses to the hapten NP were normal (16). These results suggested the hypothesis that responses
using the V1 gene would be inhibited in the DN Bright TG mice. In further support of this hypothesis, overexpression of WT Bright caused more than a 10-fold increase in PC-specific IgM (30). To determine whether anti-PC responses were impaired by DN Bright expression, antibodies were preblotted and immunized (day 0) with PC-KLH or with NP-KLH. Sera were collected at several time points after immunization and analyzed for PC- or NP-specific IgM by ELISA. DN Bright mice exhibited significant deficiencies in IgM responses to PC compared with non-TG controls at both days 7 and 25 (Fig. 7A). In addition to IgM, sera from immunized mice were analyzed at day 25 for PC-specific total Ig to detect isotype-switched Abs. Homozygous DN Bright mice also exhibited significant decreases in PC-specific Abs relative to those of control mice (Fig. 7A). These data were confirmed as representing more than a 2-fold decrease at the μg level when compared with a standard IgM (BH8) anti-PC Ab (provided by J. Kearney, Birmingham, AL). Heterozygous DN Bright TG mice exhibited intermediate responses to PC-KLH relative to control and homozygous DN Bright TG mice (data not shown). In contrast, control and DN TG mice immunized with NP-KLH showed similar levels of NP-specific Ab at both days 7 (IgM) and 25 (Ig) (Fig. 7B). Furthermore, mRNA expression from the V1 S107 gene in unimmunized mice, determined by RT-PCR, was decreased (from 33% of the control down to 3.6% for the lowest dilution) relative to levels observed in the same numbers of sorted cells in normal control mice (Fig. 7C). These data suggest that DN Bright does not globally impair all immune responses, but selectively inhibits a subset of immune responses, including that against PC.

PC is a component of the cell wall of S. pneumoniae that has been shown to elicit protective responses against this organism (31). To confirm and extend the data showing that DN Bright TG mice were deficient in anti-PC responses, mice were infected i.p. with S. pneumoniae. Mice infected with 10–100 bacterial CFU were monitored over a period of 4–7 days, and times of death postinfection were recorded (Fig. 7D). The mean survival time for control mice was 95 h, whereas DN Bright TG mice died at an average of 76 h postinfection. These experiments indicate that DN Bright TG mice are more susceptible to infection with S. pneumoniae than normal controls, but they are not as sensitive as Btk-deficient mice, in which death occurred at an average of 48 h (31). We conclude that Bright contributes to normal immune responses against this important pathogen.

Discussion

These data represent the first in vivo analyses resulting from Bright inhibition. TG mice expressing DN Bright in B lineage cells developed all B cell subpopulations, suggesting that Bright function is not critical for B cell maturation once CD19+/− B lineage commitment has occurred. However, DN Bright protein expression was not maintained beyond the immature B cell stage in the spleen, so no conclusions can be drawn regarding Bright’s role in mature or terminally differentiated splenic B cells. Nonetheless, serum IgM levels were decreased by DN Bright expression, and peritoneal B1 cells both expressed DN Bright protein and exhibited impaired function. Furthermore, our data indicate that Bright function is important for a subset of immune responses that uniquely use the S107 family V1 gene for Abs against PC and S. pneumoniae. These data suggest a mechanistic reason for observations made over 20 years ago, which indicated that xid mice were specifically deficient in responses against PC and S. pneumoniae (16, 31), and show that Bright contributes to these responses. Moreover, these data suggest that Bright function is critical for only a subset of immune responses.

Several similarities exist between DN Bright and Btk-deficient mice. Both lines exhibit reduced levels of serum IgM relative to controls, although IgG3 levels are only reduced in Btk-deficient mice. Normal IgG3 levels in DN Bright mice could be due to incomplete inhibition of Bright function in mature B cells. The majority of serum IgM is produced by peritoneal B cells, an important component of the innate immune response (37, 40). In Btk-deficient and xid mice, reduced IgM levels correlate with an absence of B1 peritoneal B cells (10–12). In contrast, DN Bright TG mice exhibited increased numbers of peritoneal cavity B cells with high expression of DN Bright protein. In other systems, increased cell numbers develop to compensate for functional defects. In support of this hypothesis, IgM secretion was impaired in DN Bright peritoneal B cells, and production of the secretory form of IgM was repressed relative to controls. Btk may also contribute to Bright function in B1 cells, as indicated by experiments with mice expressing intermediate levels of Btk, and suggesting that Btk plays a role in B1 cell maintenance once they develop (41). Because DN Bright may not have been expressed in the earliest B1 B cell progenitors, we cannot assess whether Bright is also important for B1 lineage development.

CD19−/− mice also display decreased levels of IgM (34). In this case, reduced IgM levels are thought to result from combined effects of decreased total B cell numbers in the bone marrow, spleen, and peritoneal cavity, and a decrease in proliferative responses to LPS, anti-IgM, and IL-4 (42). DN Bright splenic B cells responded normally to LPS, anti-IgM, and CD40L stimulation, suggesting that activation was not impaired in DN Bright mice. These findings were consistent with the low levels of DN Bright protein exhibited by TG follicular B cells. Although endogenous Bright is also down-regulated in follicular B cells (23), WT-TG Bright mice consistently expressed detectable levels of TG protein from the identical CD19 promoter (30). DN Bright protein expression was down-regulated in follicular B cells of homozygous DN Bright mice that expressed the highest levels of DN Bright in immature B cells, suggesting that cell survival might depend upon active down-regulation of DN Bright protein. However, constitutive expression of BCL-2 in follicular B cells was not able to overcome the down-regulation of DN Bright protein in double-TG follicular B cells. Therefore, the reasons for posttranslational down-regulation of DN Bright in these mice remain obscure.

Btk-deficient mice exhibit defects in the maturation of immature B cells to follicular B cells, and this results in production of fewer total B cells (43, 44). In these mice, immature transitional T1 B cells accumulate and comprise a higher percentage of B lymphocytes relative to normal controls, whereas follicular B cell numbers decrease and total marginal B cells remain equivalent to control numbers. Several lines of evidence support the idea that Bright functions in immature B cells. Both endogenous and DN Bright protein are abundantly expressed in those cells. DN Bright protein appears to be down-regulated specifically after the T1 transitional stage, implying that Bright function is important for T1 to T2 transition. Finally, overexpression of WT Bright in TG mice led to a 3- to 5-fold increase in total numbers of T1 cells (30). Others have shown that transitional B cells in Btk-deficient mice do not proliferate normally in vitro in response to anti-IgM signals (43, 44), and both proliferation and cell cycle abnormalities have been observed previously in xid mice (15, 45). Roles for human Bright in cell cycle regulation have been suggested (46, 47). Additional experiments will be required to elucidate how Bright functions in immature B cells.

Another similarity between xid and DN Bright TG mice is the selective impairment of responses to specific subsets of Ags. Data showing that xid mice do not produce T15 Abs after immunization
with PC-KLH, whereas normal female littermates generated robust T15 Id responses (16), established a stringent requirement for Btk in anti-PC responses. DN Bright TG mice also showed significant decreases in PC-specific Ig relative to controls, suggesting that Bright and Btk function in the same molecular pathway in this response. Likewise, both Btk-deficient (31), and DN Bright TG mice were more susceptible than control mice to death after infection with the PC-containing bacteria, S. pneumoniae. Although xid B cells can rearrange the SI07 V1 locus normally, the resulting B cells do not produce normal levels of anti-PC Abs (48). In our experiments, B cells from nonimmunized DN Bright TG mice exhibited reduced mRNA transcripts using the SI07 V1 H chain gene relative to normal controls, consistent with our in vitro data indicating the importance of Bright for transcription of the V1 gene. It is not clear in either xid or DN Bright TG B cells whether reductions in V1 expression are due to reduced numbers of V1-expressing cells, or to impaired Ig production from this specific B cell population. Nonetheless, these data indicate that Bright and Btk are both critical for responses involving the SI07 V1 gene.

One interesting possibility raised by these data is that all Ig H chain promoters are not regulated identically. Responses to Ags other than PC, such as NP-KLH, were not impaired in DN Bright mice or in xid mice (49). Although DNA-binding motifs for Bright exist within the 5′ flanking regions of the V1 gene, other V region genes are not associated with Bright binding sites (50, 51). Precedence exists for differential requirements for the transcription factor OcaB in regulation of subsets of L chain genes (52), but differential regulation of H chain genes in vivo has not been suggested. Alternatively, other data suggest that B cells expressing subsets of H chain genes are uniquely targeted toward distinct B cell subpopulations (53). Existence of independent B lineages that specifically express the V1 gene and require Bright might explain our observations. A small subpopulation (4–10%) of immature bone marrow B cells was identified that preferentially expressed the SI07 gene in BALB/c mice (54). However, it is not clear that these cells represent an independent lineage of B cells. Elegant studies using xid mice expressing an anti-PC H chain transgene resulted in severely impaired peripheral B cell development, suggesting that the PC-specific B cells were deleted or failed to exit the bone marrow properly (49), whereas TNP-specific B cells developed normally on the xid background. Our data suggest that the defects observed in those mice could have resulted from impaired Btk/Bright-dependent regulation of the BCR. It is not clear whether Bright functions solely through regulation of the Ig locus or through other gene targets, which is certainly the case in non-B cells. Bright is expressed in multiple embryonic tissues in the mouse (23), and regulates non-B cell pathways in Drosophila (25, 26). Additional experiments will be required to distinguish among these possibilities.

DN Bright associates with Btk in vitro, but does not bind DNA (27). Therefore, it was a formal possibility that high levels of DN Bright protein could simply sequester Btk and inhibit its function, producing another form of Btk deficiency in the DN Bright TG mice. This cannot explain the phenotype of the DN Bright TG mice because it differed substantially from that of xid mice. Preliminary data from Bright knockout mice also suggest that they differ phenotypically from xid mice (C. Webb and P. Tucker, unpublished results). Moreover, data from mice expressing even higher levels of WT Bright than were observed in the DN Bright TG mice were autoimmune rather than immunodeficient (30), indicating that DN Bright did not exert its effect simply by sequestering Btk. It will ultimately be important to determine whether Ig secretion and B cell differentiation of conventional B2 cells are affected by Bright inhibition as it is in B1 cells. However, these studies await additional model systems.

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Disclosures

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References


