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*J Immunol* 2006; 177:787-795; doi: 10.4049/jimmunol.177.2.787
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Disruption of Cyclin D3 Blocks Proliferation of Normal B-1a Cells, but Loss of Cyclin D3 Is Compensated by Cyclin D2 in Cyclin D3-Deficient Mice

Jennifer M. Mataraza,* Joseph R. Tumang,† Maria R. Gumina,* Sean M. Gurdak,† Thomas L. Rothstein,2‡ and Thomas C. Chiles3*

Peritoneal B-1a cells differ from splenic B-2 cells in the molecular mechanisms that control G0-S progression. In contrast to B-2 cells, cyclin D2 is up-regulated in a rapid and transient manner in phorbol ester (PMA)-stimulated B-1a cells, whereas cyclin D3 does not accumulate until late G1 phase. This nonoverlapping expression of cyclins D2 and D3 suggests distinct functions for these proteins in B-1a cells. To investigate the contribution of cyclin D3 in the proliferation of B-1a cells, we transduced p16INK4a peptidyl mimetics (TAT-p16) into B-1a cells before cyclin D3 induction to specifically block cyclin D3-cyclin-dependent kinase 4/6 assembly. TAT-p16 inhibited DNA synthesis in B-1a cells stimulated by PMA, CD40L, or LPS as well as endogenous pRb phosphorylation by cyclin D-cyclin-dependent kinase 4/6. Unexpectedly, however, cyclin D3-deficient B-1a cells proliferated in a manner similar to wild-type B-1a cells following PMA or LPS stimulation. This was due, at least in part, to the compensatory sustained accumulation of cyclin D2 throughout G0-S progression. Taken together, experiments in which cyclin D3 was inhibited in real time demonstrate the key role this cyclin plays in normal B-1a cell mitogenesis, whereas experiments with cyclin D3-deficient B-1a cells show that cyclin D2 can compensate for cyclin D3 loss in mutant mice. The Journal of Immunology, 2006, 177: 787–795.

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*Department of Biology, Boston College, Chestnut Hill, MA 02467; †Department of Medicine, Boston University School of Medicine, Boston, MA 02118 and Immunobiology Unit, Evans Memorial Department of Clinical Research, Boston University Medical Center, Boston, MA 02118; and‡Department of Microbiology, Boston University School of Medicine, Boston, MA 02118

Received for publication December 20, 2005. Accepted for publication April 19, 2006.


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1 This work was supported by U.S. Public Health Service Grants AI-49994 (to T.C.C.) and AI-60896 (to T.L.R.).

2 Current address: Center for Oncology and Cell Biology, The Feinstein Institute for Medical Research, 350 Community Drive, Manhasset, NY 11030.

3 Address correspondence and reprint requests to Dr. Thomas C. Chiles, Department of Biology, Boston College, 414 Higgins Hall, Chestnut Hill, MA 02467. E-mail address: Chiles@bc.edu

4 Abbreviations used in this paper: cdk, cyclin-dependent kinase; MZ, marginal zone; AML1, acute myeloid leukemia 1.
In adult animals, peritoneal B-1a cells are self-replenishing, giving rise to their own progeny, following establishment early during ontogeny, whereas B-2 cells arise continually from surface Ig-negative stem cell precursors and fail to proliferate following maturation in the absence of exogenous stimulation (30, 34). Ex vivo B-1a cells fail to proliferate following BCR cross-linking, which drives B-2 cells into S phase (35, 36). Conversely, B-1a cells enter S phase in response to PMA and do so unusually rapidly, whereas B-2 cells require the combination of PMA and a calcium ionophore (35, 37). These results establish that B-1a cells differ from B-2 cells in the molecular mechanisms that control G1-S phase progression. In support of this, cyclins D2 and D3 are expressed in a nonoverlapping manner during G1-S progression in PMA-stimulated B-1a cells (38, 39). Notably, cyclin D2 is up-regulated in a rapid and transient fashion in B-1a cells, whereas cyclin D3 induction and assembly into active cdk4/6-containing complexes occurs after cyclin D2 degradation and parallels peak endogenous pRb phosphorylation in late G1 phase (39). In marked distinction, mitogenetic stimulation of B-2 cells leads to coordinate induction of cyclins D2 and D3 (19, 20, 40). Thus, in B-1a cells, but not B-2 cells, stimulated expression of cyclin D2 and cyclin D3 is temporally and dose-dependently distinct. Taken together, these observations suggest that cyclin D3 is uniquely positioned in B-1a cells to mediate transition through the G1-S boundary, which may, as well, reflect its role in conventional B-2 cells where cyclin D3 expression overlaps that of cyclin D2. To investigate the need for cyclin D3-cdk complexes for G1-S phase progression in normal B-1a cells, we used protein transduction technology to specifically block assembly and activation of cyclin D3 holoenzyme complexes in normal peritoneal B-1a cells, and we examined the proliferative responses of B-1a cells from cyclin D3-deficient mice. Materials and Methods Preparation of murine B-1a and B-2 lymphocytes The generation of cyclin D3-deficient mice has been described (41). BALB/cByJ mice were purchased from The Jackson Laboratory and housed at Boston University Medical Center or Boston College. The studies described below were reviewed and approved by institutional animal care and use committees at both institutions. Mice were cared for and handled at all times in accordance with National Institutes of Health and University guidelines. Unseparated cells were obtained by peritoneal washout and splenic disruption, were stained with immunofluorescent Abs directed against B220 and CD5, and were subjected to FACS at 4°C using a flow cytometer (BD Biosciences) and the data were analyzed by ModFit LT software (Verity Software House). To measure proliferation, B cells (1–2×10^6 in 0.2 ml) were cultured in quadruplicate and stimulated as indicated in the figure legends. DNA synthesis was measured by incubating B cells with 0.5 μCi [3H]thymidine (20 Ci/mmol; New England Nuclear) during the last 6 h of culture. Cells were then harvested onto glass fiber filters and [3H]thymidine incorporation into DNA was quantitated by liquid scintillation spectroscopy. Western blotting B cells were solubilized in Triton X-100 buffer (20 mM Tris (pH 7.4), 100 mM NaCl, 0.1% Triton X-100) containing 2.5 μg/ml leupeptin/antiprotinin, 10 mM β-glycerophosphate, 1 mM PMSF, 1 mM NaF, and 1 mM Na₃VO₃. Insoluble debris was removed by centrifugation at 15,000 × g for 15 min (4°C). Lysate was separated by electrophoresis through a 10% polyacrylamide SDS gel (SDS-PAGE) and transferred to an Immobilon-P membrane. The membrane was blocked in TBS-T (20 mM Tris (pH 7.6), 137 mM NaCl, and 0.05% Tween 20) containing 5% nonfat dry milk for 5 h and then incubated overnight (4°C) with primary Ab at 1 μg/ml in TBS-T. The membrane was washed several times in TBS-T, incubated with a 1/250 dilution of anti-rabbit or anti-mouse IgG-coupled HRP Ab (60 min) and developed by ECL. Fluorescence microscopy For imaging the TAT-p16-FITC, slides were washed once with PBS and then mounted with Aqua Mount. For imaging D-type cyclins, lymphocytes were solubilized in SDS-PAGE sample buffer (60 mM Tris, 100 mM NaCl, 0.1% Triton X-100, and 2% SDS) and boiled for 5 min. Cell lysates were run on a 4–20% SDS-PAGE gel, and then transferred to a PVDF membrane. Membranes were blocked with 5% nonfat dry milk in 50 mM Tris (pH 7.6), 150 mM NaCl, 0.1% Tween 20, and 0.02% sodium azide for 1 h at room temperature. Membranes were incubated with the primary Abs diluted in blocking buffer for 1 h at room temperature. Membranes were washed three times with PBS and then incubated with the secondary Ab HRP-coupled anti-mouse or anti-rabbit Abs (Jackson ImmunoResearch Laboratories) for 1 h at room temperature. Membranes were washed three times with PBS and then incubated with ECL. Reagents and Abs Protein G agarose and anti-mouse IgG-coupled HRP, anti- mouse cdk4, anti-cyclin D2, and anti-cyclin D3 Abs (Abs) were purchased from Santa Cruz Biotechnology and used at 1:300 and 1:1000 dilutions for immunoprecipitation and Western blotting, respectively. Rabbit polyclonal Abs were obtained from Cell Signaling Technologies. Rabbit cdk4, rabbit cdk2, and rabbit pRb (Thr172) Abs were obtained from Cell Signaling Technologies. Rabbit polyclonal Abs were obtained from Cell Signaling Technologies.
respectively. Soluble rCD40L was obtained from transfected J558L cells that secrete a chimeric CD40L/CD8α fusion protein and prepared as previously described (46, 47). Anti-CD8α Ab was obtained from the supernatant of 53-6-72 hybridoma cells and was used to cross-link rCD40L (47). CD40L was used at a 1/10 dilution of supernatant and anti-CD8α Ab was used at a 1/40 dilution of supernatant.

Results

TAT-p16INK4a peptidyl mimetics are transduced into ex vivo peritoneal B-1a cells and disrupt endogenous D-type cyclin-cdk4/6 complexes

Our strategy for determining whether cyclin D3-cdk4/6 complexes are required for proliferation of B-1a cells was to precisely target cyclin D3-cdk4/6 holoenzymes by transducing p16INK4a peptidyl mimetics into ex vivo B-1a cells. Lane and coworkers (43) demonstrated that a 20-mer peptide derived from the third ankyrin-like repeat of the p16INK4a tumor suppressor protein selectively bound to cdk4 and cdk6 and inhibited cdk4/6-mediated pRb phosphorylation in vitro. To mediate efficient transduction into B-1a cells, we coupled the p16INK4a peptidyl mimic to an 11-mer peptide consisting of the NH2-terminal HIV TAT protein domain (denoted herein as TAT-p16 wild type) (44). Flow cytometry revealed transduction of nearly 100% of B-1a cells that had been incubated with TAT-p16-FITC wild-type peptide for 120 min (Fig. 1A). Confocal microscopy of parallel B-1a cells confirmed intracellular transduction of TAT-p16-FITC wild-type peptide into B-1a cells (Fig. 1B).

To demonstrate in vivo efficacy, 10 μM TAT-p16 wild-type peptides were transduced into PMA-stimulated B-1a cells, which express cyclin D3-cdk4 complexes as evidenced by immunoreactive cyclin D3 in nondenatured anti-cdk4 immunoprecipitates (Fig. 2A, Control) (39). Transduction of TAT-p16 wild-type peptide into B-1a cells before cyclin D3 induction prevented cyclin D3-cdk4 complex assembly (Fig. 2A, p16WT); a control charge-matched TAT-p16 (TAT-p16 mutant) peptide, that is unable to bind to cdk4 or cdk6, did not block cyclin D3-cdk4 complex assembly (Fig. 2A, p16Mut). Similar results were obtained when evaluating the efficacy of TAT-p16 peptides with respect to endogenous cyclin D3-cdk6 complexes (data not shown). Asynchronously growing murine Bal17 B cells were analyzed in parallel, as they constitutively express assembled cyclin D2-cdk4 complexes, whereas the TAT-p16 mutant peptide had minimal effect (Fig. 2B). As a control for specificity, we measured cdk-activating kinase-mediated Thr172 phosphorylation of cdk2; this modification occurs subsequent to the nuclear translocation of cyclin E-cdk2 and, thus, serves as an indicator for the presence of cyclin E-cdk2 complexes. Transduction of TAT-p16 wild-type peptide into PMA-stimulated B-1a cells before cyclin E-cdk2 assembly did not measurably affect PMA-induced Thr172 phosphorylation of cdk2 (Fig. 2C, PMA).

Transduction of TAT-p16 wild-type peptide into normal peritoneal B-1a cells inhibits proliferation

To directly evaluate the contribution of cyclin D3-cdk4/6 complexes in B-1a cell proliferation, we took advantage of the non-overlapping expression of cyclins D2 and D3 in PMA-stimulated B-1a cells (38, 39). Blocking the assembly of temporally expressed
cyclin D3-cdk4/6 complexes was achieved by transducing the TAT-p16 wild-type peptide into B-1a cells at 14 h after stimulation with PMA. This time corresponds to a point in the cell cycle wherein cyclin D2 protein is not detectable and cyclin D3 protein induction has not yet begun (initially detectable at 17 h). B-1a cells transduced with TAT-p16 wild-type peptide exhibited a $>70\%$ reduction in PMA-stimulated tritiated thymidine incorporation in comparison to control B-1a cells or B-1a cells transduced with TAT-p16 mutant peptide (Fig. 3A). Similar results were obtained with B-1a cells stimulated with LPS or CD40L (Fig. 3A). Of note, incubation of nonstimulated B-1a cells with TAT-p16 peptides did not alter the basal level of tritiated thymidine incorporation (Fig. 3A, Medium). To corroborate these findings, we analyzed B-1a cells for cell cycle position by propidium iodide staining and flow cytometry. Transduction of TAT-p16 mutant peptide into PMA-stimulated B-1a cells had a minimal effect on the percentage of B-1a cells in S/G2+M phases of the cell cycle in comparison to control B-1a cells (Fig. 3B, PMA). By contrast, transduction of TAT-p16 wild-type peptide reduced the percentage of S/G2+M B-1a cells by 70% in comparison to B-1a cells transduced with TAT-p16 mutant peptide. Similar results were obtained with LPS- or CD40L-stimulated B-1a cells (Fig. 3B).

To determine the status of endogenous pRb phosphorylation in B-1a cells treated with TAT-p16 wild-type peptide, whole cell extracts were prepared and immunoblotted with anti-phospho(Ser$^{807/811}$)pRb Ab that specifically detects cyclin D2-cdk4/6-mediated phosphorylation of pRb. Unstimulated B-1a cells did not express measurable pRb phosphorylation, whereas PMA-stimulated B-1a cells exhibited abundant phosphorylation of endogenous pRb on Ser$^{807/811}$ (Fig. 3C). Phosphorylation of endogenous pRb on Ser$^{807/811}$ was markedly inhibited following transduction of TAT-p16 wild-type, but not TAT-p16 mutant, peptides into B-1a cells (Fig. 3C). Total pRb protein levels were not altered by transduction of the TAT-p16 peptides. These results indicate that TAT-p16 wild-type peptide blocks endogenous pRb phosphorylation by cyclin D-cdk4/6 complexes in PMA-stimulated B-1a cells.

**Cyclin D3-deficient mice have normal peripheral B-1 and B-2 lymphocyte compartments**

Because the results above indicate that cyclin D3-cdk4/6 complexes are required for mitogenic stimulation of normal B-1a cells, we were interested in determining whether B-1a cell development and proliferation were affected by loss of cyclin D3 (41). To pursue this, we initially evaluated the splenic and peritoneal lymphoid compartments of cyclin D3-deficient mice. To confirm the absence of cyclin D3 in D3-deficient animals, total splenic lymphocytes were stimulated with a combination of mitogens known to induce cyclins D2 and D3 in both T and B lymphocyte populations (19, 20, 24, 40); whereas up-regulation of both cyclins D2 and D3 was apparent in spleen cells from wild-type mice, only cyclin D2 was induced in cyclin D3-deficient splenic lymphocytes and no cyclin D3 protein was observed (Fig. 4A). Cyclin D3-deficient spleens contained a decreased number of total lymphocytes as compared with spleens obtained from wild-type littermate animals, which immunofluorescent staining showed was largely attributable to decreased numbers of B-2 cells (Fig. 4, B and C). However, there was no difference between cyclin D3-deficient mice and wild-type littermates in the total number of peritoneal cells, and, more specifically, the numbers of B-1a cells (CD5$^+$B220$^{low}$Mac-1$^+$), B-1b
FIGURE 4. Characteristics of B-1a cell lymphoid compartments in wild-type and cyclin D3-deficient mice. A, Total splenic lymphocytes were isolated from wild-type (+/+ ) and cyclin D3-deficient mice (−/− ) and were either left untreated (M) or were stimulated (S) with a mitogenic combination consisting of 300 ng/ml PMA plus 400 ng/ml ionomycin and 25 μg/ml LPS. At 24 h, lymphocytes were collected, detergent extracts were prepared, and then Western blotting was performed with anti-cyclin D2 or anti-cyclin D3 Abs. The blot was stripped and reprobed with an anti-β-actin Ab to verify equal loading of each lane. B, Peritoneal washouts (peritoneum) and spleen cell suspensions (spleen) were obtained from cyclin D3-deficient mice (−/− ), and wild-type littermate control animals (+/+, □), and total cell numbers were determined. Mean results are shown, along with lines indicating SEs of the means (n = 6). C, Spleen cell suspensions were obtained from cyclin D3-deficient (−/− ) and littermate control mice (+/+/). The distribution of splenic T cells (T-S; B220+CD5+), B-2 cells (B-2S; B220−CD5−), and MZ cells (MZ-S; CD21hi/CD23lo) was determined by immunofluorescent staining and flow cytometric analysis and converted to cell number based on initial cell counts. Mean numbers of cells in each lymphoid population are shown, along with lines indicating SEs of the means (n = 6).

We next evaluated the role of cyclin D3 in peritoneal B-1a cell proliferation induced by PMA. In contrast to the results obtained when cyclin D3-cdk4/6 complex assembly was prevented by transduction of TAT-p16 wild-type peptide into normal B-1a cells, we found that cyclin D3-deficient B-1a cells responded comparably to wild-type B-2 cells stimulated by PMA (Fig. 5C). In addition, the proliferation of cyclin D3-deficient B-1a cells in response to LPS was similar to wild-type B-1a cells (Fig. 5C). In data not shown, similar results were obtained with B-1a cell populations stimulated with CD40L.

Deregulation of cyclin D2 expression in cyclin D3-deficient B-1a cells

To understand the molecular basis of the apparently normal proliferation of cyclin D3-deficient B-1a cells in response to PMA, we compared the gene expression pattern of cyclin D2 and cyclin E in wild-type and cyclin D3-deficient B-1a cells. These analyses revealed little difference in the expression of mRNAs encoding cyclins E1 and E2 in the PMA-stimulated B-1a cell populations (Fig. 6A). In B-2 cells, E2F-1 induction by BCR cross-linking corresponds to late G1 phase of the cell cycle and coincides with pRb hyperphosphorylation (23). The expression of E2F-1 mRNA in PMA-stimulated B-1a cells from cyclin D3-deficient mice was comparable to wild-type B-1a cells (Fig. 6A). In agreement with our previous results in normal B-1a cells stimulated with PMA (38), cyclin D2 mRNA was elevated at 2 h and reached a maximal level within 4 h, which corresponded to a 1.3- and 2-fold increase above nonstimulated B-1a cells, respectively (Fig. 6A). However, in PMA-stimulated cyclin D3-deficient B-1a cells, we detected a 4.5- and 8.3-fold increase in cyclin D2 mRNA above the levels observed in nonstimulated B-1a cells at 2 and 4 h, respectively. In keeping with this, in PMA-stimulated cyclin D3-deficient B-1a cells, the level of endogenous cyclin D2 protein measured at 4 h was greater than that of parallel PMA-stimulated B-1a cells from control wild-type mice (~4.5-fold, based on scanning densitometry of the ECL exposed film obtained after Western blot of B-1a cell lysates) (Fig. 6B). To obtain further evidence that this elevated induction of cyclin D2 may act to compensate for the loss of cyclin D3, we determined the expression of cyclin D2 protein in wild-type and mutant B-1a cells by indirect immunofluorescence microscopy. As expected, cyclins D2 and D3 were not detected in nonstimulated B-1a cells.
and cyclin D3-deficient B-1a cells (data not shown). Taken together, these results suggest that in contrast to wild-type B-1a cells, wherein cyclin D2 protein is induced in a rapid and transient manner, expression of cyclin D2 remains elevated throughout the G0-S interval in cyclin D3-deficient B-1a cells stimulated with PMA.

Evidence that cyclin D2 protein was functional in cyclin D3-deficient B-1a cells was provided by the measurable level of endogenous pRb phosphorylation on cyclin D-cdk4/6-targeted residues, detected by Western blotting of B-1a cell lysates at 21 h following PMA stimulation (Fig. 6B, lower panel); this level of pRb phosphorylation was comparable to wild-type B-1a cells stimulated with PMA. It is important to note that in addition to cyclin D3, cyclin D1 was not expressed in PMA-stimulated cyclin D3-deficient B-1a cells (data not shown). Further evidence that PMA-induced proliferation of B-1a cells in the absence of cyclin D3 may result from compensation by cyclin D2 was obtained by transduction of TAT-p16 wild-type peptide into cyclin D3-deficient B-1a cells, wherein only cyclin D2 expression is detectable, that resulted in an ~80% reduction of PMA-stimulated tritiated thymidine incorporation (in comparison to control B-1a cells or B-1a cells transduced with TAT-p16 mutant peptide) (Fig. 6D).

**Discussion**

To summarize the findings herein, the temporal disjunction between cyclin D2 and cyclin D3 expression in B-1a cells provided the means to dissect the role of late G1-phase cyclin D3 in mediating mitogenic responses. Inhibition of cyclin D3-cdk4/6 complexes with TAT-p16 wild-type peptide blocked normal B-1a cell proliferation in response to PMA, LPS, and CD40L, indicating that unfettered early and transient up-regulation of cyclin D2 is insufficient to drive normal B-1a cells into S phase of the cell cycle. However, complete loss of cyclin D3 did not adversely affect B-1a cell proliferation, because of a countervailing compensatory increase in cyclin D2 mRNA and sustained accumulation of functioning cyclin D2 protein in cyclin D3-null B-1a cells after stimulation with PMA. Thus, dysregulated cyclin D2 can compensate for the loss of cyclin D3 in fostering B-1a cell cycle progression in knock out mice. Our results also strike a cautionary note by demonstrating that compensation by cyclin D2 at the molecular or cellular level in cyclin D3-deficient mice can mask the normal function that cyclin D3 provides in the mitogenesis of B-1a cells.

Our previous findings raised the possibility that cyclin D3 is uniquely positioned to mediate G1-to-S phase progression in B-1a cells (38, 39). Notably, PMA stimulation of B-1a cells resulted in an early and transient induction of cyclin D2; the assembled cyclin D2-cdk4/6 complexes were transiently active, but only accounted for a relatively minor amount of the total endogenous pRb phosphorylation on cyclin D-cdk4/6-targeted residues, detected by Western blotting of B-1a cell lysates at 21 h, after which IgM secretion was assessed by ELISPOT assay, as described in Materials and Methods. Results are shown for two mice per group, with lines indicating the range of values. C, B-1a cells from wild-type (WT) or cyclin D3-deficient (KO) were cultured in medium alone (inset) or stimulated with 300 ng/ml PMA or 25 μg/ml LPS for the times indicated. Incorporation of tritiated thymidine was assessed for the final 6 h of culture as described in Materials and Methods. Results represent mean values of triplicate cultures with lines indicating SEs of the means. The data are representative of three independent experiments.

(Fig. 6C, lane M); the weak fluorescence signal detected in non-stimulated B-1a cells was similar in intensity to that of parallel B-1a cells stained with a control isotype mAb (data not shown). Stimulation of wild-type B-1a cells with PMA resulted in the non-overlapping expression of cyclin D2 and cyclin D3 at 4 and 21 h, respectively (Fig. 6C). In contrast, immunofluorescent staining of cyclin D2 in cyclin D3-deficient B-1a cells revealed that cyclin D2 was expressed at both 4 and 21 h post-PMA stimulation. It should be mentioned that similar results were obtained using flow cytometry to evaluate intracellular D-type cyclin expression in wild-type B-1a cells.
resulted in a loss of PMA-induced phosphorylation of endogenous pRb specifically on D-type cyclin-cdk4/6-targeted residues. Transduction of TAT-p16 peptide into normal B-1a cells results in inhibition of proliferation, as evidenced by reduced PMA-stimulated tritiated thymidine incorporation and reduced percentage of B-1a cells in S/G2 phases of the cell cycle. Similar results were obtained with B-1a cells stimulated via LPS or CD40L.

Our results also indicate that in normal B-1a cells where cyclin D3-cdk4/6 complex assembly has been selectively blocked by TAT-p16 peptide, the early and transient induction of cyclin D2 is not sufficient to mediate proliferation induced by PMA, LPS, or CD40L. This might reflect the relatively low pRb kinase activity associated with cyclin D2-cdk4/6 complexes and/or the transient nature of cyclin D2 holoenzyme activation in stimulated B-1a cells (38). In the absence of additional pRb phosphorylation (mediated via cyclin D3-cdk4/6), the activity and duration of cyclin D2/cdk-mediated pRb phosphorylation alone may not be of sufficient strength to drive progression through the G1-S transition. On a related point, it is highly unlikely that TAT-p16 peptide blocked B-1a cell proliferation by interfering with levels of cyclin D2 that may be below the level of detection, because the pRb kinase activity of cyclin D2 is relatively low as compared with cyclin D3 and no measurable cyclin D2 kinase activity has been detected at 17 h after stimulation. Alternatively, cyclin D2 function may not be directly involved in proliferation, but rather may be limited to promoting B-1a cell growth (i.e., accumulation of cell mass), occurring in early G1 phase of the cell cycle (48). It should be mentioned that while the analysis of cyclin D2-deficient mice has revealed significantly decreased numbers of peritoneal B-1a cells, the molecular mechanism(s) underlying this loss remain to be established (22).

It is recognized that although D-type cyclins show high amino acid homology within the cdk-binding region (75–78%), the extent of homology outside of this region is 39–47% (16, 17). Thus, we cannot rule out the possibility that separate from mediating pRb phosphorylation and G1-S phase progression, cyclin D3 may carry out additional functions in B-1a cells. For example, D-type cyclins exhibit cdk-independent functions that act as either negative or positive regulators of transcription factors, such as STAT3 and cyclin D-interacting myb-like protein-1, in addition to having a role in cell cycle regulation (reviewed in Ref. 24). Cyclin D3 was identified as a negative regulator of the hemopoietic transcription factor acute myeloid leukemia 1 (AML1), presumably by a mechanism that involves displacement of core-binding factor β from AML1, thereby inhibiting AML1’s DNA binding to target gene promoters (49). Recent reports by Gu and coworkers (50, 51) have served to extend the list of cyclin D3 partner proteins beyond transcriptional regulators to include the signal transduction protein kinase ERK3, and the translational regulator eIF3k. Notwithstanding, our results provide the first direct evidence that cyclin D3 in the context of assembled cyclin D3-cdk complexes is required for the G1-S phase progression in stimulated, normal B-1a cells.

We further analyzed the function of cyclin D3 by isolating and then stimulating B-1a cells from cyclin D3-deficient mice. Mice homozygous for a mutant allele containing a targeted deletion of the first two coding exons of cyclin D3 are viable, but suffer from...
defects in thymocyte development characterized by reduced CD4<sup>+</sup>CD8<sup>+</sup> double-positive T cells (41). Cyclin D3-null thymocytes fail to undergo the proliferative burst associated with the CD4<sup>+</sup>CD8<sup>+</sup> double-negative 3 to double-negative 4 transition. Our analyses herein of cyclin D3-deficient splenocytes revealed a decrease over wild-type littermates in the total cell number produced, in large part, by a decrease in the number of splenic B-2 (and not marginal zone) B cells that remains unexplained. Importantly, we found that cyclin D3 deficiency did not impact the peritoneal B-1a cell compartment, as the numbers of peritoneal B-1a and B-1b cells in cyclin D3-deficient mice were comparable to wild-type littermates. Furthermore, increased VH usage and spontaneous IgM secretion were similar for peritoneal B-1a cells in cyclin D3-deficient mice as compared with wild-type littermates. Consistent with this, we found that the serum levels of IgA and IgM were not significantly altered in cyclin D3-null mice in comparison to wild-type mice (data not shown). These results suggest that cyclin D3 is dispensable for B-1a cell development, self-renewal, and function. As noted above, this contrasts with the situation in cyclin D2-deficient mice, wherein a dramatic decrease in the number of peritoneal CD5<sup>+</sup> B cells has been reported (22). Thus, cyclin D2, but not cyclin D3 provides a nonredundant function in the development and/or self-renewal of peritoneal B-1a cells in the animal.

Our analysis of the potential for ex vivo peritoneal B-1a cells to proliferate in response to PMA indicated that cyclin D3-deficient B-1a cells proliferate at a normal tempo in comparison to wild-type B-1a cells. Although our results with the use of TAT-p16 peptides in normal B-1a cells demonstrate that cyclin D3 is important for mediating proliferative signals from several mitogens (e.g., PMA, LPS, and CD40L), the findings in cyclin D3-deficient mice suggest that cyclin D3 is dispensable for B-1a cell prolifer-ation in ex vivo primary cultures stimulated with PMA or LPS and under certain conditions. Such conditions include cyclin D2 gene expression increased 4-fold above the level found in wild-type B-1a cells stimulated by PMA. Furthermore, the elevated induction of cyclin D2 mRNA in cyclin D3-deficient B-1a cells is associated with early and sustained expression of cyclin D2 protein throughout G<sub>1</sub>-S progression. This sustained expression of cyclin D2 contrasts with the early and short-lived induction of cyclin D2 in wild-type B-1a cells stimulated with PMA.

The possibility that cyclin D2 may functionally replace cyclin D3 is strengthened by the observation that the level of endogenous pRb phosphorylation on cdk4/6-targeted residues in cyclin D3-deficient mice is comparable to that of wild-type B-1a cells. The authors have no financial conflict of interest.

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