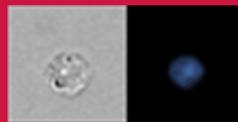


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This information is current as of May 17, 2022.

*J Immunol* 2010; 184:5678-5685; Prepublished online 16 April 2010;  
doi: 10.4049/jimmunol.0903854  
<http://www.jimmunol.org/content/184/10/5678>

**Supplementary Material** <http://www.jimmunol.org/content/suppl/2010/04/16/jimmunol.0903854.DC1>

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*The Journal of Immunology* is published twice each month by  
The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# CpG Inhibits Pro-B Cell Expansion through a Cathepsin B-Dependent Mechanism

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**TLR9 is expressed in cells of the innate immune system, as well as in B lymphocytes and their progenitors. We investigated the effect of the TLR9 ligand CpG DNA on the proliferation of pro-B cells. CpG DNA inhibits the proliferation of pro-B, but not pre-B, cells by inducing caspase-independent cell death through a pathway that requires the expression of cathepsin B. This pathway is operative in Rag-deficient mice carrying an SP6 transgene, in which B lymphopoiesis is compromised, to reduce the size of the B lymphocyte precursor compartments in the bone marrow. Thus, TLR9 signals can regulate B lymphopoiesis in vivo. *The Journal of Immunology*, 2010, 184: 5678–5685.**

**B** cell development occurs through stepwise differentiation of hematopoietic stem cells and is controlled at various checkpoints to eliminate the cells that fail to successfully rearrange their Ig genes (1). The first committed progenitors are pro-B cells, which initiate Ig H chain recombination mediated by the enzymes Rag1 and Rag2. In the subsequent developmental stage, pre-B cells express a pre-BCR composed of an H chain and the surrogate L chain and undergo Ig L chain recombination (2).

Cells that fail to produce a functional receptor undergo apoptosis, but the precise mechanisms leading to their elimination are still unclear. Major regulators of apoptotic pathways are the members of the Bcl-2 family of proteins, which can be either anti- or proapoptotic. Deficiency in the antiapoptotic protein Bcl-2 leads to a reduced number of pro-B cells (3), whereas, conversely, overexpression of this protein or deficiency in the proapoptotic family member Bim results in an enlarged pro-B cell compartment (4, 5). Activation of proapoptotic proteins of the Bcl-2 family triggers the intrinsic apoptotic pathway, with activation of the effector caspases-3, -6, and -7 (6). Caspases are also activated by

the so-called extrinsic pathway, which requires Fas-associated death domain protein and caspase-8 (6).

Other cell death pathways involve the lysosomal cysteine proteases known as cathepsins. Following some apoptotic stimuli, cathepsins and other lysosomal components are released to the cytosol where they induce cell death, either through caspase-dependent or caspase-independent pathways (7). Cathepsin B is thought to play an important role in this lysosomal pathway because its inhibition is needed for the survival of precursors of memory CD8<sup>+</sup> T cells (8), and its inactivation protects hepatocytes from apoptosis (9).

TLRs are a family of pattern recognition receptors that induce production of inflammatory cytokines by cells of the innate immune system. It was recently found that hematopoietic stem cells, as well as multipotent progenitors of human and mouse origin, express TLRs and respond to their ligands by preferentially adopting a myeloid fate (10, 11). B lymphocytes also express TLRs and respond to ligand binding by polyclonal activation, proliferation, and differentiation into Ab secretion.

TLR9 recognizes unmethylated DNA containing CpG motifs, typical of viral and bacterial DNA (12) but also found, albeit to a lesser extent, in mammals (13). Stimulation of common lymphoid progenitors through this receptor inhibits differentiation along the B lineage and stimulates differentiation of dendritic cells (DCs) (11). B cell progenitors express TLR9 (14), but little is known of its role in lymphopoiesis. We investigated whether TLR signaling regulates B cell development and show in this paper that TLR9 ligands inhibit the expansion of murine pro-B cells in vitro. Using purified populations, we demonstrate that CpG inhibits pro-B, but not pre-B, cell expansion through a direct mechanism, independent of the release of soluble mediators. Additionally, we show that CpG induces apoptosis independently of Bcl-2 family members and of caspase activation through a mechanism that requires cathepsin B. Furthermore, we show that TLR9 signaling also reduces lymphopoiesis in vivo in the SP6 transgenic (Tg) model in which B cell production is impaired. We conclude that endogenous TLR9 ligands regulate B cell development and homeostasis by inducing caspase-independent cell death in progenitor B cells.

## Materials and Methods

### Mice and bone marrow cells

C57BL/6 (B6) Ly5.1, B6 Rag2<sup>-/-</sup>, and SP6 Rag2<sup>-/-</sup> Tg mice (15) were bred in the animal facilities of Institut Pasteur (Paris, France). TLR9<sup>-/-</sup>

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Received for publication December 2, 2009. Accepted for publication March 18, 2010.

This work was supported by the Institut Pasteur and the Fondation pour la Recherche Médicale. A.I.L. was supported in part by the Arthritis Fondation Courtin.

The sequences presented in this article have been submitted to the European Molecular Biology Laboratory-European Bioinformatics Institute Array Express under accession number E-MEXP-2595.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: BM, bone marrow; DC, dendritic cell; IFNAR, type I IFN receptor; PI, propidium iodide; RACK1, receptor for activated C-kinase 1; Tg, transgenic; TSLP, thymic stromal lymphopoietin.

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(16) mice were obtained from the Centre de Distribution des Typages et d'Archivage Animal (Orléans, France) and bred in our facilities. SP6 Rag2<sup>-/-</sup> and TLR9<sup>-/-</sup> were intercrossed to generate TLR9-deficient SP6<sup>+</sup> TLR9<sup>-/-</sup> Rag2<sup>-/-</sup> and control SP6<sup>+</sup> TLR9<sup>+/+</sup> Rag2<sup>-/-</sup> mice. MyD88<sup>-/-</sup> mice were a gift from Richard Lo-Man (Institut Pasteur). Type I IFN receptor-deficient (IFNAR<sup>-/-</sup>) mice (17) were a kind gift from Dr. Matthew Albert (Institut Pasteur). Rag2<sup>-/-</sup> hBcl2 Tg (18) mice were a kind gift from Hélène Decaluwe and James P. Di Santo (Institut Pasteur). Bim<sup>-/-</sup> (19) bone marrow (BM) was a kind gift from Andreas Villunger (Innsbruck Medical University, Innsbruck, Austria). Cathepsin B<sup>-/-</sup> and cathepsin B<sup>+/+</sup> control mice (20) were a kind gift from Bénédicte Manoury (Institut Curie, Paris, France). All animal experiments were performed in accordance with the guidelines from Institut Pasteur, approved by the French Ministry of Agriculture.

### Reagents

The following TLR ligands were purchased from Invivogen (San Diego, CA) and used at the indicated concentrations: as a TLR9 ligand, we used phosphorothioate CpG 1826 at 0.5 μM; the TLR4 ligand used was ultrapure *Escherichia coli* LPS at 10 μg/ml; as a TLR2 ligand, we used PAM3CSK4 at 1 μg/ml; as a TLR3 ligand, we used polyinosinic:polycytidylic acid at 10 μg/ml; and as a TLR7 ligand, we used imidazoline CL097 at a concentration of 5 μg/ml. The caspase inhibitor Z-VAD-FMK was purchased from Sigma-Aldrich (St. Louis, MO) and used at 50 μM. Staurosporin was purchased from Sigma-Aldrich and used at 1 μM.

### Abs, flow cytometry, and cell sorting FACS analysis and FACS sorting

Flow cytometry analyses were performed in an FACSCalibur or FACSCanto cytometers (BD Biosciences, San Jose, CA). Cell sorting was performed in a MoFlo (DakoCytomation, Carpinteria, CA) or FACSaria (BD Biosciences). Fluorochrome-coupled Abs were purchased from BD Biosciences [CD19 (1D3), CD43 (S7), IgM (II/41), λ (R26-46), CD69 (H1.2F3), P-Stat5 (47), Ter-119, CD45.1 (A20), and CD45.2 (104)] or Jackson ImmunoResearch Laboratories [IgM (donkey F(ab')<sub>2</sub>; West Grove, PA). P-Stat5 (BD Biosciences) was detected following the manufacturer's instructions.

### Estimation of cell death in culture

Exposure of phosphatidylserine residues was detected by staining with propidium iodide (PI) and Annexin V (BD Biosciences) following the manufacturer's protocol. To determine the inhibition of apoptosis by Z-VAD-FMK, the PVN53 fibrosarcoma line was incubated for 48 h with or without 500 pM IFN-α2 in the presence of different doses of Z-VAD-FMK. Frequency of apoptotic cells was determined by fixing the cells in 70% ice-cold ethanol for 1 h and DNA staining in PBS containing 0.18 mg/ml PI and 0.4 mg/ml DNase-free RNase type 1-A (Sigma-Aldrich) at room temperature. Percentage of cells in sub-G<sub>1</sub> phase was determined by flow cytometry.

### Cultures of precursor B cells

BM cells were flushed from femora and tibia of mice. Cell suspensions were enriched for B lineage cells by magnetic positive selection using anti-CD19 beads (Miltenyi Biotec, Auburn, CA), and pro-B cells (CD19<sup>+</sup> CD43<sup>+</sup> IgM<sup>-</sup> IgD<sup>-</sup>) were sorted by FACS. The culture medium was Opti-MEM (Life Technologies, Rockville, MD) with 10% FCS (Eurobio, Les Ulis, France), penicillin (50 U/ml), streptomycin (50 μg/ml), and 2-ME (50 μM) (Life Technologies). Cells were cultured with saturating amounts of IL-7 or with 20 ng/ml recombinant thymic stromal lymphopoietin (TSLP) (R&D Systems, Minneapolis, MN). Individual cultures were started at 1000–5000 cells/well. After 1 wk of culture, the cells were stained for CD19 and IgM, counted, and analyzed by flow cytometry. Cells were counted using latex beads (Coulter CC Size Standard L10, Beckman Coulter, Fullerton, CA).

### TLR9<sup>-/-</sup> stromal cell lines

Primary adherent stromal cell cultures were established from the BM of TLR9<sup>-/-</sup> mice. BM cells were incubated with biotinylated Ter-119, Mac-1 (M1/70), and CD45 (30-F11) Abs (BD Biosciences) and depleted using streptavidin microbeads (Miltenyi Biotec). The cells were cultured in Opti-MEM medium (Life Technologies) with 20% FCS, penicillin (50 U/ml), and streptomycin (50 μg/ml). After 1 mo, remaining hematopoietic cells were depleted by sorting for CD45- and Mac-1-negative cells. Lines of adherent cells were then propagated in the same medium supplemented with basic fibroblast growth factor at 10 ng/ml.

### Microarray analysis

Rag2<sup>-/-</sup> pro-B cells were cultured in complete medium with IL-7, and in the absence of stroma 3, cultures were supplemented with CpG and three cultures not supplemented. After 16 h of culture, the cells were washed and lysed for RNA extraction with the RNeasy Plus Mini kit (Qiagen, Valencia, CA). A total of 100 pg RNA per sample was processed, labeled, and hybridized to the mouse GeneChip Gene 1.0 ST Array (Affymetrix, Santa Clara, CA) containing ~27 probes for each of the 28853 genes included (Plate-forme Pucés à ADN of the Institut Pasteur).

The DNA microarray data are accessible at European Molecular Biology Laboratory-European Bioinformatics Institute Array Express ([www.ebi.ac.uk/microarray-as/ae/](http://www.ebi.ac.uk/microarray-as/ae/)) with the accession number E-MEXP-2595.

### Quantitative PCR

RNA was extracted with the RNeasy Micro Kit (Qiagen), and first-strand cDNA was synthesized using Superscript III Reverse transcriptase (Invitrogen, Carlsbad, CA) and poly(dT) oligos. Quantitative PCR reactions were performed in triplicate in TaqMan Master Mix with TaqMan probes for CD36 (Mm00432403\_m1) and HPRT (Mm00446968\_m1) in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA).

### CFSE labeling

Labeling was performed with 2 μM CFSE in PBS/3% FBS for 12 min at room temperature, protected from light, and followed by two washes in culture medium.

### Western blot

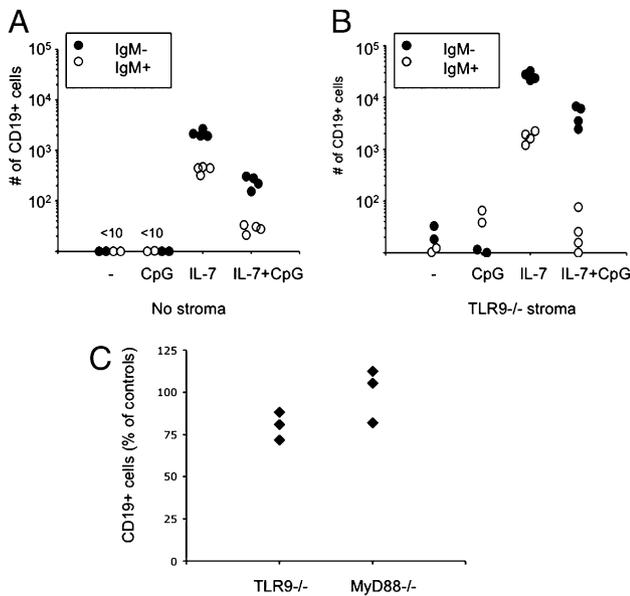
Sorted pro-B cells were lysed in RIPA buffer and the amount of protein quantitated by the Bradford assay. A total of 4 μg protein from each lysate was then separated by 15% SDS-PAGE and blotted onto nitrocellulose membranes. Immunoblots were revealed by ECL with Western Lightning Chemiluminescence Reagent Plus (PerkinElmer, Wellesley, MA). The 17-kDa band corresponding to cleaved caspase-3 and the 20-kDa band corresponding to cleaved caspase-7 were detected using the Cleaved Caspase Ab Sampler Kit (Calbiochem, San Diego, CA). The 36-kDa band of the receptor for activated C-kinase 1 (RACK1) was detected using anti-RACK1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA). The bands corresponding to caspase-3 and -7 in the Western blot were quantified with the Image J software (National Institutes of Health, Bethesda, MD), and their values were normalized to the amount of RACK1. The active caspase/RACK1 ratio was then calculated and expressed in arbitrary units. The amount of active caspase-3 postincubation with IL-7 was set at 10 arbitrary units.

## Results

### CpG inhibits the IL-7-dependent proliferation of pro-B cells

To study the effect of TLR9 signaling in the growth of pro-B cells, we sorted CD19<sup>+</sup> CD43<sup>+</sup> IgM<sup>-</sup> IgD<sup>-</sup> precursors from the BM of adult mice, cultured them with IL-7 in the presence or absence of CpG, and determined the number of CD19<sup>+</sup> cells after 1 wk. As shown in Fig. 1A, the number of both IgM<sup>+</sup> and IgM<sup>-</sup> cells was reduced by >90% in CpG-containing cultures. As shown in Supplemental Fig. 1, the inhibition was titratable, and even very low concentrations of CpG (0.02 μM) strongly inhibited the proliferation in the pro-B cell cultures. For all subsequent experiments, we used CpG at a concentration of 0.5 μM.

IL-7 cultures performed in the presence of stroma yielded more cells than cultures directly on plastic (Fig. 1B), but the addition of CpG still led to a reduction of at least one order of magnitude in the number of cells recovered. To avoid eventual secondary effects due to activation of the stromal cells by CpG, we used, as stroma, adherent cell lines derived from the BM of TLR9<sup>-/-</sup> mice (Fig. 1B). The inhibitory effect of CpG required an intact TLR9–MyD88 pathway on pro-B cells because neither TLR9<sup>-/-</sup> nor MyD88<sup>-/-</sup> pro-B cells were inhibited by CpG (Fig. 1C). We also tested other TLR ligands and show in Supplemental Fig. 2 that a TLR7 ligand, imidazoline CL097, also strongly inhibited pro-B cell cultures, but ligands to TLR2, -3, and -4 had no effect on pro-B cell proliferation.



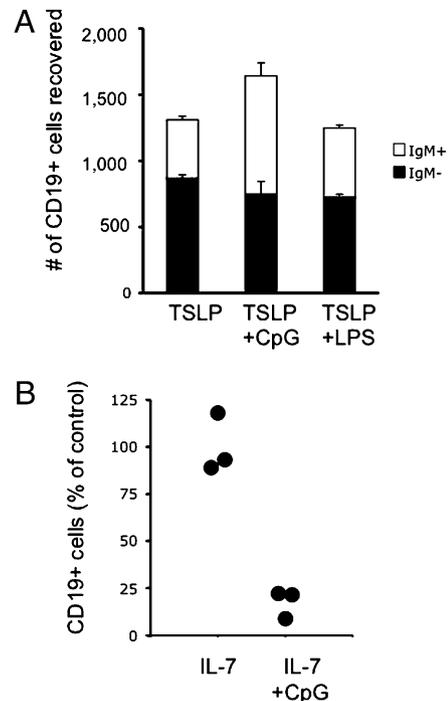
**FIGURE 1.** CpG inhibits IL-7-dependent pro-B cell expansion in vitro. One thousand sorted CD19<sup>+</sup>CD43<sup>+</sup>IgM<sup>-</sup>IgD<sup>-</sup> cells were cultured in the absence (A) or presence (B) of a TLR9<sup>-/-</sup> stromal line with or without saturating amounts of IL-7. The TLR9 ligand CpG 1826 was added to the indicated cultures at a concentration of 0.5  $\mu$ M. The figure shows the number of CD19<sup>+</sup>IgM<sup>-</sup> and CD19<sup>+</sup>IgM<sup>+</sup> cells recovered in individual wells after 7 d of culture. C, CD19<sup>+</sup>CD43<sup>+</sup>IgM<sup>-</sup>IgD<sup>-</sup> cells were isolated from TLR9<sup>-/-</sup> or MyD88<sup>-/-</sup> mice and cultured with IL-7 in the presence of CpG 1826. The figure shows the number of CD19<sup>+</sup> cells recovered after 7 d in single wells expressed as a percentage of the average number recovered in cultures of cells of the same genotype without CpG.

#### CpG selectively inhibits pro-B cells

In Rag-sufficient animals, the CD19<sup>+</sup>CD43<sup>+</sup>IgM<sup>-</sup>IgD<sup>-</sup> population contains pro-B cells as well as large cycling pre-B cells (Hardy's fraction C') (21) that proliferate in response to TSLP, whereas pro-B cells do not (22). To investigate whether CpG inhibited the expansion of cycling pre-B cells, we cultured the cells with TSLP in the absence of stroma to avoid any source of IL-7 in the culture. No significant reduction in the number of cells recovered was observed in TSLP-driven cultures in the presence of CpG (Fig. 2A), showing that proliferation of pre-B cells was not inhibited. A slight increase in the number of IgM<sup>+</sup> cells may have resulted from stimulation by CpG of the B cells formed during culture, because, as judged by their forward scatter profile, most of the IgM<sup>+</sup> cells found in the absence of CpG were of small size, whereas in cultures with CpG, the IgM<sup>+</sup> cells were of large size, suggesting that they were actively engaged in cell cycles (not shown). Deficiency of Rag2 prevents rearrangement of the Ig genes, and cells are blocked at the pro-B stage (23). Fig. 2B shows that proliferation in IL-7 of Rag-deficient BM-derived pro-B cells was strongly inhibited by CpG. Fetal liver pro-B cells, which proliferate in response to either IL-7 or TSLP (24), were also strongly inhibited by CpG (Supplemental Fig. 3).

#### The effect of CpG is not mediated by soluble factors

CpG induces production of type I IFN, which also inhibits proliferation of pro-B cells (25). To investigate whether the effect observed was mediated by type I IFN produced in response to CpG, either by contaminating cells or by the pro-B cells themselves, we isolated pro-B cells from IFNAR<sup>-/-</sup> mice (17) and studied the effect of CpG on their IL-7-dependent proliferation. We found that pro-B cells from IFNAR<sup>-/-</sup> mice are inhibited by CpG to the same extent as control wild-type cells (Fig. 3A).



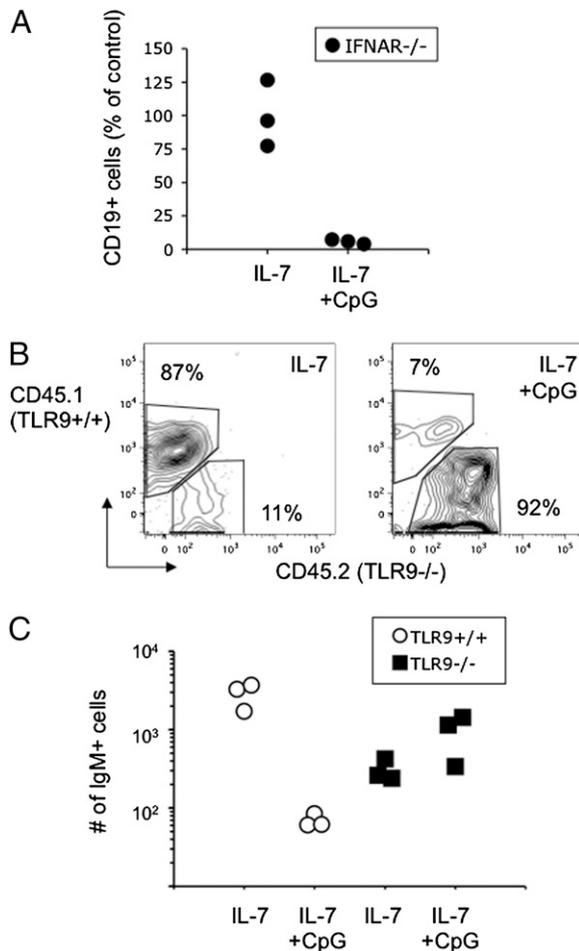
**FIGURE 2.** Pro-B but not pre-B cells are inhibited by CpG. A, Wild-type CD19<sup>+</sup>CD43<sup>+</sup>IgM<sup>-</sup>IgD<sup>-</sup> cells were cultured, without stromal cells, with 20 ng/ml TSLP in the presence of CpG 1826 (0.5  $\mu$ M) or LPS (10  $\mu$ g/ml). The figure shows the mean number of CD19<sup>+</sup> IgM<sup>-</sup> (black bars) and IgM<sup>+</sup> (white bars) cells recovered after 4 d in quadruplicate cultures. Error bars indicate the SD. This result is representative of two experiments. B, Number of CD19<sup>+</sup> cells recovered from Rag2<sup>-/-</sup> pro-B cells after 7 d of culture in IL-7 in the absence or presence of CpG 1826 (0.5  $\mu$ M). The figure shows the number of cells recovered in single wells expressed as a percentage of the average number recovered in triplicate cultures without CpG.

Moreover, expression of Daxx, which mediates the inhibitory effect of type I IFN (26), is not increased in pro-B cells cultured for 6, 14, or 22 h with CpG, as determined by quantitative RT-PCR (data not shown).

To address whether other soluble mediators might be responsible for the inhibitory effect of CpG, we cocultured TLR9<sup>+/+</sup> and TLR9<sup>-/-</sup> pro-B cells. If the TLR9<sup>+/+</sup> cells responded to CpG by secreting molecules capable of inhibiting pro-B cell proliferation, then the TLR9<sup>-/-</sup> cells in the same culture would be similarly inhibited. To distinguish between the genotypes, we used the congenic markers CD45.1 and CD45.2, and we restricted our analysis to IgM-expressing B lymphocytes because the level of CD45 expressed in B cells facilitates the discrimination of cells expressing either allele. We cocultured TLR9<sup>+/+</sup> CD45.1 and TLR9<sup>-/-</sup> CD45.2 pro-B cells to give a ratio of 90%:10%, respectively, among IgM<sup>+</sup> cells after 7 d of culture in IL-7 (Fig. 3B). Addition of CpG to this coculture resulted in an inversion of the ratio of TLR9<sup>+/+</sup>/TLR9<sup>-/-</sup> cells, to 7%:90%, respectively. The number of TLR9<sup>-/-</sup> cells recovered was unaffected by the presence of CpG in the coculture (Fig. 3C), indicating that TLR9 expression on the pro-B cell was necessary for the inhibitory effect to be observed.

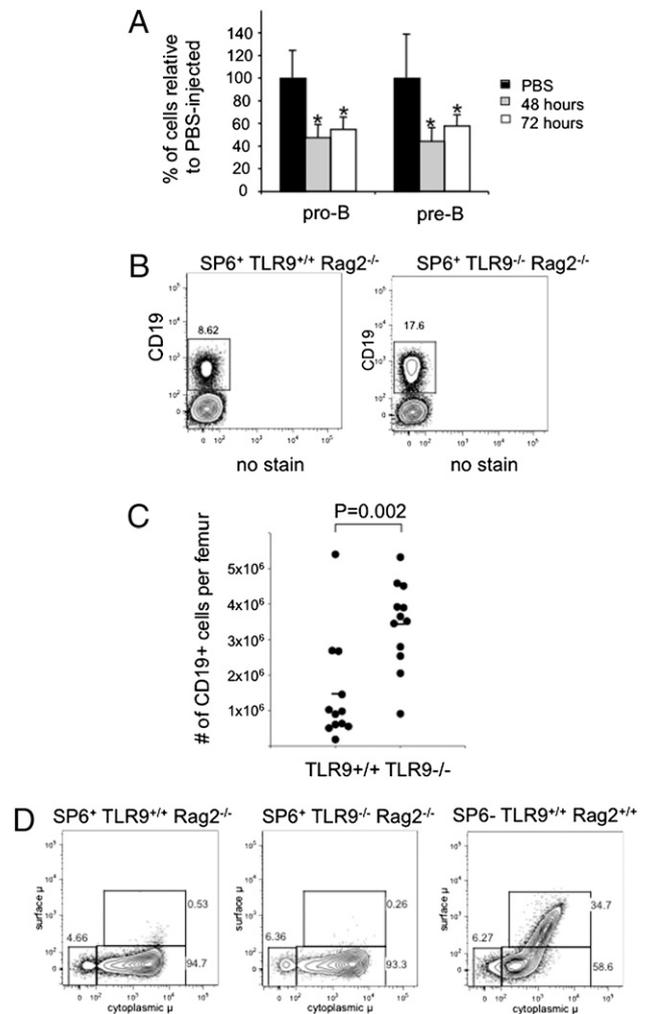
#### TLR9 signaling reduces the number of pro-B cells in vivo

Adult TLR9<sup>-/-</sup> mice do not have a significant increase in the number of pro-B cells (16) (A.I. Lalanne and P. Vieira, unpublished observations), but administration of CpG to adult mice reduced the number of B cell precursors (Fig. 4A), indicating that TLR9 signaling could regulate the size of the precursor compartments in vivo.



**FIGURE 3.** Soluble factors are not responsible for the inhibition of pro-B cells. *A*, Number of cells recovered after 7 d of culture of CD19<sup>+</sup>CD43<sup>+</sup>IgM<sup>-</sup>IgD<sup>-</sup> precursors from IFNAR<sup>-/-</sup> mice in the presence of IL-7 alone or with IL-7 plus CpG. The figure shows the number of CD19<sup>+</sup> cells recovered in individual wells expressed as a percentage of the average number recovered in triplicate cultures done in the presence of IL-7 alone. *B*, TLR9<sup>+/+</sup> CD45.1<sup>+</sup> and TLR9<sup>-/-</sup> CD45.2<sup>+</sup> precursors were cocultured with IL-7 in absence (*left panel*) or presence (*right panel*) of CpG. After 7 d, the cells were recovered and analyzed for surface expression of IgM, CD45.1, and CD45.2. Numbers represent the percentage of cells in each gate among IgM<sup>+</sup> cells. *C*, Number of TLR9<sup>+/+</sup> (CD45.1<sup>+</sup>) or CD45.2<sup>+</sup> (CD45.2<sup>+</sup>) IgM<sup>+</sup> cells recovered from single wells of the cocultures shown in *B*.

We reasoned that putative effects of endogenous TLR9 signaling on the size of the progenitor population would be more readily detected in animals in which B cell production is compromised. Therefore, to analyze the role of TLR9 *in vivo*, we intercrossed TLR9<sup>-/-</sup> and SP6<sup>+</sup>Rag2<sup>-/-</sup> mice (15) to generate SP6<sup>+</sup>TLR9<sup>+/+</sup>Rag2<sup>-/-</sup> and SP6<sup>+</sup>TLR9<sup>-/-</sup>Rag2<sup>-/-</sup> mice. SP6<sup>+</sup> mice are Tg for a BCR that binds to ssDNA and dsDNA (27) and in a Rag2<sup>-/-</sup> context have a strong reduction in the number of B lineage cells (15). In these mice, B cell development is blocked at the pre-B (cytoplasmic IgM<sup>+</sup> surface IgM<sup>-</sup>) to the immature B cell (surface IgM<sup>+</sup>) transition due to the self-reactivity of the BCR and the inability to change BCR specificity (editing) because of the absence of Rag2. Thus, in SP6<sup>+</sup>Rag2<sup>-/-</sup> BM, all CD19<sup>+</sup> cells belong to either the pro-B or the pre-B populations. We found an increased frequency (Fig. 4*B*) and number (Fig. 4*C*) of CD19<sup>+</sup> cells in the BM of SP6<sup>+</sup>TLR9<sup>-/-</sup>Rag2<sup>-/-</sup> when compared with SP6<sup>+</sup>TLR9<sup>+/+</sup>Rag2<sup>-/-</sup> mice, showing that the abrogation of TLR9 signaling partially rescued the lymphopenia in SP6<sup>+</sup>Rag2<sup>-/-</sup> mice. To determine if the inhibition of B lymphopoiesis by TLR9

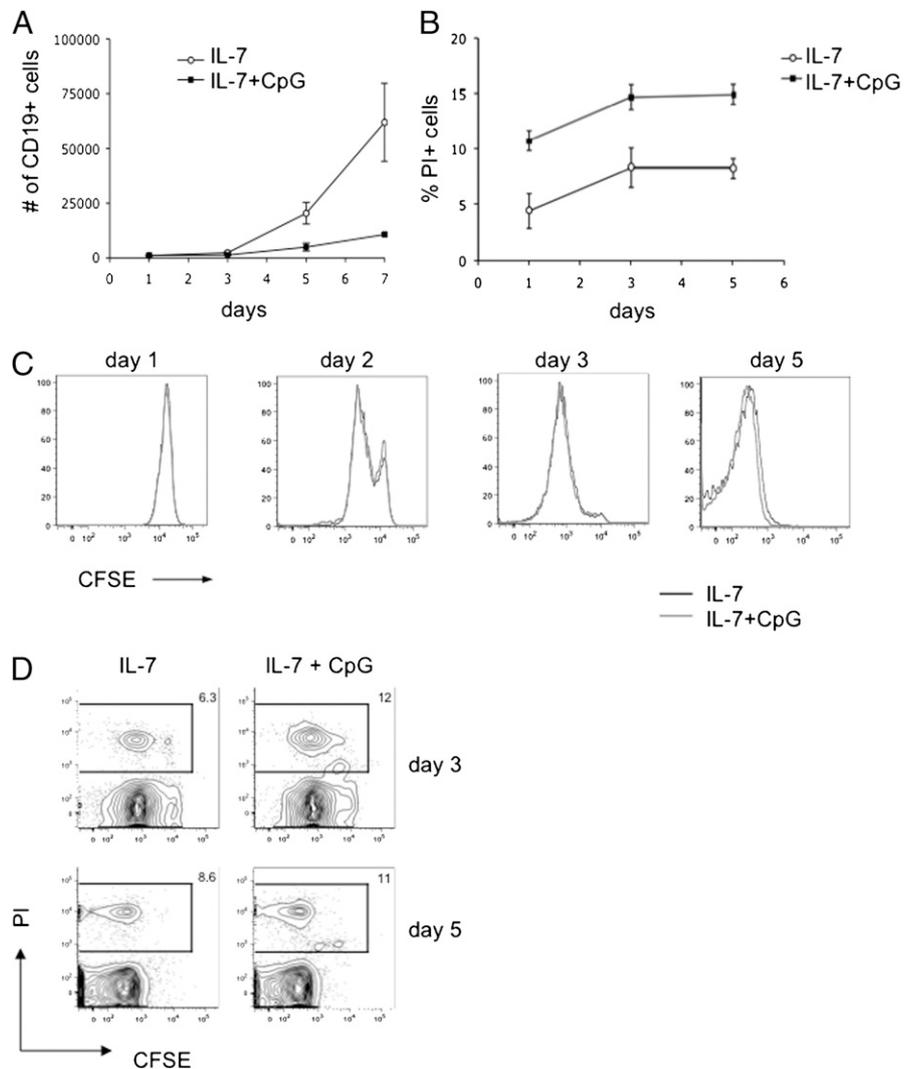


**FIGURE 4.** TLR9 signaling regulates B cell lymphopoiesis *in vivo*. *A*, Pro-B (CD19<sup>+</sup>CD43<sup>+</sup>IgM<sup>-</sup>) and pre-B (CD19<sup>+</sup>CD43<sup>+</sup>IgM<sup>-</sup>) cells in the BM of C57BL/6 mice 48 or 72 h after i.p. injection of 30  $\mu$ g CpG 1826. The figure shows the number of cells expressed as a percentage of the number recovered from control PBS-injected mice. Four mice were analyzed in each group. This result is representative of three experiments. *B*, Expression of CD19 among BM cells of SP6<sup>+</sup>TLR9<sup>+/+</sup>Rag2<sup>-/-</sup> (*left panel*) and SP6<sup>+</sup>TLR9<sup>-/-</sup>Rag2<sup>-/-</sup> (*right panel*) adult mice. Numbers represent the percentage of CD19<sup>+</sup> among total nucleated cells in the BM. The staining is representative of at least 10 mice of each genotype. *C*, Number of CD19<sup>+</sup> cells in individual femurs of SP6<sup>+</sup>TLR9<sup>+/+</sup>Rag2<sup>-/-</sup> and SP6<sup>+</sup>TLR9<sup>-/-</sup>Rag2<sup>-/-</sup> adult mice. *D*, Expression of surface and cytoplasmic IgM among BM CD19<sup>+</sup> cells of representative SP6<sup>+</sup>TLR9<sup>+/+</sup>Rag2<sup>-/-</sup>, SP6<sup>+</sup>TLR9<sup>-/-</sup>Rag2<sup>-/-</sup>, and wild-type (SP6-TLR9<sup>+/+</sup>Rag2<sup>+/+</sup>) adult mice. The numbers in the plots indicate the percentage of cells within each gate. \**p* < 0.05.

in SP6<sup>+</sup>TLR9<sup>+/+</sup>Rag2<sup>-/-</sup> took place before or after the expression of the Tg BCR, we stained BM of the two genotypes for surface and cytoplasmic IgM. We found that the proportions of cytoplasmic  $\mu^-$  and cytoplasmic  $\mu^+$  cells was comparable in the two genotypes (Fig. 4*D*), showing that both pro-B and pre-B cell numbers are reduced in the presence of TLR9. Taking all our findings into account, we concluded that the reduction in the size of the B cell compartment in SP6<sup>+</sup>TLR9<sup>+/+</sup>Rag2<sup>-/-</sup> was due to inhibition of pro-B cell expansion.

#### CpG induces apoptosis in pro-B cells

The dynamics of pro-B cell expansion in the presence and absence of CpG was investigated by counting the number of cells at different time points after the onset of the culture. As shown in Fig. 5*A*, in the cultures with IL-7 alone, pro-B cells accumulated and



**FIGURE 5.** CpG increases the frequency of apoptosis and reduces expansion of pro-B cells. **A**, Time course of progenitor expansion in cultures done in the presence or absence of CpG. A total of  $5 \times 10^3$  Rag2<sup>-/-</sup> pro-B cells were cultured with TLR9<sup>-/-</sup> stroma and IL-7 with or without CpG. The plot shows the average number of CD19<sup>+</sup> PI<sup>-</sup> cells counted at the indicated days. Bars show SD of triplicate cultures. **B**, Percentage of PI<sup>+</sup> cells within the CD19<sup>+</sup> population in the same cultures as in **A**. **C**, CFSE-stained Rag2<sup>-/-</sup> pro-B cells cultured in the presence of TLR9<sup>-/-</sup> stroma and IL-7 with or without CpG. At different time points, the cells were recovered, stained with PI, and analyzed by flow cytometry. Histograms show PI<sup>-</sup> cells in the cultures. **D**, Detection of CFSE and PI permeability in cells from **C** at 3 or 5 d of culture. The numbers in the plots indicate the percentage of cells in the gates.

expanded exponentially, whereas when CpG was present, very little expansion was observed. In parallel, the fraction of dead cells was constantly increased in CpG cultures at all time points (Fig. 5B). CpG did not prevent cell division, as indicated by the dilution of CFSE staining (Fig. 5C). The presence of CpG in the culture led to a consistently increased frequency of PI<sup>+</sup> cells, even among the ones that had diluted CFSE (i.e., had undergone cell division) (Fig. 5D). Therefore, the inhibition of pro-B cell expansion by CpG was due to an increased rate of cell death detectable at all time points postinitiation of culture.

#### *CpG inhibits pro-B cell proliferation by a mechanism independent of caspase activation*

CpG does not block the IL-7 signaling pathway because phosphorylated Stat5 (28) was easily detected in pro-B cells after 4 h of incubation with IL-7 either in the absence or in the presence of CpG (Supplemental Fig. 4).

Overexpression of Bcl-2 in Rag2<sup>-/-</sup> hBcl2 Tg pro-B cells (18) did not abolish the inhibitory effect of CpG, whereas pro-B cells deficient for the proapoptotic protein Bim (5, 19) were also strongly inhibited (Fig. 6A). In Supplemental Fig. 5, we show that Rag2<sup>-/-</sup> hBcl2 Tg pro-B cells are indeed resistant to apoptosis induced by cytokine deprivation. Furthermore, as determined by quantitative RT-PCR in pro-B cells, the expression of the antiapoptotic genes Bcl-2, Mcl-1, and Bcl-XL, as well as the pro-

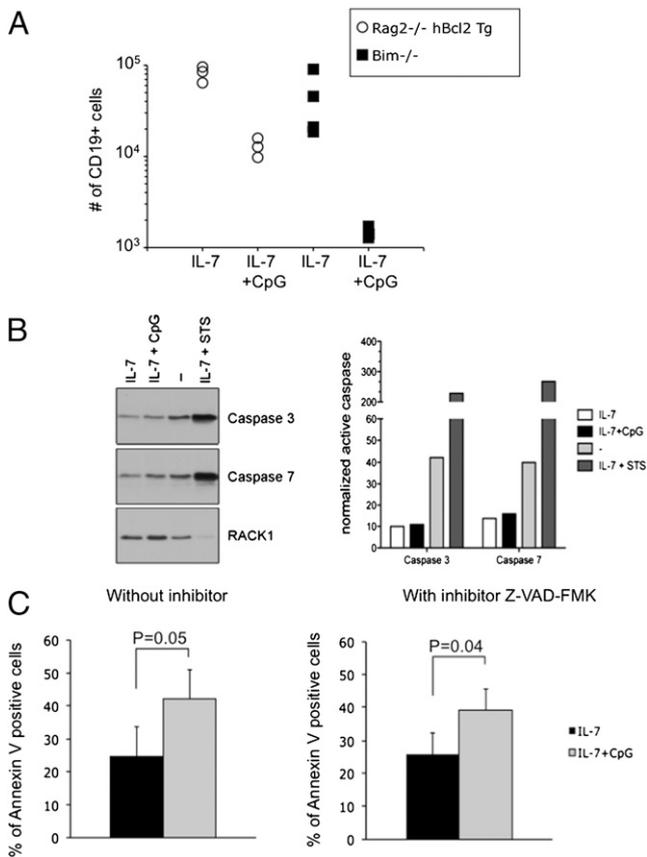
apoptotic members Bim, Bak, and Bax, were not changed by the presence of CpG (not shown).

To determine if CpG triggered apoptosis via caspase activation, we analyzed the presence of the active form of the effector caspases-3 and -7 in pro-B cells and found that the basal level of caspase activation in culture was not measurably increased by the presence of CpG (Fig. 6B). To further investigate the involvement of caspases in the cell death pathway induced by CpG, pro-B cells were cultured in the presence of the caspase inhibitor Z-VAD-FMK, which inhibited caspase-dependent apoptosis (Supplemental Fig. 6). As shown in Fig. 6C, this inhibitor did not prevent the increased apoptosis caused by CpG in pro-B cells.

#### *The inhibition of pro-B cells is cathepsin B dependent*

Cell death can also be induced by cathepsins released from the lysosomes, which can trigger caspase-independent apoptotic pathways (7, 29, 30). We isolated pro-B cells from cathepsin B<sup>-/-</sup> mice (20) and found that their proliferation was not affected by the addition of CpG to the culture (Fig. 7A), indicating that the effect of the TLR9 signal required the expression of cathepsin B.

Endosomal proteases, such as cathepsins, process TLR9 to generate a functional receptor able to bind CpG with high affinity (31). Nevertheless, the early activation marker CD69 was upregulated in cathepsin B<sup>-/-</sup> cells (Fig. 7B). Although in pro-B cells the upregulation of CD69 is modest, this result indicates that



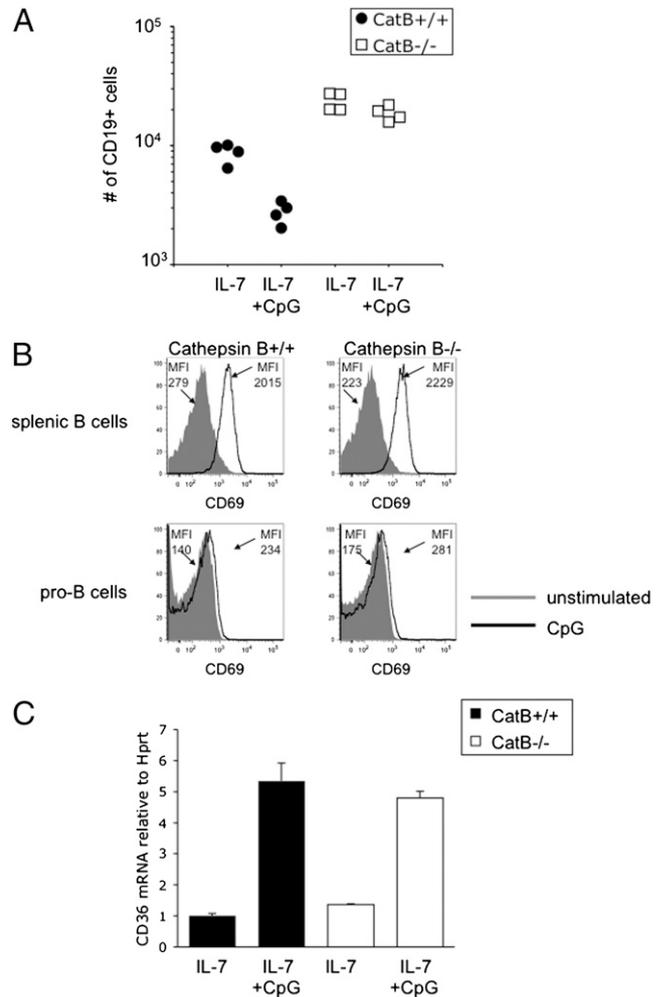
**FIGURE 6.** Apoptosis induced by CpG does not involve Bcl-2, Bim, or caspase activation. *A*, Number of CD19<sup>+</sup> cells recovered postculture in IL-7 of precursors isolated from Rag2<sup>-/-</sup> hBcl2 Tg (white symbols) or Bim<sup>-/-</sup> (black symbols) in the presence or absence of CpG as indicated. The figure shows the number of CD19<sup>+</sup> cells recovered in individual wells after 7 d of culture. *B*, *left panel*, Detection of cleaved caspase-3 and -7 in wild-type pro-B cells postincubation with IL-7 (white bars), IL-7 + CpG (black bars), no cytokine (light gray bars), or IL-7 + staurosporin (STS; dark gray bars). Total RACK1 was used as a protein loading control. *Right panel*, Normalized amount of active caspases-3 and -7 for each condition expressed as arbitrary units in which the amount of caspase-3 postincubation with IL-7 is fixed as 10 U. *C*, Percentage of Annexin V-positive cells after 16 h incubation with IL-7 or IL-7 + CpG of Rag2<sup>-/-</sup> pro-B cells in the absence (*left panel*) or presence (*right panel*) of Z-VAD-FMK (50 μM). Bars represent the mean and SD of three independent experiments.

cathepsin B deficiency does not impair TLR9 signaling. Furthermore, in a microarray analysis, we found that mRNA for CD36 was prominently upregulated by culture of pro-B cells with CpG (Supplemental Fig. 7). CD36 is similarly induced in follicular B cells by stimulation with a TLR4 ligand (32). We therefore quantitated the upregulation of CD36 as a marker of TLR9 signaling in pro-B cells and found that cathepsin B<sup>-/-</sup> pro-B cells increased CD36 mRNA to the same extent as wild-type cells (Fig. 7C), demonstrating that TLR9 signaling is unaffected in pro-B cells by the absence of cathepsin B.

**Discussion**

This work shows that TLR9, best known as a sentinel of pathogen invasion and trigger of immune responses, can directly regulate B cell development by preventing expansion and inducing caspase-independent cell death at the pro-B stage of development.

CpG selectively inhibits pro-B but not pre-B cells. It is possible that survival signals emanating from the pre-BCR override the inhibitory TLR9 pathway, because it is known that TLR9 and BCR



**FIGURE 7.** CpG-induced apoptosis in pro-B cells is dependent on cathepsin B. *A*, Number of CD19<sup>+</sup> cells recovered in individual wells after 7 d of culture in IL-7 of cathepsin B<sup>+/+</sup> control (black symbols) or cathepsin B<sup>-/-</sup> (white symbols) progenitors in the absence or presence of CpG, as indicated. The result is representative of three experiments. *B*, Total splenic cells (*top panels*) or sorted pro-B cells cultured in the presence of IL-7 (*bottom panels*) were isolated from cathepsin B<sup>+/+</sup> (*left panels*) or cathepsin B<sup>-/-</sup> (*right panels*) mice. Histograms show the expression of CD69 among CD19<sup>+</sup> cells after 16 h of culture with (black lines) or without (gray fill) CpG. *C*, CD36 mRNA expression in pro-B cells from cathepsin B<sup>+/+</sup> (black bars) or cathepsin B<sup>-/-</sup> (white bars) mice after 16 h of culture in IL-7 with or without CpG. Data are mean ± SD of triplicate PCRs.

signals can synergize or antagonize each other depending on the cell context. For example, in splenic B cells, these signals synergize to induce proliferation, but in immature B cells, BCR signals promote apoptosis and TLR9 signals promote survival (33).

Pro-B cells are continuously produced and die unless they express a pre-BCR. In the BM, a high pre- to pro-B cell ratio is typically found because cells carrying nonproductive rearrangements are constantly removed (34), and expression of the pre-BCR is accompanied by a proliferative burst (35).

How are the cells that fail at V(D)J rearrangement eliminated? It could be argued that early B lineage cells are programmed to die unless survival signals are received through the pre-BCR, but this is unlikely because IL-7 promotes the continuous proliferation and survival of pro-B cells (36, 37). It has also been hypothesized that movement of B cell progenitors into niches with reduced availability of IL-7 leads to the selective elimination of failed pro-B cells (38). It is, however, likely that additional signals exist to

actively promote pro-B cell elimination because the pro-B compartment of IL-7 Tg mice, in which the putative IL-7 gradient would be disrupted, does not progressively increase in size, and an even higher ratio of pre-B to pro-B cells is observed (39). Additionally, this compartment does not progressively become larger, even when apoptosis is inhibited by overexpression of Bcl-2 [A.I. Lalanne and P. Vieira, unpublished observations; Janani et al. (40) and Strasser et al. (41)].

We propose that TLR9 signaling is a mechanism that controls the homeostatic balance in BM B lineage compartments by favoring the expansion of the pre-B cell compartment at the expense of pro-B cells. This ensures a permanent renovation of the pro-B cell pool from earlier precursors while sparing the cells that successfully rearranged the H chain and became pre-B cells. This mechanism can operate under physiologic conditions through the action of TLR9 ligands that are available in vivo. A candidate endogenous ligand for TLR9 is the DNA released either by apoptotic cells or maturing erythrocytes.

The mechanisms implicated in the transport of DNA from the extracellular compartment to the endolysosome to activate the receptor are not fully known, although coreceptors that can mediate this transport have been presumed (42, 43). Recently, in plasmacytoid DCs and B lymphocytes, high mobility group box 1 has been shown to deliver, via receptor for advanced glycation end products, extracellular DNA to compartments where TLR9 is present (44). Because pro-B cells, like most cell types, express receptor for advanced glycation end products (Supplemental Fig. 6), this receptor is a candidate to mediate the uptake of DNA by pro-B cells.

The SP6 transgene encodes a BCR with affinity for self-Ags (15, 27), and, in a Rag<sup>-/-</sup> context, B cell development in these mice is blocked at the pre-B stage due to the inability of the self-reactive cells to escape negative selection by rearranging their endogenous L chain loci (15). When SP6<sup>+</sup>Rag2<sup>-/-</sup> mice were further made TLR9 deficient, we observed an increase in the number of BM CD19<sup>+</sup> cells, although the developmental block was still existent. The reduction in B lymphopoiesis caused by signaling through TLR9 affected the number of cytoplasmic  $\mu^-$  pro-B cells (and, naturally, also the downstream pre-B cells), which indicates that the effect is not due to the autoreactivity of the Ig, although it may be especially evident in this particular line because premature expression of this transgene may cause a more severe block in B cell development. Thus, receptor-negative cells encountered TLR9 ligands in the BM, and signaling through this receptor resulted in a reduced number of B lineage cells generated in vivo. It was only when B cell development was severely compromised that the inhibitory effect of TLR9 signaling resulted in a measurable reduction in the number of precursor cells. However, B cell development is a highly dynamic system, and the various homeostatic mechanisms controlling it are likely to be redundant, such that invalidation of a single one may not suffice to cause a detectable alteration in the number of precursor cells. It is of note in this context that mice deficient in TSLPR or IFNAR have normal adult B cell development (17, 45, 46) despite the clear effects that these signaling systems have on the generation of precursor B cells.

During infection, profound modifications take place in the BM with inhibition of lymphopoiesis and increased differentiation of granulocytes (47). TLR signaling is known to interfere with lymphocyte development, and CpG, in particular, directs differentiation of DCs from common lymphoid progenitors at the expense of B cells (10, 11). Our observation that TLR9 signals directly inhibited pro-B cells is consistent with those findings and suggests that, in pro-B cells, the only alternative to further differentiation is death by apoptosis. Another study found, contrary to our observations, that TLR2 and TLR4 ligands inhibit pro-B

cell proliferation (48). Differences in the populations analyzed are likely to explain the discrepancy; we used purified CD19<sup>+</sup> pro-B cells, whereas Nobrega's group [Hayashi et al. (48)] used an enriched B220<sup>+</sup> IgM<sup>-</sup> population containing other cells (such as macrophages, NK cells, and DCs) capable of producing factors that inhibit B cell progenitor expansion.

We observed a constantly increased fraction of apoptotic pro-B cells during culture with CpG in stark contrast to the effect on mature B cells, in which CpG, a well-known mitogen (12), strongly reduces the number of apoptotic cells in the culture. Annexin V also marks positively selected B cells in the BM (49). It should be noted, however, that in our experiments, we used Rag<sup>-/-</sup> pro-B cells and also that we obtained similar results when estimating apoptosis with PI, 7-aminoactinomycin D, and TO-PRO-3 (not shown). The IL-7-dependent Stat5 phosphorylation, essential for survival and proliferation of B cell precursors, was not affected by stimulation with CpG. The intrinsic (or mitochondrial) apoptotic pathway was not responsible for the induction of apoptosis, because neither overexpression of Bcl-2 nor deficiency in Bim rescued the cells. Furthermore, stimulation of pro-B cells with CpG did not induce the effector caspases-3 or -7, and a caspase inhibitor did not prevent apoptosis of pro-B cells.

Cathepsins, such as cathepsin B, can induce cell death (9) through caspase-independent pathways (7, 29, 30). CpG did not inhibit pro-B cells from cathepsin B<sup>-/-</sup> mice, although TLR9 activation was not affected. Deficiency in cathepsin B leads to reduced permeabilization of the lysosomal membrane, and, in contrast, this protease is released to the cytosol after lysosomal destabilization (50). It is tempting to speculate that developmental progression leads to the expression of cathepsin B inhibitors, allowing the cells to survive CpG activation. A similar process has been implicated in the rescue of CD8<sup>+</sup> memory T cell precursors from programmed cell death with inhibition of cathepsin B by the serine protease inhibitor 2A (8).

In conclusion, the data presented in this study reveal that TLR9 signaling directly inhibits expansion of pro-B cells via a cathepsin B-dependent mechanism that promotes caspase-independent cell death. This activity regulates lymphopoiesis in vivo and participates in the homeostatic control of the precursor compartments.

## Acknowledgments

We thank James P. Di Santo, Sandra Pellegrini, António Bandeira, and Sheila Dias for critical reading of the manuscript. We also thank Guillaume Soubigou (Plate-forme Puces à ADN-Genopole de l'Institut Pasteur) for expert technical help with the gene array experiments.

## Disclosures

The authors have no financial conflicts of interest.

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