

CDK Inhibitor p18^{INK4c} Is Required for the Generation of Functional Plasma Cells

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Summary

B cell terminal differentiation is associated with the onset of high-level antibody secretion and cell cycle arrest. Here the cyclin-dependent kinase (CDK) inhibitor p18^{INK4c} is shown to be required within B cells for both terminating cell proliferation and differentiation of functional plasma cells. In its absence, B cells hyperproliferate in germinal centers and extrafollicular foci in response to T-dependent antigens but serum antibody titers are severely reduced, despite unimpaired germinal center formation, class switch recombination, variable region-directed hypermutation, and differentiation to antibody-containing plasmacytoid cells. The novel link between cell cycle control and plasma cell differentiation may, at least in part, relate to p18^{INK4c} inhibition of CDK6. Cell cycle arrest mediated by p18^{INK4c} is therefore requisite for the generation of functional plasma cells.

Introduction

The generation of a primary antibody response requires activation and expansion of antigen-specific B cells by cell division, followed by terminal differentiation to antibody-secreting plasma cells (Jacob et al., 1991; Liu et al., 1991; Smith et al., 1997). In successive immunizations, the antibody response is accelerated and amplified by the activation of long-lived, antigen-specific memory B cells (Ahmed and Gray, 1996). Plasma cells are permanently withdrawn from the cell cycle and mem-

ory cells cycle infrequently, if at all. Although timely cell cycle entry and exit is undoubtedly crucial for this dynamic process, the precise mechanism is not known. The fact that plasma cells and memory cells are both arrested in the G1 phase of the cell cycle further suggests that cell cycle arrest may be a requisite for differentiation of activated B cells to plasma cells or memory cells, or both.

The mammalian cell cycle is regulated primarily by a family of serine/threonine protein kinases, consisting of regulatory cyclin subunits and catalytic cyclin-dependent kinases (CDK) (Hunter and Pines, 1994). Two CDK enzymes (CDK4 and CDK6) in combination with three D-type cyclins (D1, D2, and D3), and CDK 2 in association with cyclin E, play distinct and integral roles in regulating G1 progression (Weinberg, 1995). The CDK activity is in turn negatively controlled by seven inhibitors of the INK4 (p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}) and Cip/Kip (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}) families (Sherr and Roberts, 1999). In vitro, INK4 family members specifically form inactive stable binary complexes with CDK4 and CDK6, whereas the CIP/KIP inhibitors have a broader substrate specificity and inhibit kinase function through the formation of ternary cyclin/CDK complexes (Sherr and Roberts, 1999). Targeted gene disruption in mice reveals a requirement for CDK inhibitors in some aspects of development (Sherr and Roberts, 1999), but their physiological functions remain largely uncharacterized.

Cell cycle control of B cell development and differentiation is a complex but poorly understood process. Several CDK inhibitors (p19^{INK4d}, p21^{Cip1}, and p27^{Kip1}), CDKs, and D-type cyclins have been shown to be regulated by IL-4 and anti-IgM in primary mouse splenic B cells in vitro (Lam et al., 2000; Solvason et al., 1996; Tanguay and Chiles, 1996). However, the relevance of these findings to cell cycle control in B cell immunity has not been defined. Previously, we demonstrated that inhibition of CDK6 by the CDK inhibitor p18^{INK4c} (p18) (Guan et al., 1994; Hirai et al., 1995) was coincidental with cell cycle arrest during terminal differentiation of human IgG lymphoblastoid cells to plasma cells by IL-6 (Morse et al., 1997). Moreover, the p18 level was increased in response to IL-6, and forced expression of p18 was sufficient to cause G1 arrest of IgM lymphoblastoid cells by inhibition of CDK6 (Morse et al., 1997). These results suggest that p18 may play a critical role in terminating the cell cycle following B cell activation. Consistent with this possibility, we showed that mice deficient in p18 had increased numbers of B and T cells that displayed elevated CDK6 activity (Franklin et al., 1998). The absence of p18 augmented the activation of resting B cells and expansion in response to CD40L (Franklin et al., 1998) as well as the proliferation of primary lymphocytes induced by PMA and concanavalin A in vitro (Latres et al., 2000). These results suggest that through negative cell cycle control, p18 participates in the regulation of B cell immunity. It is not known whether B cell terminal differentiation requires cell cycle arrest or, if it does,

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PRIMARY NP-CGG RESPONSE

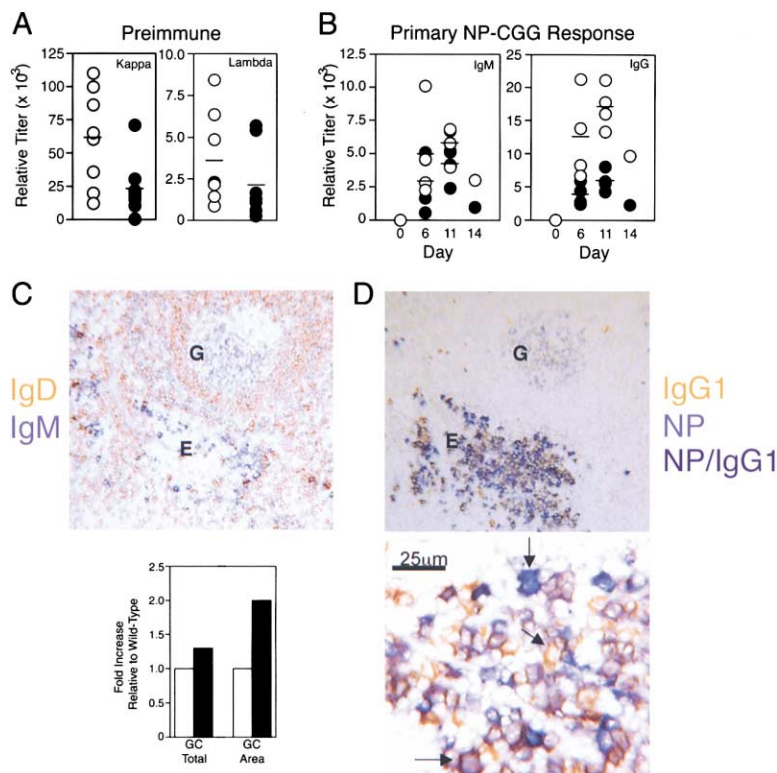


Figure 1. Impaired Primary NP-CGG Antibody Secretion Despite Normal Germinal Center Formation in p18^{-/-} Mice

(A) ELISA of preimmune serum IgG_κ and IgG_λ titers in 4-month-old p18^{+/+} (open circle) and p18^{-/-} (filled circle) mice. The horizontal bars represent mean values of eight mice.

(B) Serum NP-specific IgM (left) and IgG (right) titers in p18^{+/+} and p18^{-/-} mice on indicated days after immunization with NP-CGG. The horizontal bars represent mean values of four mice.

(C) and (D) Immunohistochemical analysis of serial spleen sections showing germinal centers "G" and an extrafollicular focus of plasmacytoid cells "E" from a p18^{-/-} mouse 6 days postimmunization. In (C), top panel shows a switched germinal center and a few IgM⁺ plasmacytoid cells. Lower panel indicates the total number and area of germinal centers in p18^{-/-} mice relative to p18^{+/+} mice as deduced from histomorphometric analysis of PNA and IgM stained sections. (D) Many NP, IgG1-specific plasmacytoid cells are seen in the top panel. Lower panel shows NP/IgG (dark blue, vertical arrow), IgG (brown, oblique arrow), and NP (light blue, horizontal arrow)-specific plasmacytoid cells present in an extrafollicular locus in a higher magnification.

what role p18 plays in the plasma cell generation process.

In this study, we demonstrate that p18-mediated cell cycle arrest is essential for the final maturation of plasmacytoid cells to functional, antibody-secreting plasma cells in the T-dependent humoral immune response. This requirement is differentiation stage specific, because p18 is dispensable for Ig switch recombination and variable region hypermutation or differentiation of antigen-specific, switched B cells to plasmacytoid cells. This provides direct evidence for the control of B cell immunity by a specific cell cycle regulator.

Results

Impaired Antibody Secretion in the Absence of p18 in a Primary T-Dependent Immune Response

Enhanced expansion of activated B cells in the p18-deficient mice (Franklin et al., 1998) suggests that they may develop autoimmunity, if p18 is dispensable for terminal differentiation of activated B cells to antibody-secreting plasma cells. However, a screen of serum immunoglobulin levels showed that both κ and λ antibody titers were markedly reduced in unimmunized p18-deficient mice compared with congenic wild-type mice (Figure 1A). This led us to test the requirement for p18 in the T-dependent antibody response to (4-hydroxy-3-nitrophenyl) acetyl linked to chicken γ-globulin (NP-CGG) in p18-deficient mice. The serum NP-specific IgM and IgG antibody levels increased with time after immunization in the wild-type mice but lower titers were in-

duced in the p18^{-/-} mice; the effect was particularly marked in the switched antibody response (Figure 1B).

The stage or stages in the primary response that were affected by p18 deficiency were studied immunohistologically. Splenic B cells were shown to proliferate both in follicles, where they formed germinal centers, and in extrafollicular foci as plasmacytoid cells (Figures 1C and 1D). These plasmacytoid cells contained NP- or CGG-specific antibody and many had switched to IgG1 production (Figure 1D). The p18-deficient mice generated more germinal centers than the control mice, as shown by costaining for IgM and PNA (Figure 1C, lower panel; data not shown), and the proportion of germinal center B cells that had Ig switched confirmed that there was no defect in switch recombination (Figures 1C and 1D). Thus, the impaired primary antibody response in the p18^{-/-} mice cannot be attributed to defective germinal center formation, Ig switch recombination, or plasmablast formation. The data point to defective terminal differentiation of antibody-secreting plasma cells in the absence of p18.

Defective Cell Cycle Arrest in p18-Deficient Plasmacytoid Cells

There were comparable numbers of total and NP-specific antibody-containing cells formed in splenic extrafollicular foci of p18-deficient and control mice along with similar numbers of switched NP-specific cells (Figure 2A). To address the relationship between cell cycle control and B cell terminal differentiation, we characterized the expression of Ki67, which marks cycling cells,

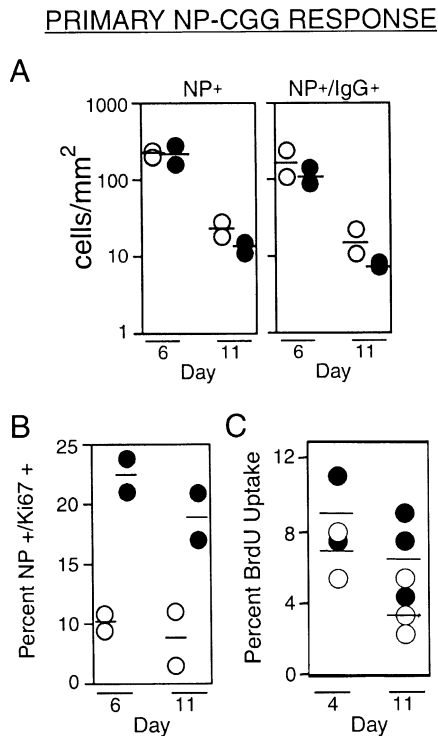


Figure 2. Normal Ig Switching and Enhanced Replication in NP-Specific Extrafollicular Plasmacytoid Cells in p18^{-/-} Mice

(A) The numbers of NP⁺ or NP⁺IgG1⁺ plasmacytoid cells per mm² of spleen sections in p18^{+/+} and p18^{-/-} mice 6 days after immunization.

(B) Percentage of NP-specific extrafollicular splenic plasmacytoid cells expressing Ki67.

(C) Percentage of B220-positive splenic B cells that have taken up BrdU in the 12 hr before the tissue was taken on days after immunization with NP-CGG. p18^{+/+} (open circle) and p18^{-/-} (filled circle).

in extrafollicular plasmacytoid cells. This revealed that at least twice as many NP-positive p18^{-/-} cells were cycling, suggesting that p18 is required for efficient cell cycle arrest of extrafollicular antibody-containing cells during the primary response (Figure 2B). Corroborating this result, twice as many B220⁺ splenic B cells from p18^{-/-} mice had taken up 5-bromo-2-deoxyuridine (BrdU) within the 12 hr prior to the removal of spleen on days 4 and 11 after immunization (Figure 2C). p18 is therefore required for efficient cell cycle arrest of B cells and plasmacytoid cells in vivo.

The Secondary Response Is Virtually Absent in p18^{-/-} Mice

On reimmunization, the defect in the antibody response was even more pronounced in p18^{-/-} mice. The serum antibody titers of either NP or CGG were virtually absent (Figure 3A), and the frequency of NP-IgG-secreting cells in the spleen, as determined by ELISPOT, was drastically reduced (Figure 3B, left). Thus, the secondary NP-CGG response is virtually absent in p18^{-/-} mice.

The absence of serum antibody titers suggests that antibody secretion must also be defective in bone marrow plasmacytoid cells. ELISPOT confirmed this possi-

bility, since no NP-specific IgG-secreting plasma cells were detected in the bone marrow by day 6 of reimmunization (Figure 3B, right). To further assess whether the secretion defect is due to the absence of p18^{-/-} plasmacytoid cells or failure of plasmacytoid cells to secrete antibody in the bone marrow, the number of bone marrow IgM and IgG cells was enumerated during NP-CGG responses (Figures 3C and 3D). IgM or IgG cells were present in comparable numbers in the bone marrow of p18-deficient and control mice in primary and secondary immunizations, and more IgG cells were detected before reimmunization (Figure 3C). Most of these cells were plasmacytoid expressing a high level of intracellular Ig (Figure 3D; data not shown). p18 is therefore dispensable for homing of plasmacytoid cells to the bone marrow but required for plasmacytoid cells to terminal differentiate to Ig-secreting plasma cells as observed in the spleen.

On reimmunization, comparable numbers of plasmacytoid cells containing NP and CGG-specific antibody were again found in the splenic extrafollicular foci in the p18^{-/-} and wild-type mice (Figure 4A). The early onset and time course of expansion of NP-specific IgG plasmacytoid cells indicates that the p18-deficient mice were able, at the cellular level, to initiate a secondary response (Figure 4A). However, a higher proportion of the Syndecan-1-positive plasmacytoid cells in p18-deficient mice were in cell cycle as indicated by Ki67 expression (Figure 4B), suggesting that they are defective in cell cycle arrest as observed in the primary response. The absence of a secondary response in p18-deficient mice is therefore due to defective cell cycle termination and antibody secretion of both splenic and bone marrow plasmacytoid cells.

p18 Expression Is Increased in B Cell Terminal Differentiation

The coordinated defects in cell cycle arrest and Ig secretion suggest that p18 mediates a critical link between cell cycle termination and final differentiation of plasmacytoid cells to Ig-secreting plasma cells. Furthermore, p18 may be regulated during plasma cell differentiation as observed in IL-6 differentiation of human IgG plasma cells in vitro (Morse et al., 1997). The p18 protein level was extremely low in both resting or activated splenic B cells before immunization and increased in splenic B cells by day 3 of reimmunization (Figure 4D), coincidental with the expansion of NP-specific IgG plasmacytoid cells and the onset of Ig secretion (Figures 4A and 3B). p18 expression is therefore increased during B cell terminal differentiation in vivo.

BLIMP-1 is thought to be important for the commitment of B cell terminal differentiation (Piskurich et al., 2000). The expression of Syndecan-1, a target of BLIMP regulation (Turner et al., 1994) on p18^{-/-} plasmacytoid cells, (Figure 4B) predicts that the BLIMP-1 expression is not contingent on p18. This was confirmed; the BLIMP-1 RNA levels were significantly higher in Syndecan-1 positive plasmacytoid cells compared to Syndecan-1 negative B cells on day 6 of reimmunization, regardless of the presence of p18 (Figure 4E). p18 may therefore function downstream or complementary to BLIMP-1. In either case, p18 is increased during plasma cell differentiation

SECONDARY NP-CGG RESPONSE

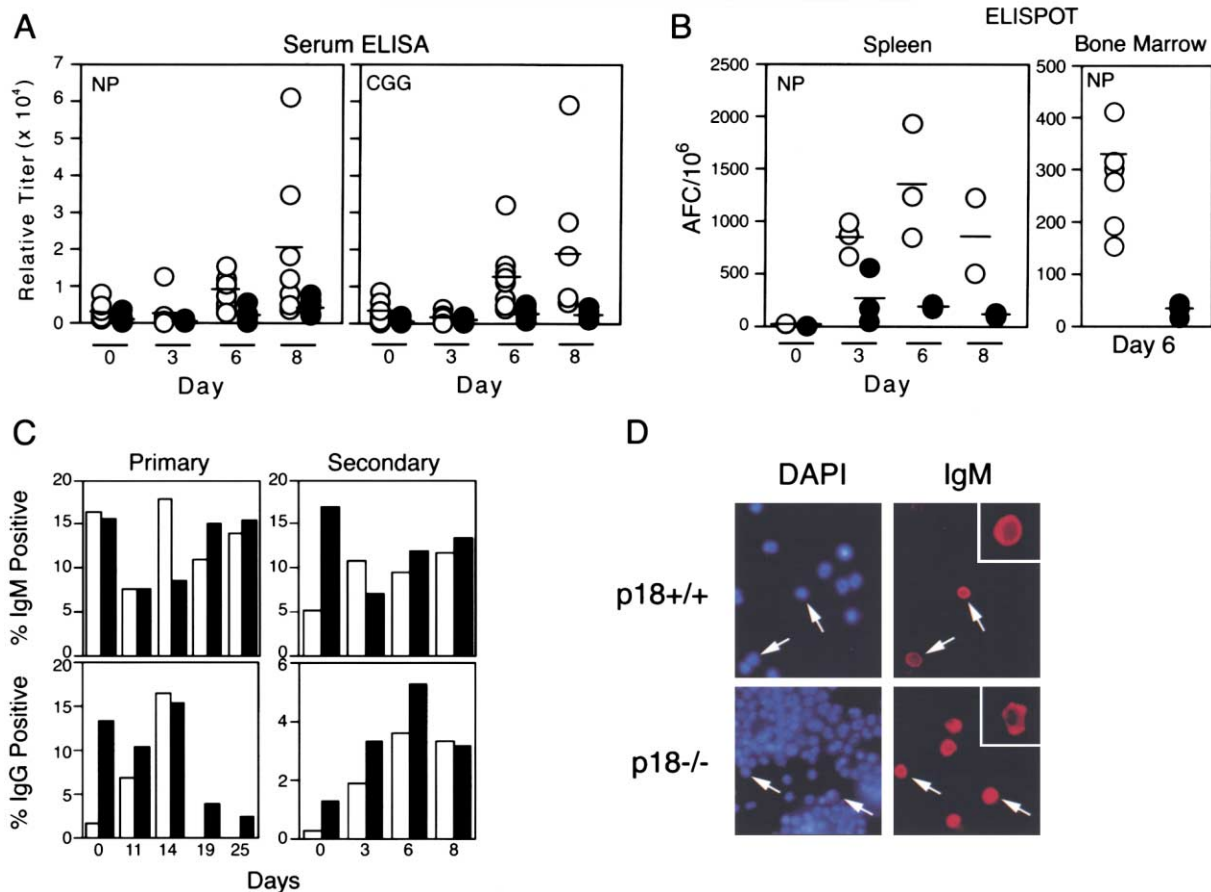


Figure 3. Severe Reduction of Serum Antibody Titers and Antibody-Secreting Plasma Cells in Secondary NP-CGG Response in $p18^{-/-}$ Mice
 (A) ELISA of serum NP (left)- and CGG-specific (right) IgG titers.
 (B) ELISPOT of the number of plasma cells secreting NP-specific IgG (AFC) per 10^6 splenic B cells (left) and total bone marrow cells (right) in $p18^{+/+}$ (open circle) and $p18^{-/-}$ (filled circle) mice on indicated days after reimmunization. The horizontal bars are mean values of 8 mice in the ELISA and 4–6 mice in the ELISPOT analyses.
 (C) The percentage of IgM (open bar) and the percentage of $p18^{+/+}$ (open bar) and $p18^{-/-}$ (filled bar) IgM and IgG cells present in the bone marrow during and between primary and secondary immunization on day 42 (indicated as “0”). Each bar represents the mean of three mice. The result is representative of three experiments.
 (D) Plasmacytoid cells containing high levels of IgM revealed by immunofluorescence staining. The arrows indicate the corresponding cell by DAPI staining.

and required for both cell cycle termination and final differentiation to Ig-secreting plasma cells.

p18 Deficiency Does Not Impair Hypermutation in Germinal Centers but Enhances Plasmacytoid Cell Apoptosis

These data link impaired cell cycle arrest to defective maturation of plasmacytoid cells to antibody-secreting plasma cells in $p18^{-/-}$ mice in both primary and secondary responses. The time course of expansion of NP-specific plasmacytoid cells (Figure 4A) further suggests that p18 deficiency is unlikely to affect V region hypermutation in germinal centers. To confirm this possibility, cells were scraped from an NP-specific germinal center identified in sections of spleens taken from mice on day 14 of the primary response to NP-CGG. The Ig variable region DNA was amplified by RT-PCR, cloned, and sequenced. Twelve independent sequences were isolated

from $p18^{-/-}$ mice (Figure 5A) and six from wild-type controls (data not shown). The V region sequences from the $p18^{-/-}$ mice show extensive mutation with evidence of serial acquisition of mutations. The pattern of mutations is comparable to those published previously (Jacob et al., 1993; Weiss et al., 1992), including the high-affinity tryptophan to leucine mutation at codon 33. p18 deficiency therefore has no effect on the induction of Ig-directed hypermutation in germinal centers.

A closer inspection of the NP-specific IgG-containing plasmacytoid cells in the extrafollicular foci of $p18^{-/-}$ mice indicates that although the time course of their expansion is similar to the wild-type mice, there were fewer numbers of cells at each time point (Figure 4A). The increased proliferation of these antibody-containing cells (Figure 4B) without a corresponding increase in cell number (Figure 4A) implies a higher death rate in $p18^{-/-}$ plasmacytoid cells. This was confirmed by

SECONDARY NP-CGG RESPONSE

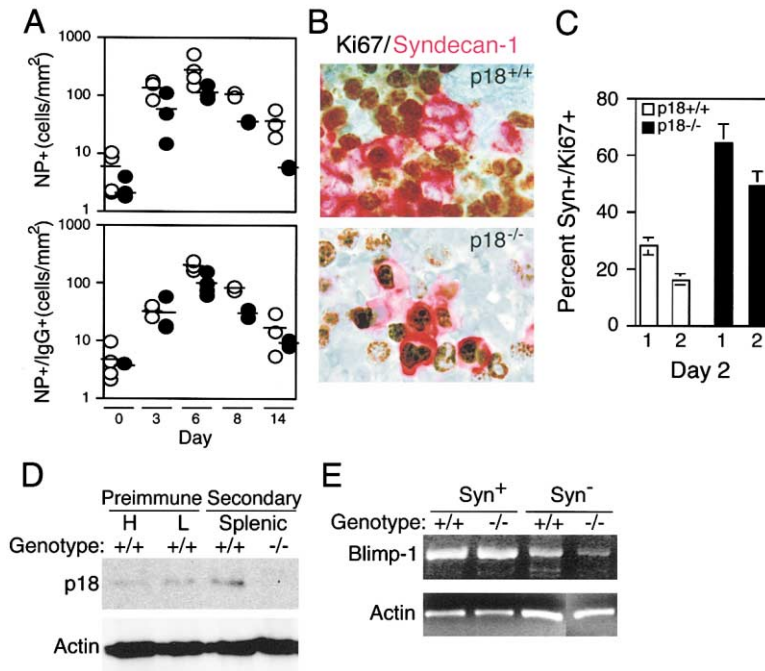


Figure 4. Enhanced p18 Expression in Plasmacytoid Cells and Hyperproliferation of NP-Specific Plasmacytoid Cells upon Reimmunization in p18-Deficient Mice

(A) The number of NP-, NP-, and IgG-specific extrafollicular plasmacytoid cells per mm² of spleen sections of p18^{+/+} (open) and p18^{-/-} (filled) mice on indicated days after reimmunization. Horizontal bars are the mean values of each time point.

(B) Immunohistochemical analysis of splenic extrafollicular plasmacytoid cells expressing Ki67 (brown) and Syndecan-1 (red) in p18^{+/+} and p18^{-/-} mice on day 2 after reimmunization.

(C) The percentage of Ki67-positive cells among Syndecan-1 positive cells.

(D) Immunoblot analysis of p18 protein present in total cell lysates (80 μg) prepared from resting (H) and activated (L) splenic B cells of preimmune p18^{+/+} mice, and splenic B cells of p18^{+/+} and p18^{-/-} mice on day 3 after reimmunization. The blot was reprobed with an actin antibody as a control for protein loading.

(E) RT-PCR analysis for BLIMP-1 and actin RNA levels in Syndecan-1-positive and -negative splenic B cells isolated from p18^{+/+} and p18^{-/-} mice on day 6 after reimmunization.

TUNEL staining of IgG-containing plasmacytoid cells on day 2 after reimmunization (Figures 5B and 5C). Thus, while B cells are not prone to apoptosis in the absence of p18 in vitro (Franklin et al., 1998), p18-deficient plasmacytoid cells, which fail to differentiate to antibody-secreting plasma cells, are more rapidly eliminated by apoptosis in splenic extrafollicular foci.

p18 Is Required within B Cells for Negative Cell Cycle Control and Ig Secretion during B Cell Terminal Differentiation

Consistent with an essential role in the T-dependent response, p18 is required for negative control of splenic B cell expansion mediated by CD40 in vitro (Franklin et al., 1998). Whether p18 is required within B cells to cou-

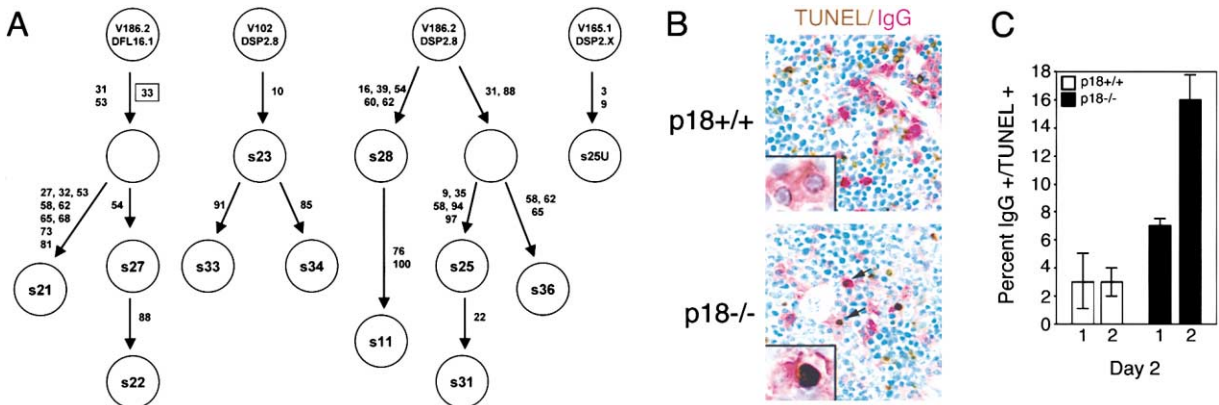


Figure 5. Unimpaired Ig V Region Hypermutation and Enhanced Plasmacytoid Cell Apoptosis in p18^{-/-} Mice

(A) The evolution of four clones from a germinal center isolated from the spleen of a p18^{-/-} mouse on day 14 after primary NP-CGG immunization. Each clone was identified by its common CDR3 sequences; the V and D segment origins of clones are shown in the four top circles. The numbers in the other circles are the arbitrary sequence number prefixed with an "S." Circles with no numbers are deduced intermediate members of the clone that were not isolated. Numbers beside the arrows of each tree indicate the codons in which mutations are found. Codon numbers are assigned according to Kabat et al. (1991). The tree on the left is based on the canonical V186.2-DFL16.2-Jh2 combination found in C57BL/6 mice in responses to NP (Cumano and Rajewsky, 1985; Weiss et al., 1992). This clone developed the typical high-affinity mutation in codon 33 (squared). The sequence data have been submitted to GenBank/EMBL/DBJ under accession numbers AJ427323-AJ427334.

(B) TUNEL (brown) analysis of IgG⁺ (red) cells on day 2 of secondary response in spleen sections prepared from p18^{-/-} and p18^{+/+} mice (400×, insert at 1000×).

(C) The histogram represents the average percentage of TUNEL⁺ cells among the IgG⁺ cells per spleen on day 2 after secondary immunization. The bars represent the values derived from counting 250 IgG⁺ cells over four individual sections. Results from individual animals are represented by open (p18^{+/+}) or closed bars (p18^{-/-}).

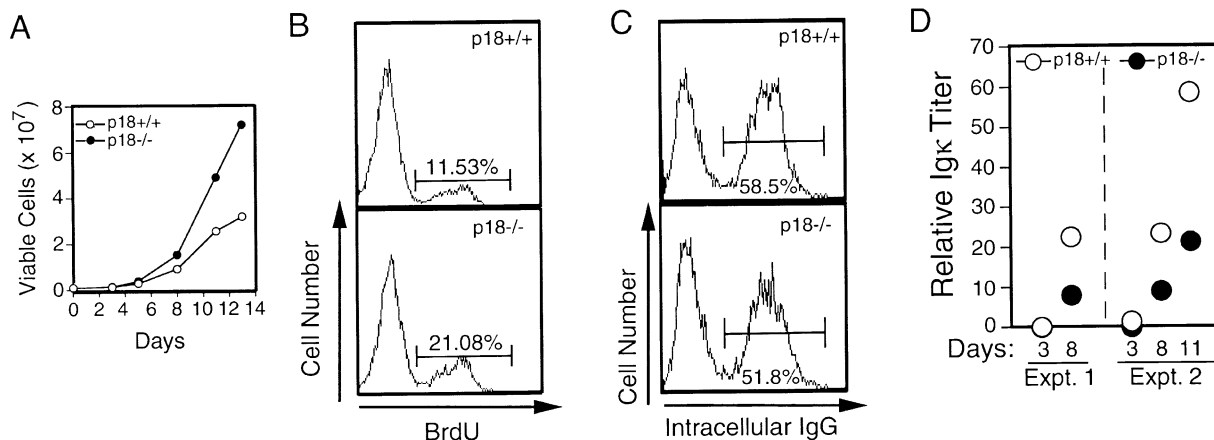


Figure 6. p18 Is Required within B Cells for Negative Cell Cycle Control and Ig Secretion but Not for Ig Switch Recombination during Terminal Differentiation

(A) Expansion of viable p18^{+/+} (open circle) and p18^{-/-} (filled circle) cells on days of in vitro differentiation of resting splenic B cells by CD40L, IL-4, IL-6, and OX40.

(B) Flow cytometric analysis of BrdU uptake by p18^{+/+} (upper) or p18^{-/-} (lower) B cells on day 8 of in vitro differentiation.

(C) Flow cytometric analysis of p18^{+/+} (top panel) and p18^{-/-} (lower panel) B cells expressing high levels of intracellular IgG1 on day 13 of in vitro differentiation.

(D) ELISA of Ig κ secreted into the media by p18^{+/+} (open circle) or p18^{-/-} (filled circle) B cells on indicated days of in vitro differentiation. The values represent cumulative IgG titers of the preceding 3 days, including the day shown (days 0–3, days 5–8, and days 8–11). The results of two representative experiments are shown.

ple cell cycle control to Ig secretion was addressed by terminal differentiation of primary B cells in vitro (Figure 6). Primary plasma cells were generated in vitro by coculturing resting splenic B cells, recovered from the 60–70% interface of a discontinuous Percoll gradient, with mitomycin C-arrested CD40L-expressing L cells at a 10:1 ratio (Do et al., 2000). The majority of cells (85%) entered the cell cycle by day 3 as determined by BrdU uptake and continued to proliferate until the onset of G1 arrest around day 6. The addition of IL-6, IL-4, and OX40 prolonged cell expansion, induced efficient Ig switch recombination and terminal differentiation to Ig-secreting plasma cells by day 8 (Figures 6A–6D).

It is evident that p18 is required to negatively control the aggregated cell cycle signals mediated by CD40 and cytokines, because twice as many live p18^{-/-} cells accumulated by day 13 (Figure 6A). This was due to enhanced cell replication in the absence of p18, as indicated by the enhanced BrdU uptake within 2 hr on day 8 (21.1% versus 11.53%) (Figure 6B). As predicted from the NP-CGG response in vivo, the absence of p18 does not affect Ig switch recombination since comparable fractions of p18^{+/+} and p18^{-/-} plasmacytoid cells (58% versus 52%) express a high level of intracellular IgG1 by day 13 (Figure 6C). However, IgG secretion was profoundly impaired in the absence of p18, 2-fold by day 8 and 3-fold by day 11 (Figure 6D). p18 is therefore required within B cells for both negative cell cycle control and efficient Ig secretion, but not for Ig switch recombination.

p18 Inhibits CD40-Signaled Cell Cycle Progression by Preferential Inhibition of CDK6

To address the biochemical mechanism of p18 action in B cells, we first confirmed that hyperproliferation of

p18^{-/-} splenic B cells induced by CD40L alone was due to an increase in S phase cells (Figure 7A). Analysis of CDK inhibitor expression by immunoblotting following saturating immunoprecipitation indicated sustained expression of p18 and the closely related p19^{INK4d} (Chan et al., 1995; Guan et al., 1996; Hirai et al., 1995) during cell cycle activation (S) and the subsequent onset of G1 cell cycle arrest (Figure 7B). By contrast, while both p21^{Cip1} and p27^{Kip1} were abundantly present in resting B cells, p21 was progressively and markedly reduced during cell cycle entry and arrest, thus unlikely to play a key role in mediating cell cycle arrest during B cell terminal differentiation. The p27 expression diminished in response to CD40L but was restored in G1-arrested cells to a level approaching that of resting B cells. Thus, CDK inhibitors are differentially regulated in primary B cells in CD40 signaling, and despite their expression in G1 arrested cells, p19 and p27 cannot functionally compensate for the loss of p18 in B cells.

p18 preferentially inhibits CDK6 activity in cultured myogenic cells and primary T cells in vitro (Franklin and Xiong, 1996; Kovalev et al., 2001). CDK6 was regulated by CD40 independent of p18, being barely detectable in resting B cells, prominently elevated in activated B cells, and slightly reduced in G1-arrested cells (Figure 7C). By contrast, CDK4 was highly expressed in resting B cells and only marginally regulated by CD40 (Figure 7C). Immunoblots of p18 immune complexes show that p18 binds a substantial and constant amount of both CDKs in cycling and G1-arrested B cells (Figure 7D). Analysis of p18- and p19-associated CDK4 and CDK6 on the same blot indicate that p18 preferentially binds CDK6 while p19 binds both CDKs comparably (Figure 7D). CDK4 and CDK6 are therefore differentially regulated by CD40L, and p18 is likely to attenuate CD40-

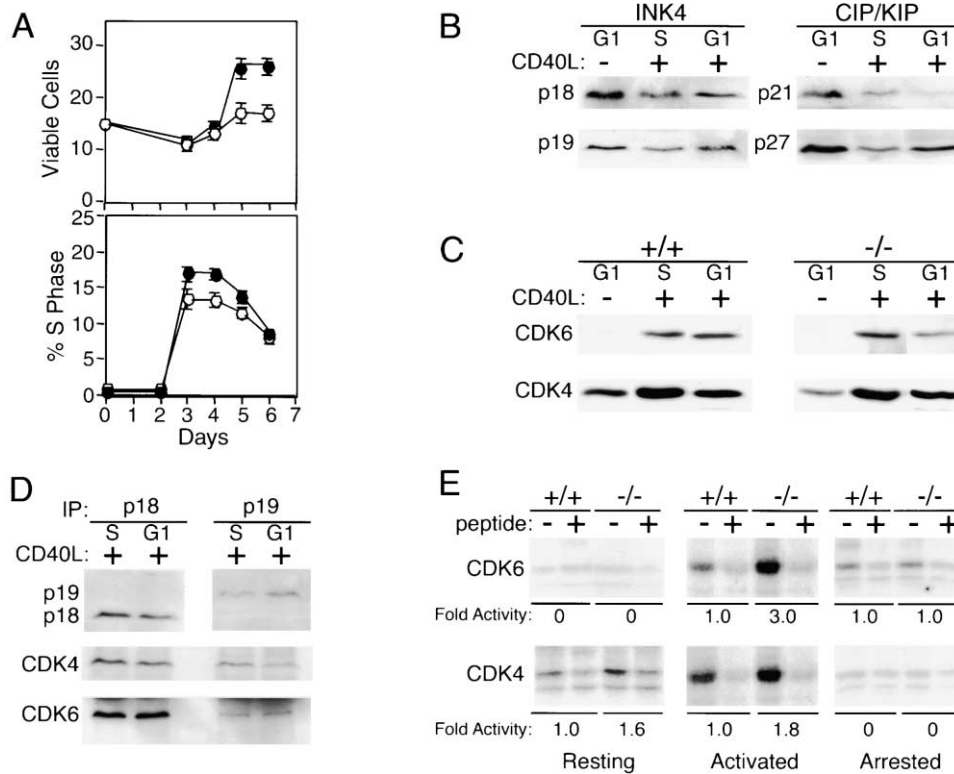


Figure 7. Differential Regulation of CDK Inhibitors and CDKs by CD40L

(A) Upper panel shows the expansion of viable p18^{+/+} (open circle) and p18^{-/-} (filled circle) cells, and lower panel shows the percentage of S phase cells on days of in vitro differentiation of resting splenic B cells by CD40L alone.

(B) Immunoblotting analysis of p18, p19, p21, and p27 in immunoprecipitates of resting splenic B cells without activation (G1, -), CD40L-activated cycling B cells (S, +), and cells entering G1 arrest following activation (G1, +).

(C) Immunoblots of CDK6 and CDK4 expression in the same cells shown as in (B).

(D) Immunoblots of CDK6 and CDK4 levels present in the p18 and p19 immune complexes shown in (B).

(E) Analysis of CDK6 and CDK4 catalytic activities in resting, CD40-activated, and G1 arrested cells. The fold kinase activity represents the ratio of CDK activity in p18^{-/-} cells relative to that in p18^{+/+} cells, after subtraction of nonspecific GST-Rb phosphorylation activity revealed in the presence of blocking peptides.

mediated cell cycle progression primarily through inhibition of CDK6.

To test this, the CDK activities were determined by assaying phosphorylation of RB by the CDK immune complexes (Figure 7E). The CDK6 activity correlates tightly with the protein levels: undetectable in resting B cells, elevated in cycling B cells, and reduced in G1-arrested cells. CDK4 was already detected in resting B cells and showed modest changes. Importantly, the absence of p18 led to a 3-fold increase in the CDK6 activity in cycling B cells and a 1.6-fold increase in CDK4 activity in the same cells (Figure 7E). Given the prominent regulation of CDK6 by CD40L (Figure 7C), these results support that p18 inhibits CD40-signaled cell cycle progression by preferential inhibition of CDK6.

Discussion

Plasma cell differentiation is a multistep process that is not well understood. In this study, we have demonstrated a requirement of the CDK inhibitor p18 for the generation of functional, antibody-secreting plasma cells. In its absence, antibody secretion is notably reduced during, and even before, primary immunization

(Figure 1) and is virtually absent in the secondary response due to defective secretion in both spleen and bone marrow (Figure 3). Increased replication of extrafollicular plasmacytoid cells (Figures 2 and 4) in the context of normal germinal center formation (Figure 1), Ig switch recombination, and V region hypermutation (Figures 1–6) suggests that p18 is specifically required for plasmacytoid cells to terminate the cell cycle and differentiate into Ig-secreting plasma cells. This requirement is intrinsic to B cells, because the defects in cell cycle arrest and IgG secretion are recapitulated during terminal differentiation of p18-deficient B cells by CD40 and cytokines in vitro (Figures 6 and 7). Together with the essential role of p18 in negative control of TCR-mediated T cell proliferation (Kovalev et al., 2001), this study provides direct evidence that a cell cycle regulator controls the T-dependent humoral immune response, affecting both B and T lineage cells, and suggests an underlying biochemical mechanism.

p18 Is Required within B Cells for Cell Cycle Arrest and Differentiation to Ig-Secreting Plasma Cells

p18 controls B cell terminal differentiation by mediating a critical link between cell cycle control and generation

of functional plasma cells: plasmacytoid cells expressing abundant antigen-specific intracellular IgM and IgG are present as replicating, nonsecreting intermediates in the extrafollicular foci and bone marrow of p18^{-/-} mice (Figures 3 and 4). These p18^{-/-} plasmacytoid cells are otherwise indistinguishable from Ig-secreting plasma cells based on the expression of known plasma cell surface markers such as Syndecan-1 and the loss of others such as B220 (Figure 4B; M.R.T. and S.C.-K., unpublished data).

Contrasting its requirement in cell cycle control, the absence of p18 does not affect the survival of CD40L-activated B cells in vitro (Franklin et al., 1998). However, inappropriately cycling p18^{-/-} plasmacytoid cells are more rapidly eliminated by apoptosis in the splenic extrafollicular foci early during the secondary NP-CGG response (Figures 5, 4C, and 4D). The severe defect in antibody secretion (compare Figures 3 and 1) implies that extrinsic factors uniquely present in the secondary response may augment cell cycle progression, thereby increasing the need for p18 to maintain negative cell cycle control. While the signals that control cell cycle-directed homeostasis of plasmacytoid cells in the extrafollicular foci are unknown, our data suggest a model in which p18-mediated cell cycle arrest induces differentiation of plasmacytoid cells to Ig-secreting plasma cells within a restricted time, and failure to do so leads to their removal by apoptosis. This model is consistent with the modulation of plasmablast survival by extrinsic constraints as we have previously proposed (Sze et al., 2000).

The requirement for p18 in Ig secretion is not limited to splenic plasmacytoid cells, given the severe defect in Ig secretion by bone marrow IgM and IgG plasmacytoid cells in p18^{-/-} mice (Figures 4B–4D). Thus, while p18 is dispensable for homing of plasmacytoid cells to bone marrow and their survival in the bone marrow microenvironment, it is required for terminal differentiation to functional plasma cells. Thus, the requirement for p18 in the differentiation of plasmacytoid cells to Ig-secreting plasma cells is extraordinarily specific and independent of their microenvironment.

p18 Is a Physiologic Regulator of B Cell Terminal Differentiation

Little is known about gene regulation in primary plasma cells. However, the p18 protein level is elevated in Syndecan-1-positive plasmacytoid cells in the secondary NP-CGG response (Figure 4D), while remaining constant in response to CD40 in vitro (Figure 7B). Given the increase in p18 protein during terminal differentiation of human IgG plasma cells by IL-6 (Morse et al., 1997), IL-6 is likely to be one physiologic signal that regulates p18 expression in the humoral immune response. IL-6 is known to be essential for IgG and IgA antibody secretion in vivo (Kopf et al., 1994; Ramsay et al., 1994) and functions as a potent survival factor for B cells and plasma cells (J.U.-S. and S.C.-K., unpublished data). p18 may therefore integrate IL-6 signals to facilitate cell cycle termination, as well as plasma cell differentiation and survival in vivo.

This raises the question of the relationship between p18 and two transcription factors that are important

for B cell terminal differentiation, BLIMP-1 and XBP-1. BLIMP was identified as a zinc finger-containing transcription factor capable of inducing IgM secretion and Syndecan-1 expression when ectopically expressed in mature B cell lines (Turner et al., 1994). It has been postulated to promote the commitment of plasma cell differentiation based on its ability to activate or repress multiple genes that are expressed or extinct in plasma cells in vitro (Piskurich et al., 2000). However, BLIMP-1 expression is not restricted to B lineage cells (Chang et al., 2000), and its essential role in B cell terminal differentiation remains to be confirmed by gene targeting studies. Clearly, p18 is not required for the expression of BLIMP-1 in B cells or its activation in Syndecan-1-positive plasmacytoid cells during the NP-CGG response (Figure 4E). Nonetheless, it is possible that through transcriptional regulation and cell cycle control, BLIMP and p18 may function in concert in the commitment and final maturation of functional plasma cells at two distinct stages of B cell terminal differentiation.

The absence of XBP-1, a basic leucine zipper-containing transcription factor (Liou et al., 1990), results in defective plasma cell generation and antibody production, but germinal centers form in reconstituted RAG2-deficient mice (Reimold et al., 2001). Independent investigations of the unfolded protein response have further revealed that GRP78 (Bip) (Haas and Wabl, 1983), a stress-induced endoplasmic reticulum chaperone protein (Lee, 2001; Lee et al., 1981), is regulated by XBP-1 in model cell line systems in vitro (Calfon et al., 2002; Yoshida et al., 2001). GRP78, along with related ER stress-induced proteins, have been shown to facilitate the assembly of nascent Ig heavy and light chains in the endoplasmic reticulum (Melnick et al., 1994). Whether the p18 defects relate to impaired functions of XBP-1 and GRPs poses an exciting question that remains to be addressed.

The Specificity and Biochemical Mechanism of p18 Action in B Cells

p18 is expressed in many tissues (Guan et al., 1994) but is dispensable for development, including the B and T lineages (Franklin et al., 1998). We now show that p18 is required specifically for the final step of B cell terminal differentiation in the T-dependent antibody response. This requirement is specific among CDK inhibitors since the p18 deficiency cannot be functionally compensated by another CDK inhibitor in vivo, not even its closest relative p19, which has a similar expression pattern in B cells (Figure 7B).

Biochemical dissection of p18 action in B cells in vitro has confirmed that p18 is required within B cells to counter CD40-mediated cell cycle progression (Figure 7) and that hyperproliferation of p18^{-/-} B cells is not secondary to elevated CD40 expression (H.L. and S.C.-K., unpublished data). This requirement for p18 is specific, given that resting splenic B cells isolated from mice lacking p16^{INK4a}, p21^{CIP1}, or p27^{KIP1} do not hyperproliferate under the same conditions in vitro (H.L. and S.C.-K., unpublished data). The p18 requirement may in part be due to differential regulation of CDK inhibitors and CDKs by CD40L and IL-6 and preferential interaction between p18 and CDK6. p18 is regulated by IL-6 but

not CD40L whereas CDK6 is prominently regulated by CD40L but not IL-6 (Figures 4 and 7; Morse et al., 1997). In response to CD40L alone, p18 attenuate G1 progression of primary B cells by binding and inhibiting a constant amount of CDK6, and less CDK4, thereby limiting the availability of free CDK6. During a physiologic NP-CGG response in vivo, p18 levels are elevated presumably by IL-6 (Figure 4C), leading to more efficient inhibition of CDK6 and G1 arrest. The absence of p18 would then lower the threshold for CDK6 activation and permit cell cycle progression.

This hypothesis does not exclude the cooperation between p18 and other CDK inhibitors expressed in B cells, because CD40L-activated B cells eventually enter G1 arrest despite the residual CDK6 activity, and the presence of CDK4 activity alone in resting B cells is insufficient to initiate cell cycle entry (Figure 7E). The augmented germinal center formation in p18^{-/-} mice in the primary NP-CGG response (Figure 1) may result from both increased availability of CD40L as a consequence of enhanced T cell expansion (H.L. and S.C.-K., unpublished data) and lack of negative cell cycle control by p18 in B cells. It is unknown whether p18 is required for negative cell cycle control in a T-independent immune response. If so, p18 would negatively control cell cycle signals mediated by distinct physiologic signals for a successful antibody response. The way cell cycle control relates to the onset of efficient Ig secretion poses a fascinating challenge to future research.

Experimental Procedures

Immunization and Isolation of B Cells

p18^{-/-} and p18^{+/+} mice (Franklin et al., 1998) (8- to 14-week-old, age-matched) were immunized intraperitoneally with 75 µg alum-precipitated NP-CGG (21:1; Biosearch Technologies, Inc.) (Toellner et al., 1996). Five to six weeks later, secondary responses were elicited by injecting tail veins with 15 µg NP-CGG in PBS. The number of mice used in each time point during the primary and secondary responses is as indicated. High-density (resting B cells) and low-density (activated B cells and plasma cells) cells were isolated from the 60%–70% and 50%–60% interface, respectively, of a discontinuous Percoll gradient as previously described (Do et al., 2000).

Terminal Differentiation of Primary IgG-Secreting Plasma Cells In Vitro

Resting splenic B cells were isolated and differentiated in vitro by coculturing with mitomycin-C arrested mouse CD40L-expressing L cells essentially as previously described (Do et al., 2000). In some cultures, baculovirus-produced recombinant human IL-6 (40 U/ml) (Morse et al., 1997) and the soluble human gp80 subunit of the IL-6 receptor, which enhances IL-6 signaling, were added from the beginning. Recombinant mouse IL-4 (200 U/mL, a gift from Cindy Watson and Bill Paul) and OX40-Ig (1:30 dilution) (Stuber et al., 1995) were added from day 4 onward to promote Ig switch recombination, cell survival, and terminal differentiation. Determination of viable cells by trypan blue exclusion and the percentage of S phase cells by flow cytometry were carried out as described (Do et al., 2000).

To detect intracellular IgG by flow cytometry, the in vitro differentiated B cells and plasma cells were collected, rinsed twice in phenol red-free RPMI, and fixed in 4% paraformaldehyde for 5 min at 37°C. They were then rinsed twice again with PBS containing 0.1% BSA, permeabilized in a PBS buffer containing 10 mM Hepes, 0.1% BSA, and 0.1% saponin (Sigma), and stained with an FITC-conjugated anti-mouse IgG1 antibody (Pharmingen) for 30 min on ice. After two rinses in the permeabilization buffer, cells were analyzed by flow cytometry using a FACS Calibur (Becton Dickinson) and CellQuest software.

ELISA and ELISPOT

ELISA was carried out as previously described (Do et al., 2000) to determine the total serum κ and λ titers in 4-month-old unimmunized mice, the relative NP- and CGG-specific serum Ig titers after immunization with NP-CGG, and IgG secreted into the media during in vitro terminal differentiation of primary B cells. The only modification was that wells were coated with 1 µg of CGG (Sigma) or 2.5 µg of a goat-anti mouse κ or λ antibody (Southern Biotech). Detection of plasma cells secreting NP- or CGG-specific IgG by ELISPOT was performed also as described (Do et al., 2000), using low-density splenic B cells isolated on indicated days after immunization or bone marrow cells flushed from the femur and tibia of mice on day 6 after secondary immunization with NP-CGG.

In Vivo and In Vitro BrdU Labeling and Detection

For in vivo bromodeoxyuridine (BrdU) labeling studies, mice were injected intraperitoneally with 2.5 mg BrdU in 200 µl PBS at the indicated times after immunization and sacrificed 12 hr later. Low-density splenic B cells were cytospun onto slides and stained with a FITC-conjugated anti-BrdU mouse monoclonal antibody (Boehringer Mannheim) and a rhodamine-conjugated anti-B220 antibody (PharMingen) (Morse et al., 1997). The cells were counterstained with DAPI (Boehringer Mannheim); the number of BrdU-positive cells is expressed as a percentage of B220-positive cells. Cells were counted in triplicate from each mouse; at least 500 cells were counted in each sampling.

Analysis of BrdU uptake in vitro was carried out as previously described (Do et al., 2000), with the exception that propidium iodide was not added to the final samples. In brief, cells were incubated with BrdU in regular RPMI media (5 µg/ml) for 2 hr during in vitro differentiation, washed twice in phenol red-free RPMI containing 5% fetal calf serum, and fixed overnight in 70% ethanol at 4°C. After centrifugation, cells were resuspended in 2 N HCl-0.5% Triton-X-100 and incubated for 30 min at room temperature, neutralized with 0.1 M Tetraborate (pH 8.5), and rinsed twice in RPMI containing 5% fetal calf serum and 0.5% Tween-20. The cells were stained with the above-mentioned anti-BrdU antibody and analyzed by flow cytometry.

Immunohistochemistry and Immunofluorescence Staining

Immunohistochemical detection of intracellular antibodies specific for NP, or the expression of IgG1, IgM, IgD, or the proliferation marker Ki67 (Starborg et al., 1996) in splenic cells was carried out on cryosections (5 µm) and enumerated as previously described (Luther et al., 1997; Sze et al., 2000). The proportion of NP-positive extrafollicular cells expressing Ki67 shown in Figure 2 was determined by counting 500 NP positive cells, or as many as were on the section.

Detection of proliferating and apoptotic plasmacytoid cells shown in Figures 4 and 5 was carried out on 5 µm sections of formalin fixed, paraffin-embedded spleens. For simultaneous detection of Syndecan-1 and Ki67, the sections were stained first with anti-Ki67 (Becton Dickinson, San Jose, CA) using an immunoperoxidase detection system (Ventana Medical Systems, Inc., Tuscon, AZ) and then with an anti-mouse Syndecan-1 antibody (Pharmingen) using a ChemMate alkaline phosphatase detection system (Ventana, Tucson, AZ). Cells were then counterstained with methyl green (Aldrich, Milwaukee, WI). TUNEL-positive cells were detected using the ApopTag kit (Intergen, Purchase, NY) according to the manufacturer's specifications. The sections were then stained with anti-mouse IgG (Vector, Burlingame, CA, Cat#BA-9200) and counterstained with methyl green. The number of Syndecan-1 positive cells that were Ki67-positive (Figures 4B and 4C) and intracellular IgG-positive cells that were TUNEL-positive (Figures 5B and 5C) were counted, 500 cells in each counting, from four different regions of the extrafollicular foci of each spleen using an eight channel Diffcount (Modulus Data Systems, Santa Clara, CA).

To detect IgM and IgG cells in the bone marrow, total bone marrow cells were collected by flushing the femurs of mice with RPMI at indicated days after primary NP-CGG immunization, or reimmunization day 42 later. The cells were cytospun onto slides, stained with rhodamine-anti-mouse IgM and FITC-anti-mouse IgG, and counter stained with DAPI. The IgM- or IgG-positive cells present at each

time point in each mouse were counted three times, 500 cells per sampling. Three to five mice were used for each time point.

Sequencing of V Region Genes in NP-Specific Germinal Center Cells

NP-specific germinal center cells were detected on cryosections of spleen by staining for NP binding and IgD (Toellner et al., 1996). Following microdissection using a micromanipulator needle, V region genes were amplified by nested PCR as described (Sze et al., 2000). PCR products were cloned (Jacob et al., 1993) and sequenced, and mutations were verified by comparing sense and anti-sense strand sequences (Sze et al., 2000). The frequency of PCR errors was checked by sequencing nonmutated cells and was found to be insignificant (Sze et al., 2000).

Immunoprecipitation and Immunoblotting

Total cell lysates were prepared from high-density and low-density splenic B cells or CD40L-activated B cells (day 3), and G1 arrested B cells late in the course of *in vitro* differentiation (day 6), and analyzed by immunoblotting and immunoprecipitation essentially as previously described (Franklin et al., 1998). The rabbit polyclonal antisera used in these studies were specific for amino acids 155-168 of p18^{INK4c} (11255), 153-166 of p19^{INK4d} (12076), 2-15 of mouse p21^{Cip1} (12075), and 183-197 of mouse p27^{Kip1} (11845) (Franklin et al., 1998; Franklin and Xiong, 1996). Rabbit anti-human CDK4 (sc-177, 1:1000) and CDK6 (sc-260, 1:1000) antibodies were purchased from Santa Cruz. To detect p18, a rabbit polyclonal anti-p18 antibody was used (1:1000, N20, Santa Cruz). Immunoblots were probed with the indicated antisera, then incubated with an HRP-conjugated donkey anti-rabbit antibody (Amersham Life Science) and developed with an enhanced chemiluminescent system (ECL, Amersham).

In vitro kinase assays, immunoprecipitations of CDK4 and CDK6 present in 300 μ g of total protein lysate were carried out in the presence or absence of blocking peptides as previously described (Franklin et al., 1998). The CDK4 and CDK6 antibodies (Santa Cruz) were used at a 1:100 dilution, while the CDK4 (cat sc-260P) and CDK6 (cat sc-177P) peptides were added at a 1:25 dilution. The immune complexes were incubated with GST-Rb in the presence of γ -³²P ATP as previously described (Franklin et al., 1998). Proteins were resolved by SDS PAGE and the relative levels of phosphorylated GST-Rb were determined by phosphorimager analysis.

RT-PCR Analysis

Splenic B cells were isolated from p18^{+/+} and p18^{-/-} mice on day 6 after reimmunization and incubated with a biotinylated anti-Syndecan-1 antibody (1:500 dilution; Pharmingen) and then with streptavidin microbeads (Miltenyi Biotec). Syndecan-1-positive and -negative cells were separated using an automated magnetic bead separator (AutoMacs, Miltenyi Biotec). Total RNA was isolated using Trizol (Gibco BRL) and reverse-transcribed according to manufacturer's specification (Gibco BRL). Polymerase chain reactions were subsequently performed under the following conditions (94°C for 40 s; 60°C for 1 min; 72°C for 1 min; 30 cycles) using the following primer pairs: BLIMP-1 (forward primer, GTTCTGACAGGCAGCCA GGTTTTG; reverse primer, GTGAAGTTTCAAGGACTGGCAGAGAC) and actin (forward primer, CACCGATCCACACAGACTTGTG; reverse primer, ACCGAGGCCCCCTGAACCCCTAAG).

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