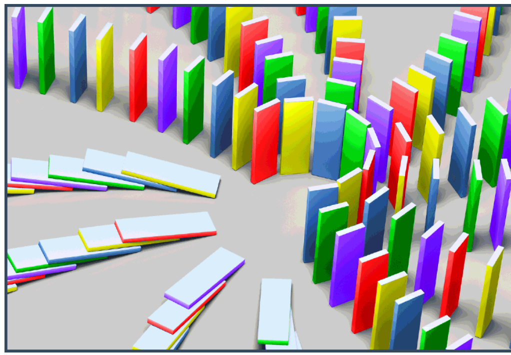
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A Pivotal Role of Cyclin D3 and Cyclin-Dependent Kinase Inhibitor p27 in the Regulation of IL-2-, IL-4-, or IL-10-Mediated Human B Cell Proliferation[1]

Eric F. Wagner, Marija Hleb, Nazeeh Hanna, and Surendra Sharma[2]

The functional differences between IgD^{high}CD38^{-} naive and IgD^{-}CD38^{-} memory (M) or IgD^{low}CD38^{+} germinal center (GC) B cells may stem from their variable response to signals that regulate activation, proliferation, and differentiation. In this report, we provide evidence for differential induction of cell cycle regulators in tonsillar human B cell subpopulations that were activated with anti-IgM and anti-CD40 in the presence or absence of IL-2, IL-4, or IL-10. Naive (IgD^{high}) B cells exhibited a significant proliferative response to IL-4, but not to IL-2 or IL-10, whereas these cytokines triggered variable levels of growth in the combined GC/M subpopulation (referred to as IgD^{low}), as measured by [^{3}H]thymidine incorporation. Induction of growth by cytokines in B cell subpopulations strictly correlated with the increased levels of cyclin D3 and cyclin-dependent protein kinase (cdk) 6. Moreover, only cyclin D3/cdk6 complexes were functional as observed in both naive and GC/M B cells stimulated in the presence of IL-4. In addition, active growth was associated with cytokine-mediated elimination of the cell cycle inhibitor p27. The significance of p27 in human B cell cycle was further demonstrated by rapamycin-mediated growth inhibition of IL-4-dependent proliferation, which resulted in strikingly increased p27 levels. Taken together, our findings suggest that cyclin D3, cdk6, and p27 play key roles in IL-2-, IL-4-, and IL-10-mediated human B cell proliferation. Furthermore, these results may provide a molecular basis for different cycling characteristics of naive and GC/M B cell subpopulations. *The Journal of Immunology,* **1998, 161: 1123–1131.**

The key events in the generation of humoral immune responses are mediated by cell-cell cross-talk and by signal-transducing interactions between cytokines and their receptors (1–4). Recently, these events have been extensively studied by using purified human tonsillar B cell subpopulations and CD40-mediated activation in combination with cytokines or Ig cross-linking. Importantly, it has been shown that IgD^{-}CD38^{-} memory (M) and IgD^{-}CD38^{+} germinal center (GC)[3] B cells exhibit different growth and differentiation functions as compared with IgD^{+}CD38^{-} naive B cells when activated through Ag receptor and/or CD40 Ag (5–7). Furthermore, these effector functions vary after costimulation with IL-2, IL-4, or IL-10 (8, 9). It has also been shown that memory, but not naive, peripheral blood B lymphocytes differentiate into Ig-secreting cells after CD40 ligation and costimulation with IL-4 or a combination of IL-2, IL-10, and IL-3 (10). It is then possible that Ag- or CD40-dependent activation and cytokine-mediated signaling may differ amply to exert disparate effects on B cell growth and differentiation events. The molecular picture of how B cell growth and differentiation occur is far from completion, but a new facet of cytokine-mediated signaling or autocrine growth involves the functional induction or downmodulation of key cell cycle regulators (11–14). Thus, in the context of intrinsic differences between naive and GC/M B cells, it can be hypothesized that these subpopulations may undergo differential induction of cell cycle regulators in response to activators and cytokines.

The restrictive nature of the cell cycle is in part regulated through an interplay between cyclins, serine/threonine cyclin-dependent protein kinases (cdks), and cyclin-dependent kinase inhibitors (CKIs) (15–18). The primary cyclin/cdk complexes associated with the G_1 phase of the cell cycle include cyclin D/cdk4 or 6 and cyclin E/cdk2 (19). These complexes regulate cell cycle progression from G_1 into S phase by interacting with the retinoblastoma gene product (pRB), leading to its phosphorylation (20). Recent studies have suggested that there is a differential usage of D-type cyclins in various immune cell types. For example, induction of cyclins D2 and D3, but not cyclin D1, has been detected in mitogen-activated human T cells and anti-IgM + IL-4-stimulated murine B cells (13, 21). Conversely, a murine macrophage cell line has been shown to express cyclin D1 (22). In the context of human B cells, induction and overexpression of cyclins D1 and D2 have been shown to occur in mantle zone B cell tumors and in EBV-positive B cells, respectively (12, 23–25). However, it remains to be determined whether differential induction and utilization of key cell cycle regulators is associated with the B cell growth in general and with intrinsic growth characteristics of naive and GC/M B cell subpopulations in response to cytokines.

The enzymatic activity of cyclin/cdk complexes can be altered by the action of a variety of CKIs with mechanisms of action that are not yet fully understood. These inhibitors can be separated into two groups. The first group (p16, p19, etc.) acts in a specific manner by sequestering monomeric cdk4 or cdk6 to prevent their interaction with D-type cyclins (26, 27). The second group (p21, p27, etc.) is capable of inhibiting all G_1 cyclin/cdk complexes

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[3] Abbreviations used in this paper: GC, germinal center; cdk, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; pRB, retinoblastoma gene product; ECL, enhanced chemiluminescence; USF, upstream stimulating factor.

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(28–30). Interestingly, p27 protein levels have been shown to fluctuate throughout the cell cycle, with the highest levels occurring during growth arrest (15, 16). It is believed that a mechanism of translational or postranslational control is in part responsible for the alteration in p27 protein levels (31, 32). Moreover, it has been demonstrated that anti-IgM + IL-4 stimulation results in a rapid down-regulation of p27 in both murine and human B cells (13, 14). These findings suggest an important role for this protein in normal B lymphocyte growth in response to cytokines.

In the present study, we have utilized anti-IgM + anti-CD40-activated naive and GC/M B cell subpopulations isolated from human tonsils to demonstrate that IL-2, IL-4, and IL-10 exert their growth-promoting activities by regulating the induction and/or down-modulation of cyclin D3, cdk6, and p27. Furthermore, it is shown that naive and GC/M human B cells differ in their potential to proliferate in response to distinct stimuli by virtue of their ability or failure to regulate these cell cycle regulators.

Materials and Methods

Reagents and cell culture

All cell culture experiments were performed in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with L-glutamine (2 mM), penicillin (50 U/ml)/streptomycin (50 μg/ml) and 10% FCS (all from Life Technologies). Cells were seeded at a density of 1×10^6 cells/ml and incubated at 37°C under 5% CO_2. B cell activation was achieved by using rabbit anti-human IgM specific for μ-chains (Dako, Carpinteria, CA), and anti-CD40 derived from hybridoma cells secreting the anti-human CD40 Ab G28.5 (American Type Culture Collection, Manassas, VA). Anti-human CD40 Ab was further purified by passing the hybridoma-conditioned medium through a protein G-Sepharose column (Bio-Rad, Hercules, CA), and the purified Ab was evaluated for its ability to activate resting human B cells to respond to IL-4 or IL-10. These preliminary experiments suggested that a concentration of 1 μg/ml was capable of stimulating significant [^{3}H]thymidine incorporation in the presence of these cytokines. Recombinant human IL-2 and IL-4 were obtained from Genzyme, Cambridge, MA. Recombinant human IL-10 was purchased from R&D Systems, Minneapolis, MN. Rapamycin was obtained from BioMol (Plymouth Meeting, PA), and cyclosporin A was a kind gift from Sandoz (East Hanover, NJ). Baculovirus recombinant cyclin D1, D2, and D3 lysates were purchased from PharMingen (San Diego, CA). The Jurkat T cell line was obtained from the American Type Culture Collection and was routinely maintained in RPMI 1640 + 10% FCS growth medium.

Isolation of tonsillar B cells

Human tonsillar B cells were isolated as previously described (6, 33). Briefly, tonsils were obtained from routine tonsillectomy patients and finely minced, and the resulting cell suspension was depleted of T cells by rosetting with neuraminidase-treated SRBC and subsequent Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient centrifugation. Non-B cells were removed through adherence to plastic for 2 h to overnight at 37°C in a humidified atmosphere of 5% CO_2. B cell populations were further purified on Percoll gradients (Pharmacia) according to density as described (34); however, a Percoll mix (2.5× PBS, 0.01 N HCl) was used in place of HBSS. Briefly, B cells were passed through a discontinuous Percoll gradient (50%, 60%, 66%, 70%). Visualization of gradients was aided by the addition of phenol red to the 50% and 66% layers. Phenotyping by FACScan (Becton Dickinson, Mountain View, CA) of cells collected at the 60% gradient (representing low density B cells enriched for IgD^-CD38^- memory and $IgD^{low}CD38^+$ GC B cells) showed that 97% of the cells were $CD19^+$, <28% IgD^+, >61% $CD38^+$, and >41% $CD95^+$. Cells collected at the 70% gradient (representing high density B cells enriched for IgD^+CD38^- naive B cells) showed that 98% of the cells were $CD19^+$, >75% IgD^+, <16% $CD38^+$, and <9% $CD95^+$. Cells sedimented at the 50% Percoll gradient represented dead and apoptotic cells as determined by trypan blue exclusion and morphology. The IgD^+ fraction in low density cells, which was repeatedly observed, most likely represents a IgD^+CD38^+ subpopulation of GC cells (35). Accordingly, combined GC/M population has been referred to as IgD^{low}.

Proliferation assays

Purified B cells (1×10^6 cells/ml) were activated with 1/250 dilution rabbit anti-human IgM and 1 μg/ml anti-CD40 and further stimulated with either 20 U/ml recombinant human IL-4, 100 U/ml recombinant human IL-2, or 10 ng/ml recombinant human IL-10. An aliquot of 200 μl containing 2×10^5 stimulated cells was placed in a 96-well, round-bottom microtiter plate (Falcon Labware, Oxford, CA) at times indicated and pulsed for 1 h with 1 μCi of [^{3}H]thymidine (1 Ci/mmol; ICN, Irvine, CA). Cells were harvested, and incorporated radioactivity was measured. All assays were repeated at least three times, and all measurements were in triplicate. It is important to point out that we observed variability in response to cytokines, in particular IL-2 and IL-10, among different tonsillar preparations. For the effect of rapamycin on the growth of B cells, 1×10^5 purified unfractionated B cells were seeded in 96-well round-bottom microtiter plates in a final volume of 200 μl. Cells were grown in 10% FCS and activated with anti-IgM (1/250 dilution), anti-CD40 (1 μg/ml), and IL-4 (20 U/ml) and cultured in the presence or absence of various concentrations of rapamycin or cyclosporin A. After 72 h in culture, cells were pulsed with 1 μCi/well [^{3}H]thymidine (1 Ci/mmol, ICN) for an additional 16 h, and incorporated radioactivity was measured.

Immunoblot analysis

Purified B cells were stimulated as described above for various time periods. Total cell extract preparation and immunodetection were performed as previously described (12). Briefly, 5 or 10×10^6 cells per time point were washed once in ice-cold PBS and harvested in 0.5 ml of lysis buffer (20 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5% Nonidet P-40, 4 mM NaF, 100 μM Na_3VO_4, 20 mM β-glycerophosphate, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF, 50 mM HEPES, 20 mM DTT). Protein concentration was quantified by the Bradford assay and by Coomassie blue staining of protein extracts separated on 12% SDS-PAGE. Total extract (50 μg/lane) was separated on a 12% SDS-PAGE, transferred to Hybond-enhanced chemiluminescence (ECL) nitrocellulose membranes (Amersham, Arlington Heights, IL), and blocked in TBS-T (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.2% Tween 20) containing 5% nonfat dried milk for 1 h at room temperature. Filters were then incubated overnight at 4°C with either anti-cdk2 (residues 287–298, Upstate Biotechnology, Lake Placid, NY), anti-cdk4 (Upstate Biotechnology), anti-cdk6 (clone C-21, Santa Cruz, Santa Cruz, CA), anti-cyclin D (residues 285–295, Upstate Biotechnology), anti-cyclin D2 (clone C-17, Santa Cruz), anti-cyclin D3 (clone C-16, Santa Cruz), anti-cyclin E (residues 1–128, Upstate Biotechnology), anti-p27 (clone 57, Transduction Laboratories, Lexington, KY), or anti-upstream stimulating factor (USF) (clone C-20, Santa Cruz) at a concentration of 1 μg/ml. Baculovirus recombinant cyclins D1 and D2 were used to differentiate between the different D-type cyclins. The cyclin D Ab recognizes both human cyclin D1 (36 kDa) and D2 (34 kDa). The membranes were incubated for 1 h at room temperature using appropriate horseradish peroxidase-conjugated secondary Abs and processed using the ECL detection system (Amersham).

Immunoprecipitations and in vitro kinase assays

Cell extracts were prepared as described above. Cell extracts containing 300 μg of total protein were immunoprecipitated by incubating with either rabbit polyclonal Ab against cdk2 (5.5 μg), cdk4 (2 μg), cdk6 (2 μg), or cyclin D2 (2 μg) or cyclin D3 Ab (2 μg) for 1 h at 4°C with rocking. Twenty-five microliters of protein A-Sepharose beads (Pharmacia, Milwaukee, WI) were then added, and the samples were rocked overnight at 4°C. After centrifugation, the pelleted beads were washed twice in lysis buffer and three times in kinase buffer (50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM DTT, 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM NaF) and suspended in a total reaction volume of 50 μl. Phosphorylation of histone H1 or pRB was determined by incubating the beads for 30 min at 30°C in the presence of 1 μM cold ATP (Stratagene, La Jolla, CA), 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; Andotek, Irvine, CA), and 2.5 μg of histone H1 (Boehringer Mannheim, Indianapolis, IN) or 0.5 μg of truncated retinoblastoma p56RB protein (QED Bioscience, San Diego, CA). The reaction was terminated by boiling the samples in 2× SDS sample buffer for 10 min. The phosphorylated substrates were then identified by resolution in 12% SDS-PAGE followed by drying and autoradiography.

Results

IL-4 induces proliferation in both IgD^{high} and IgD^{low} B cells

Naive, GC, and memory (M) B cells, distinguished on the basis of IgD and CD38 expression and separated into high and low density fractions on a Percoll gradient have been shown to exhibit differential maturation properties (6, 10). Distinct tonsillar B cell subpopulations were purified as described in *Materials and Methods*. For the purpose of this study, naive B cells are referred to as

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IgD^{high}, whereas GC/M combined population is referred to as IgD^{low}, since GC population consists of a IgD^{+} subpopulation (35). Consequently, we have used these subpopulations to evaluate their response to cytokines in a coactivation (anti-IgM + CD40) system. Simultaneous activation through IgM and CD40 receptor was utilized since coactivation of B cells with anti-IgM and anti-CD40 Abs has been shown to enhance their response to cytokines (36). Cell aliquots were harvested at 0, 6, 18, 30, 60, 72, and 96 h after stimulation and DNA synthesis measured by [^{3}H]thymidine incorporation. Figure 1*A* shows a typical experiment performed with a number of tonsillar IgD^{high} and IgD^{low} B cell preparations stimulated in the presence of IL-4. IL-4-dependent stimulation resulted in abundant proliferation in both IgD^{high} and IgD^{low} B cells, with maximal proliferation occurring around 60 h. It appears that both cell subpopulations enter S phase between 30 and 42 h, a finding consistent with the kinetics of S phase entry in anti-IgM + IL-4 stimulated murine low density B cells (13). Furthermore, the high [^{3}H]thymidine incorporation detected at 60 to 72 h in IgD^{high} B cells and at 42 to 60 h in IgD^{low} B cells indicates that a significant proportion of these cells undergoes two or three rounds of DNA synthesis. The extended kinetics of [^{3}H]thymidine incorporation in IgD^{high} B cells was observed in a number of experiments, suggesting that this B cell subpopulation may have a higher percentage of cycling cells in response to IL-4 than IgD^{low} B cells.

Identification of G_1 cyclins, cdks, and CKIs in IL-4-stimulated B cell subpopulations

The results shown in Figure 1*A* clearly suggest that both IgD^{high} and IgD^{low} subpopulations are competent in responding to IL-4 under the activation conditions used here. Since these subpopulations have been shown to differ in their long term proliferating abilities in response to CD40 and IL-4 or other cytokines (10, 37, 38), it is important to evaluate whether these subpopulations intrinsically differ in their induction and utilization of G_1 cyclins, cdks, and CKIs. Thus, we first investigated the expression of cyclins D1 and D2 by immunoblot analysis in IgD^{high} and IgD^{low} B cells stimulated in vitro for different time periods with anti-IgM + anti-CD40 + IL-4. Both subpopulations failed to express cyclin D1, even after treatment for 96 h (Fig. 1*B*). Cyclin D2, however, was detected as a doublet at low levels in both B cell populations (Fig. 1*B*). There appeared to be an up-regulation of cyclin D2 in IgD^{low} B cells between 24 and 48 h after stimulation. Total cell extract from the Jurkat T cell line was used as a positive control because of its documented expression of cyclin D2, but not of cyclin D1 (39). The Abs were also tested against baculovirus recombinant cyclins D1 and D2 and were found to detect both cyclin D1 (36 kDa) and D2 (34 kDa). It is important to point out that Abs from different vendors were used to ensure that a low level detection of cyclins D1 and D2 was not due to the poor quality of Abs. Also, to ensure equal protein loading, the blots were probed for the ubiquitously expressed cellular USF. The absence of cyclin D1 and low level of cyclin D2 expression are in agreement with the previous data from our laboratory and others indicating that both normal, unfractionated human B cells and EBV-negative Burkitt's lymphoma B cells express little or no cyclin D1 or D2 (12, 24, 25).

Although we cannot rule out the involvement of cyclin D2 in human B cell proliferation, it is highly unlikely that this cyclin plays an important role since it was not detected in abundance in proliferating IgD^{high} B cells (Fig. 1*B*). We next investigated the presence and induction of cyclin D3 and other G_1 cyclins and cdks by Western blotting using total cell extracts from IgD^{high} and IgD^{low} human B cells stimulated as described above. Cyclin D3 could be detected in unstimulated B cells, albeit at lower levels; this may be due to in vivo activation of a fraction of these cells.

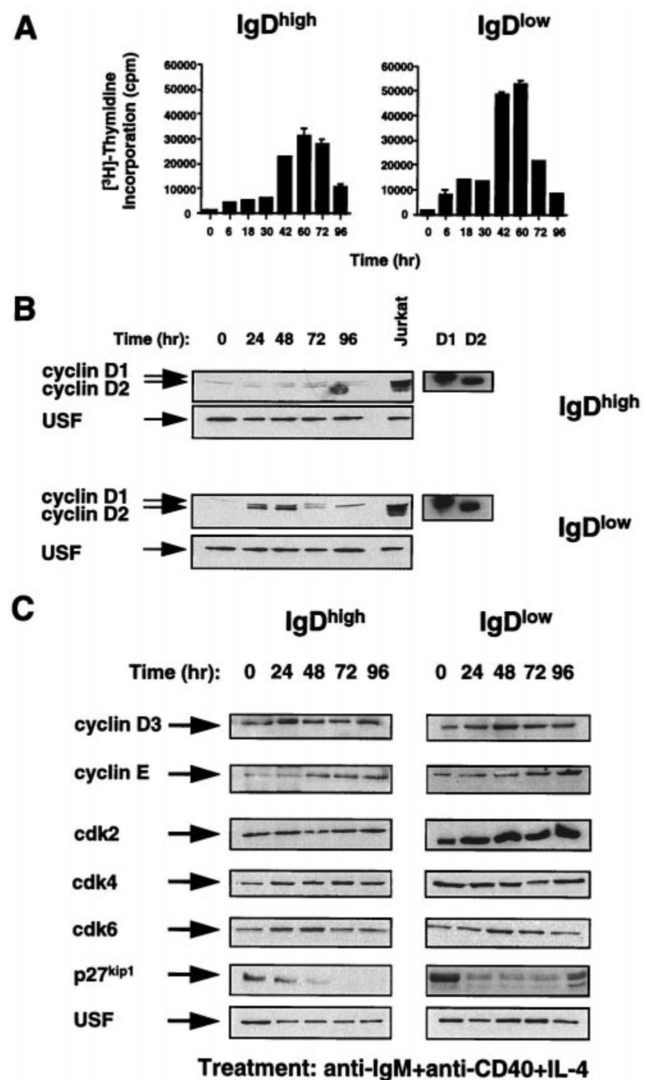

FIGURE 1. *A*, Effect of IL-4 on anti-IgM + anti-CD40-activated IgD^{high} and IgD^{low} human B cell proliferation. IgD^{high} and IgD^{low} human tonsillar B cells were cultured in the presence of anti-IgM (1/250 dilution), anti-CD40 (1 μg/ml), and IL-4 (20U/ml). At the times indicated, aliquots (200 μl) were removed and pulsed with 1 μCi [^{3}H]thymidine for 1 h. Cells were harvested, and incorporated radioactivity was measured. Each experiment was repeated several times and each time point was performed in triplicate. *B*, Cyclin D1 and D2 expression in IgD^{high} and IgD^{low} activated human tonsillar B cells cocultured with IL-4. IgD^{high} and IgD^{low} human B cells were stimulated as described in *A*, and cell lysates were isolated at the times indicated. Cyclins D1 and D2 were detected by immunoblotting with a combination of Abs against cyclins D1 and D2. Total cell extract from the Jurkat T cell line was included as a positive control for cyclin D2 expression. The amount of protein loaded in each lane was assessed by rehybridization of the filter with a specific Ab for the ubiquitously expressed transcription factor USF. *C*, IL-4-mediated induction of cell cycle proteins in activated IgD^{high} and IgD^{low} B cells. IgD^{high} and IgD^{low} B cells were stimulated as described above, and total cell extracts were prepared at 0, 24, 48, 72, and 96 h as described in *Materials and Methods*. Western blotting was performed as previously described and incubated with rabbit antisera against cyclin D3. In different experiments, the same membrane was successively stripped and reprobed with Abs against cdk2, cdk4, cdk6, cyclin E, p27, and USF.

Importantly, stimulation in the presence of IL-4 resulted in further induction of cyclin D3 which peaked at 24 to 48 h (Fig. 1*C*). We further examined the expression of cdk4 and cdk6, the catalytic

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partner(s) of D-type cyclins. cdk4 was expressed at detectable levels but showed no significant induction in either cell fraction during the time period examined (Fig. 1*C*). On the other hand, there was an increase in cdk6 expression, which was maximum around 48 h in both B cell subpopulations (Fig. 1*C*). Next, the expression of cyclin E and cdk2 was evaluated. Cyclin E induction was detectable in both IgD^{high} and IgD^{low} B cells and increased until 96 h after IL-4 stimulation (Fig. 1*C*). cdk2 expression was significant in both subpopulations; however, it was somewhat inducible in IgD^{low} B cells with little or no change in IgD^{high} B cells (Fig. 1*C*).

The cell cycle can be negatively regulated through the action of CKIs, including p16, p21, or p27 (15, 16). The CKI p16 was below the level of detection, and p21 remained unchanged at detectable but not significant levels in both IgD^{high} and IgD^{low} subpopulations during the time observed (data not shown). On the other hand, the levels of p27 were highest before IL-4 stimulation, correlating with growth arrest, and quickly decreased by 48 h in IgD^{high} fraction and 24 h in IgD^{low} fraction (Fig. 1*C*). The protein levels remained below detection in IgD^{high} B cells, whereas p27 began to return to original levels by 96 h in IgD^{low} B cells (Fig. 1*C*). As a control for equal protein loading, immunoblot analysis of USF was performed. Furthermore, the same immunoblot was stripped and reprobed with different Abs. Therefore, the unique regulation of p27 during IL-4-mediated B cell growth suggests an important role played by this protein.

Cyclin D3, cdk6, and p27 regulate IL-4-mediated stimulation of growth in human B cells

Given the induction profile of cell cycle regulators in IgD^{high} and IgD^{low} B cells (Fig. 1, *B* and *C*), it is of importance to evaluate which cyclin/cdk complexes are functionally active. Since cyclin D2 was seen to be induced in at least IgD^{low} B cells (Fig. 1*B*), we investigated the ability of cyclin D2/cdk or cyclin D3/cdk complexes to phosphorylate the pRB protein in vitro. Purified, fractionated B cells were activated with anti-IgM, anti-CD40, and IL-4, and total cell lysates were prepared at various time points. Lysates were immunoprecipitated with either a polyclonal cyclin D2 Ab or a polyclonal cyclin D3 Ab. Immunoprecipitates were then incubated in the presence of pRB substrate, γ-[^{32}P]ATP, and ATP and kinase activity analyzed by autoradiographic analysis. The cyclin D3 immune complexes elicited a strong kinase activity which could be detected at 12 h after IL-4 stimulation and remained constant in both IgD^{high} and IgD^{low} subpopulations throughout the observed 96 h time period (Fig. 2*A*). In contrast, cyclin D2 immune complexes failed to mediate any significant pRB phosphorylation in either B cell subpopulation, suggesting that cyclin D3, but not cyclin D2, was the key D-type cyclin regulating human B cell proliferation.

Next, we sought to examine whether cdk4 or cdk6 was associated with cyclin D3. Cyclin D3 immune complexes from B cells stimulated for 48 h as described above were probed with an Ab specific for either cdk4 or cdk6. The kinase cdk6, but not cdk4, was detected in immunoblots in both IgD^{high} and IgD^{low} B cells, indicating that the major active complex in the early G_1 phase is cyclin D3/cdk6 (Fig. 2*B*). To determine the efficiency of immunoprecipitations, total cell extracts from IgD^{low} B cells stimulated for 48 h with anti-IgM, anti-CD40, and IL-4 were immunoprecipitated and probed with either cdk4 or cdk6 Ab. cdk6 immunoprecipitated by its own Ab comigrated with cdk6 complexed with cyclin D3 (Fig. 2*B*). Importantly, while cdk4 was not part of cyclin D3-cdk complexes, it could be detected in immunoprecipitates obtained with its own Ab.

Since the mechanism of growth inhibition by p27 involves binding to and inhibiting the activity of the cyclin E/cdk2 complex (40)

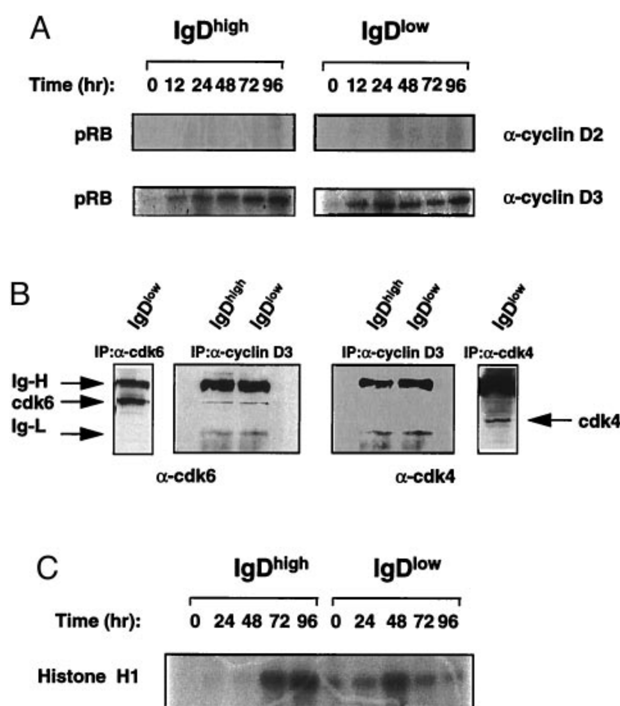

FIGURE 2. Cyclin D3-dependent pRB kinase activity and cdk2-dependent histone H1 kinase activity in IL-4-stimulated IgD^{high} and IgD^{low} B cells. *A*, Cell lysates from fractionated human B cells stimulated with anti-IgM (1/250 dilution), anti-CD40 (1 μg/ml), and IL-4 (20 U/ml) were immunoprecipitated with either anti-cyclin D2 (α-cyclin D2) or anti-cyclin D3 (α-cyclin D3) Ab, and kinase activity was assayed in vitro using pRB as a substrate as described in *Materials and Methods*. Time in hours after stimulation is indicated. *B*, IgD^{high} and IgD^{low} B cells were stimulated with anti-IgM, anti-CD40, and IL-4 for 48 h as described in *A*. Cell lysates were immunoprecipitated with anti-cyclin D3 Ab, and the immune complexes were probed with either anti-cdk4 (α-cdk4) Ab or anti-cdk6 (α-cdk6) Ab. To ensure efficient immunoprecipitation, IgD^{low} extracts were also immunoprecipitated and probed with either anti-cdk4 Ab or anti-cdk6 Ab. *C*, IgD^{high} and IgD^{low} human B cells were activated as described in *A*. cdk2-dependent kinase activity of the histone H1 protein was analyzed as described in *Materials and Methods*. The stimulation time is indicated in hours.

and because p27 was somewhat differentially regulated in IgD^{high} and IgD^{low} populations (Fig. 1*C*), cyclin E/cdk2 kinase activity was evaluated in vitro in the extracts from these B cell subpopulations. To monitor the activity of the cyclin E/cdk2 complex in IL-4-stimulated B cells, in vitro kinase assays were performed using the histone H1 protein as a substrate. Purified IgD^{high} and IgD^{low} human B cells were cultured for various times in the presence of anti-IgM + anti-CD40 + IL-4, and cell lysates immunoprecipitated with anti-cdk2 Ab. Since cdk2 complexes with cyclin E, it has been shown that in vitro phosphorylation of histone H1 is due in significant part to cyclin E/cdk2 complexes (27, 30). The kinase reactions were conducted as described in *Materials and Methods*. A strong histone H1 phosphorylation activity was observed in extracts from stimulated IgD^{high} cells at 72 to 96 h (Fig. 2*C*), which correlated with the elimination of p27 in these cells (Fig. 1*C*). However, in IgD^{low} B cells, maximum kinase activity occurred at 48 h when p27 levels were lowest. These data suggest that down-modulation of p27 is important for G_1-S transition, which correlates with active [^{3}H]thymidine incorporation in both B cell subpopulations (Fig. 1*A*) and with functional cyclin E/cdk2 complexes. Interestingly, p27 down-modulation appears to be kinetically different between naive and GC/M human B cells under

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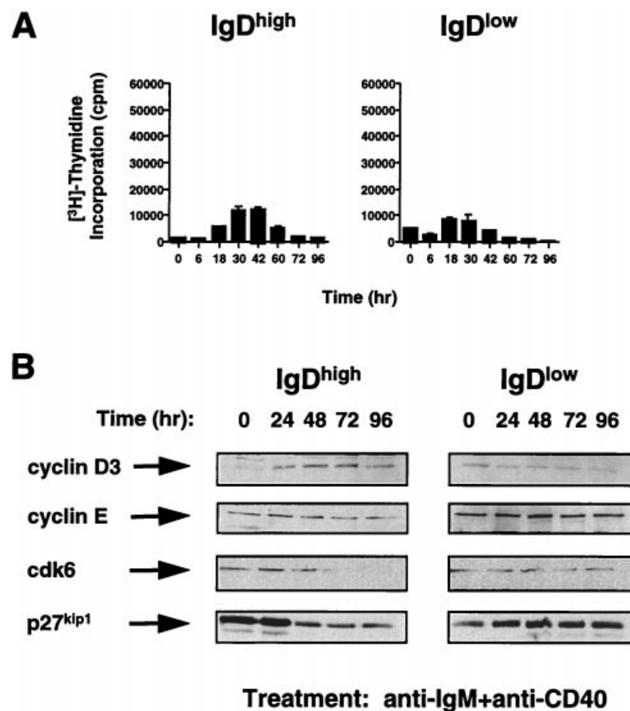

FIGURE 3. Effect of anti-IgM + anti-CD40 activation on the proliferation and induction of G_1 cyclins, cyclin-dependent kinases, and p27 in IgD^{high} and IgD^{low} human B cells. *A*, Human B cell subpopulations were activated with anti-IgM and anti-CD40 as described Figure 1. DNA synthesis was quantified by measuring [^{3}H]thymidine incorporation at various time points after activation. All assays were repeated at least three times, and all measurements were in triplicate. *B*, Fractionated human B cells were activated with anti-IgM + anti-CD40 as described in *A*, and total cell extracts were prepared at various times as described in *Materials and Methods*. Cyclin D3 was detected using a rabbit polyclonal anti-cyclin D3 Ab. In different experiments, the same membrane was sequentially stripped and reprobed with Abs against cdk6, cyclin E, and p27. Protein concentrations were normalized by Coomassie blue staining (data not shown).

similar activation conditions. An extended cyclin E/cdk2 kinase activity until 96 h might explain the proportionately higher [^{3}H]thymidine incorporation observed in IgD^{high} B cells in response to IL-4 at 72 to 96 h (Fig. 1*A*).

Anti-IgM and anti-CD40-mediated activation alone results in a poor down-modulation of p27 and a weak induction of cyclin D3 and/or cdk6

Next, we evaluated the extent of B cell proliferation as well as induction of cell cycle regulators in B cells activated with anti-IgM + anti-CD40 alone. The data in Figure 3*A* revealed that anti-IgM + anti-CD40-mediated activation triggered a minor proliferative response as compared with IL-4 costimulation, with maximum [^{3}H]thymidine incorporation (12,000 cpm for IgD^{high}; ~7500 cpm for IgD^{low}) occurring between 30 and 42 h in both cell fractions.

Since earlier results suggested that IL-4-mediated growth involved cyclin D3/cdk6 complexes in combination with p27 down-regulation, we next sought to determine whether the minor proliferation observed after activation with anti-IgM + anti-CD40 was due to inadequate expression of cyclin D3 and/or cdk6 or possibly the inability to down-modulate p27. Immunoblot analyses of proteins from total cell extracts isolated from IgD^{high} and IgD^{low} B cells activated with anti-IgM + anti-CD40 were conducted for cyclin D3, cdk6, cyclin E, and p27. In IgD^{high} B cells, cdk6 expression remained unchanged until 48 h when levels decreased to below detection (Fig. 3*B*). Cyclin D3 expression was inducible at low levels initially (~24 h); however, its levels did not appear to change thereafter (Fig. 3*B*). Cyclin E levels remained unchanged throughout the time examined. Interestingly, the levels of p27 began to decrease at 48 h after activation but remained at significant levels throughout the culture period (Fig. 3*B*). The presence of cyclin D3 and cdk6 until 48 h along with a slight down-modulation of p27 could possibly account for the minor proliferation observed in Figure 3*A*. On the other hand, IgD^{low} B cells failed to show any induction of either cyclin D3 or cdk6, although they could be detected at low levels (Fig. 3*B*). Cyclin E was expressed at significant levels but, like IgD^{high} B cells, failed to be induced. Curiously, there was no modulation of p27 throughout the time examined, perhaps accounting for the poor proliferation observed in this subpopulation (Fig. 3*A*). Since these cells begin to undergo apoptosis without further stimulation (E. Wagner and S. Sharma, unpublished observations), probing with USF to ensure equal protein loading was not possible. As an alternative approach, protein concentrations were normalized by Coomassie blue staining (data not shown). The inability of anti-IgM + anti-CD40 treatment to induce a threshold expression of cyclin D3 and cdk6 and to significantly decrease the levels of p27 may explain the minor proliferation observed in these cells.

IL-10 differs from IL-4 in that it fails to stimulate a sustained expression of cyclin D3 and cdk6 in IgD^{high} cells

IL-10 is another potent B cell maturation factor that exerts varying effects depending on the origin of a cell and the presence of other cytokines (41–43). To examine the response of IgD^{high} and IgD^{low} human tonsillar B cells to IL-10, fractionated B cells were activated with anti-IgM + anti-CD40 + IL-10 and harvested at various times; and DNA synthesis was measured by [^{3}H]thymidine incorporation. IgD^{low} B cells displayed a robust proliferation in response to IL-10, with maximum proliferation occurring 60 h poststimulation (Fig. 4*A*). In contrast, the addition of IL-10 to IgD^{high} B cells resulted in only minor proliferation, which was comparable to that observed in cells activated with anti-IgM and anti-CD40, except for a shift in the kinetics of peak proliferation (60 h vs 30–42 h). These results indicate that IgD^{high} and IgD^{low} human B cells exhibit differential proliferation in response to IL-10 (42, 43).

To determine whether the varied proliferation responses between IgD^{high} and IgD^{low} human B cells to IL-10 was mediated through differential regulation of cyclin D3, cdk6, or p27, Western blot analysis was performed using total cell extracts from fractionated B cells activated with anti-IgM, anti-CD40, and IL-10. IgD^{high} B cells showed an inducible expression of cdk6 with maximum levels detected around 60 h poststimulation which decreased to below detection by 96 h (Fig. 4*B*). Cyclin D3, however, was induced between 24 and 48 h, and its levels began to decline thereafter (Fig. 4*B*). Curiously, cyclin E was detectable throughout the time examined but failed to be induced (Fig. 4*B*). p27, however, remained unchanged until 60 h poststimulation but decreased below detectable levels (Fig. 4*B*) after this time period. On the other hand, in IgD^{low} B cells, cyclin D3 was induced at ~24 h and reached maximum levels between 48 and 60 h after treatment with IL-10 (Fig. 4*B*). cdk6 expression was also induced at 24 h, and its levels remained constant until 96 h (Fig. 4*B*). Cyclin E reached maximum levels between 72 and 96 h (Fig. 4*B*). The p27 levels were characteristically highest at 0 h but dropped significantly by 24 h. The original levels, however, were restored by 96 h (Fig. 4*B*). The growth of IgD^{low} B cells in response to IL-10 correlates with the disappearance of p27 and the presence of cyclin D3 and cdk6 between 24 and 60 h. On the other hand, in IgD^{high} B cells, the presence of p27 up to 60 h poststimulation and the absence of cdk6

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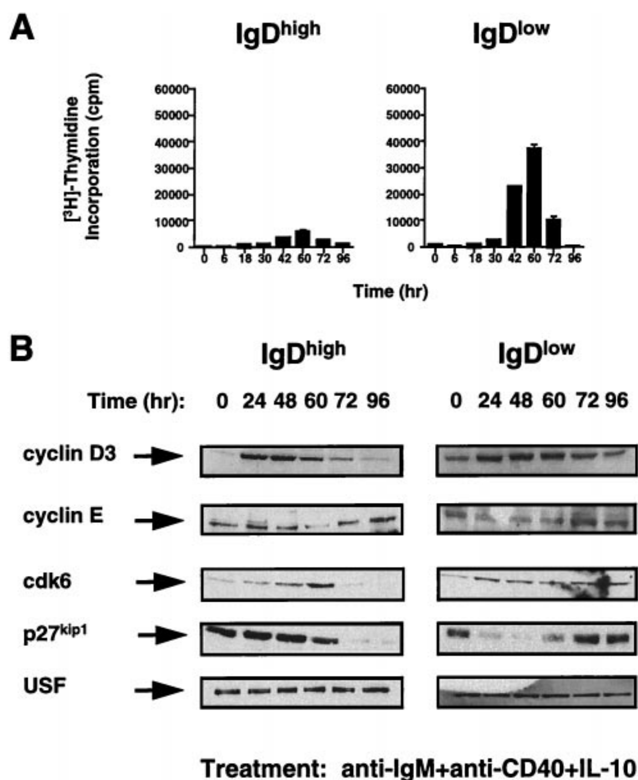

FIGURE 4. Effect of IL-10 on the growth of B cells and induction of G_1 cyclins, kinases, and the inhibitor p27 in IgD^{high} and IgD^{low} human B cells. *A*, Fractionated human B cells were stimulated with anti-IgM (1/250 dilution), anti-CD40 (1 μg/ml), and IL-10 (10ng/ml). Cell aliquots (200 μl) were removed at various times after stimulation, and DNA synthesis was quantified by measuring [^{3}H]thymidine incorporation. For accuracy, all experiments were repeated several times, and each point was performed in triplicate. *B*, IgD^{high} and IgD^{low} human B cells were stimulated as described in *A*, and total cell lysates were prepared as described in *Materials and Methods*. Cyclin D3 was detected in cell lysates (50 μg) by immunoblotting with anti-cyclin D3 Ab. The blot was subsequently stripped and reprobed with anti-cdk6, anti-cyclin E, anti-p27, and anti-USF Abs.

and cyclin D3 at later time points may account for the poor proliferation observed in Figure 4*A*. The varying expression of key cell cycle regulators in IgD^{high} and IgD^{low} B cells is not due to unequal loading of protein, because the USF signal was of approximately the same intensity in each lane. Taken together, our data suggest that the differential modulation of cyclin D3, cdk6, and p27 could possibly explain the varying proliferative responses between IgD^{high} and IgD^{low} B cells in response to IL-10.

IL-2 fails to significantly down-modulate p27 expression and induces a poor proliferative response in both IgD^{high} and IgD^{low} cells

The major growth factor that stimulates proliferation and functional differentiation of T cells is IL-2 (44). In T cells, IL-2 has been shown to induce proliferation by eliminating p27 (45). In the context of B cells, IL-2 has been shown to act as a growth and differentiation factor, albeit with variable effects depending on the stage of maturation and/or activation state of the B cell (46–48). To examine the response of IgD^{high} and IgD^{low} human tonsillar B cells to IL-2, anti-IgM + anti-CD40-activated IgD^{high} and IgD^{low} B cells were cocultured with IL-2. Cell aliquots were taken at various times, and DNA synthesis was measured by [^{3}H]thymidine incorporation. In IgD^{low} B cells, maximum proliferation occurred between 42 and 60 h poststimulation (Fig. 5*A*). However, the peak

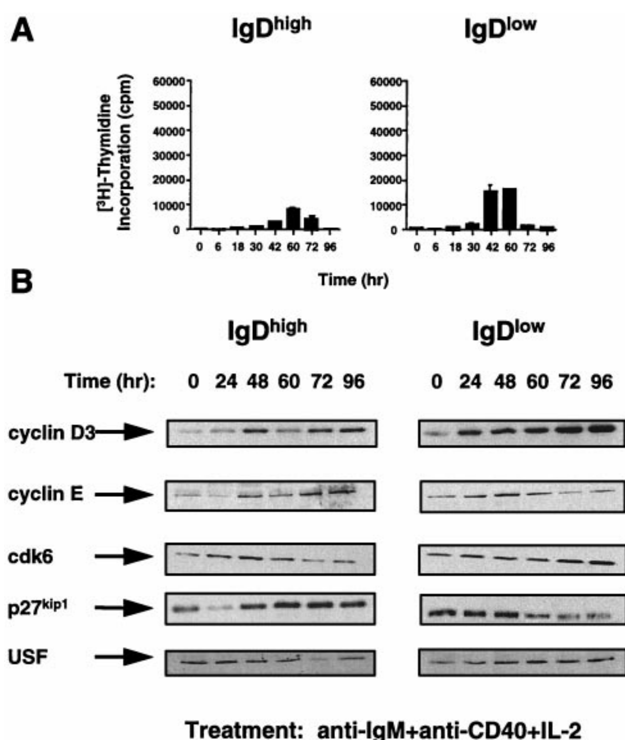

FIGURE 5. IL-2-mediated cell proliferation and modulation of G_1 phase cell cycle-regulatory proteins in IgD^{high} and IgD^{low} human B cells. *A*, IgD^{high} and IgD^{low} human B cells were activated with anti-IgM (1/250 dilution), anti-CD40 (1 μg/ml), and IL-2 (100U/ml). Aliquots (200 μl) were removed at various times after stimulation, and DNA synthesis was quantified by measuring [^{3}H]thymidine incorporation. Each point was performed in triplicate, and each experiment was repeated several times. *B*, Cyclin D3 was detected by immunoblotting with anti-cyclin D3 Ab in cell lysate (50 μg) of fractionated human B cells. The blots were stripped and reprobed with Abs against cdk6, cyclin E, p27, and USF.

[^{3}H]thymidine incorporation levels were only marginally higher (18,000 cpm) than those seen in response to anti-IgM + anti-CD40 alone (~7,500 cpm). In contrast, IgD^{high} B cells stimulated with IL-2 resulted only in a minor proliferation (Fig. 5*A*). Similar to treatment with IL-10, IL-2 stimulation resulted in a shift in the kinetics of peak proliferation (60 h vs 30–42 h) as compared with anti-IgM + anti-CD40-activated IgD^{high} B cells.

To correlate the overall poor response of IgD^{low} and weaker response of IgD^{high} B cells to IL-2 with an inadequate regulation of cyclin D3, cdk6, ad, p27, immunoblot analyses were performed using total cell extracts from fractionated B cells cultured in the presence of anti-IgM, anti-CD40, and IL-2 (Fig. 5*B*). In IgD^{high} B cells, cdk6 was induced and reached maximum levels by 60 h; thereafter its levels began to decline (Fig. 5*B*). Cyclin D3 levels increased throughout the time of examination; peak levels were obtained between 72 and 96 h poststimulation. p27 levels, however, remained almost constant at high levels throughout that time. IgD^{low} B cells, on the other hand, showed an inducible expression of both cdk6 and cyclin D3. Interestingly, cyclin E was induced in both subpopulations with levels somewhat declining at 72 and 96 h in IgD^{low} B cells. p27 levels were highest at time 0 h and began to decline between 48 and 60 h. However, while p27 levels declined, they never fell below the level of detection as observed in IL-4- and IL-10-stimulated IgD^{low} B cells. Again, quantitation of USF protein suggested an equal protein loading in each lane. Taken together, our results suggest that IL-2 stimulation is capable of inducing the expression of cyclin D3, cdk6, and cyclin E in both

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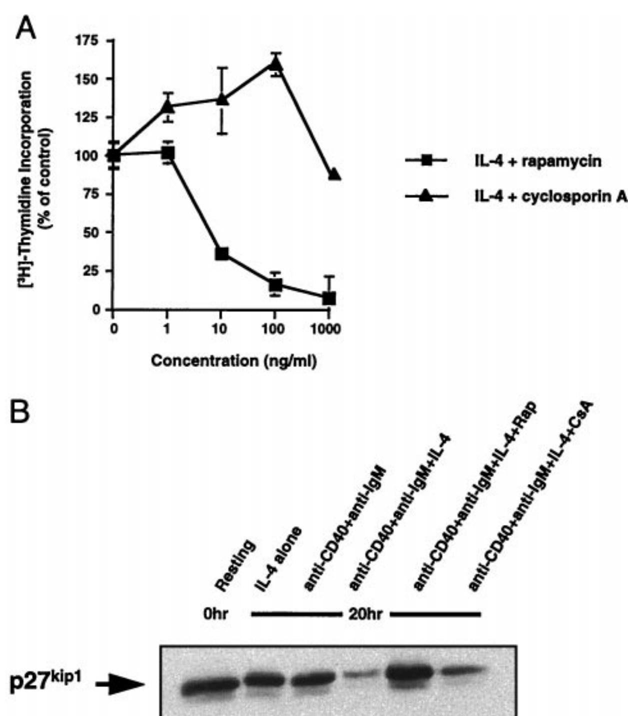

FIGURE 6. Growth inhibition of tonsillar B cells by rapamycin (Rap) is associated with restoration of p27 to threshold levels. *A*, Cell proliferation was measured as [^{3}H]thymidine incorporation and expressed as percentage of control (no rapamycin + 10% serum only). Control [^{3}H]thymidine incorporation values were 17,370 cpm (rapamycin treatment) and 15,156 cpm (cyclosporin A (CsA) treatment). *B*, Unfractionated, purified B cells were activated with anti-IgM (1/250 dilution), anti-CD40 (1 μg/ml), and IL-4 (20 U/ml) and treated with either rapamycin (100 ng/ml) or cyclosporin A (100 ng/ml) for 20 h. Total cell extracts were prepared as described in *Materials and Methods* and resolved by 12% SDS-PAGE. p27 protein expression was detected using a mouse mAb and an appropriate horseradish peroxidase-conjugated secondary Ab followed by ECL detection.

IgD^{high} and IgD^{low} B cells; however, it fails to efficiently down-modulate p27, possibly explaining the poor proliferative response in these cells (Fig. 5*A*).

Rapamycin inhibits IL-4-mediated growth in human tonsillar B cells by restoring p27 levels

Rapamycin is a potent immunosuppressant that blocks cell growth in the mid- to late G$_1$ phase of the cell cycle (49). In T cells, rapamycin blocks IL-2-mediated T cell growth by restoring p27 to threshold levels (45). Moreover, rapamycin-mediated growth inhibition in normal B cells or in the Burkitt's lymphoma B cell line BJAB (50, 51) suggests its ability to act in B cells as well. Since IL-4 induced significant proliferation in both IgD^{high} and IgD^{low} subpopulations of B cells (Fig. 1*A*) and decreased p27 levels (Fig. 1*B*), we wanted to investigate whether rapamycin would inhibit IL-4-mediated proliferation and restore p27 levels. As a control, cyclosporin A was used in these assays. Specifically, anti-IgM + anti-CD40 + IL-4-stimulated unfractionated tonsillar B cells were sensitive to the growth-inhibitory effects of rapamycin, showing >50% inhibition at the dose of 10 ng/ml (Fig. 6*A*). In contrast, cyclosporin A had no effect on IL-4-mediated B cell growth at the comparative doses (Fig. 6*A*). To determine whether the rapamycin-mediated growth inhibition in IL-4-stimulated B cells was due to an increase in p27 levels, Western blot analysis was performed with unfractionated tonsillar B cells treated for 20 h with various combinations of anti-IgM, anti-CD40, and IL-4 and grown in the absence or presence of cyclosporin A or rapamycin. Cells activated with anti-IgM, IL-4, CD40, or anti-IgM + anti-CD40 did not exhibit decreased levels of p27 (Fig. 6*B*). However, addition of IL-4 either alone or in combination with cyclosporin A to activated B cells (anti-IgM + anti-CD40) showed a significant decrease in the amounts of p27 (Fig. 6*B*), which is indicative of growth-promoting conditions. In contrast, addition of rapamycin resulted in restoration of p27 back to almost original levels (Fig. 6*B*), which correlates with the growth inhibition observed in Figure 6*A*. Taken together, these results suggest a crucial role of p27 in regulating human B cell cycle progression in response to growth promoting cytokines.

Discussion

In the present study using purified naive (IgD^{high}) and combined GC/M (IgD^{low}) B cell subpopulations, we have investigated the roles of cell cycle proteins in regulating human B cell proliferation in response to anti-IgM + anti-CD40 and costimulation with IL-2, IL-4, or IL-10. In a series of related experiments, it is demonstrated that these cytokines exert their growth-promoting activities through the induction of cyclin D3 and cdk6 and concomitant down-modulation of p27. Moreover, we have shown that the differential regulation of these molecules is in part responsible for the ability or inability of naive and GC/M B cells to proliferate in response to these cytokines.

It is well documented that IL-2, IL-4, and IL-10 exhibit a redundant characteristic in that these cytokines stimulate proliferation in activated B cells, and the extent of the proliferative responses to these cytokines has been shown to vary depending on the mode of activation and the maturation stage of the B cell (5, 36, 38, 48, 52). For example, in the reconstituted CD40 system, which depends on coculturing B cells with CD32-transfected fibroblasts coated with anti-CD40 mAb and cytokines, IL-4 induces proliferation in both naive and GC/M subpopulations and supports long term, factor-dependent growth preferentially in IgD$^+$ B cells, whereas IL-10 induces terminal differentiation possibly in both populations (38, 42). Curiously, IL-2 fails to exert any major activity in this system; however, it is reasonably effective in promoting growth in B cells activated with anti-IgM (46, 53). Corroborating these observations, purified peripheral naive (IgD$^+$) B cells have also been shown to preferentially cycle in response to anti-CD40 and IL-4, whereas memory (IgD$^-$) B cells proliferate when stimulated with a combination of IL-2, IL-10, and IL-3 (10). In the present study, IL-4 was found to be a potent inducer of proliferation in both IgD^{high} and IgD^{low} B cells (Fig. 1*A*), suggesting a unique ability of this lymphokine to stimulate both naive and GC/M B cells. IL-10 was able to induce proliferation only in IgD^{low} (GC/M) B cells, but not in IgD^{high} B cells (Fig. 4*A*). Finally, IL-2 proved to be a poor inducer of B cell proliferation, even in an IgD^{low} B cell subpopulation (Fig. 5*A*). Despite the disparity in their abilities to induce proliferation, all of these cytokines mediated their effects through the modulation of cyclin D3, cdk6, and p27, suggesting a possible link for their redundant activity in the context of B cell proliferation. Together, these findings point to intrinsic differences between naive and GC/M B cell subpopulations in their responses to different cytokines.

Although the molecular mechanisms underlying these variances remain to be elucidated, our results strongly suggest that active human B cell growth is tightly controlled by cyclin D3/cdk6 complexes and the cell cycle inhibitor p27. In the case of IgD^{high} (naive) B cells, their failure to respond to anti-IgM + anti-CD40 or to these activators in the presence of IL-2 or IL-10 clearly correlated

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with a cryptic induction of cyclin D3 and cdk6 and a poor down-modulation of p27 (Figs. 3 to 5). Interestingly, Galibert et al. have shown that IgD^{+}, but not IgD^{-}, B cells preferentially undergo long term proliferation in response to CD40 and IL-4 (38). In this regard, our data on the regulation of p27 protein levels in IgD^{high} B cells in response to anti-IgM + anti-CD40 + IL-4 are of significance in that p27 remained below detection beyond 96 h in these cells as compared with its restoration in IgD^{low} B cells at this time point (Fig. 1*C*). Although our experiments were not conducted beyond 96 h, it is tempting to speculate that the presence of active cyclin D3/cdk6 complexes and the absence of p27 for an extended time in IL-4-treated IgD^{high} B cells will provide long term growth advantages.

The finding that cyclin D3, but not cyclin D2, is the key D-type cyclin involved in human B cell cycle progression provides further evidence for cell type-specific utilization of D-type cyclins (Fig. 2). Although cyclin D2 was marginally expressed at least in IgD^{low} B cells (Fig. 1*B*), it is possible that its expression was below the threshold level required to form complexes with cdk4 or 6. In this regard, it is important to point out that EBV has been shown to up-regulate cyclin D2 expression in B cells (12, 24). However, it is not yet known whether this cyclin forms functionally active complexes with its kinase partners in EBV-positive B cells. On the other hand, PHA-activated human T cells and anti-IgM + IL-4-treated murine B cells have been shown to express and utilize cyclin D2 (13, 21). In this context, our data corroborate the findings obtained with T cell hybridomas and IL-7-stimulated precursor B cells where cyclin D3 plays a key role in their cell cycle progression (54, 55). Despite the evidence provided here for the unique participation by cyclin D3 and cdk6 in human B cell cycle, cyclin E/cdk2 complex activity seemed to pattern other cell types. For example, histone H1 phosphorylation by cdk2 immunoprecipitates occurred when p27 protein levels were found to be negligible (Figs. 1*C* and 2*C*). Similarly, cyclin E was inducible and detectable in response to IL-2, IL-4, and IL-10 under the activation conditions used here. Thus, the major differences in cell type-specific cell cycle regulation might be attributed to D-type cyclins and their catalytic kinase partners. Furthermore, it is also tempting to speculate that although cdk4 and cdk6 may both be coexpressed at significant levels, only one of them will be selectively recruited to complex with its cyclin D partner. In this regard, cdk4 was significantly expressed in both IgD^{high} and IgD^{low} B cells, but it failed to complex with cyclin D3 (Figs. 1*C* and 2*B*). It is possible that different cyclin D-cdk complexes would function in cells of different origin or stage of development. For example, cdk4 is primarily associated with cyclin D3 in precursor B cells and cdk6 complexes with both cyclin D2 and cyclin D3 in activated T cells (54, 56).

The importance of CKIs in regulating the cell cycle has been a major interest in both normal and malignant cells (18, 57). By virtue of its ubiquitous presence in growth-arrested cells and elimination during active growth, p27 has become a major focus in understanding cell cycle progression. G_1 arrest can be induced by the ectopic expression of p27 cDNA, and there appears to be a correlation between p27 levels and growth arrest under different mitogenic conditions (29). Recently, it has been suggested that cell cycle progression is dependent on the balance of sequestered p27 by cyclin D/cdk complexes and free p27 which is able to bind and inhibit cyclin E/cdk2 complex activity (40). In this regard, cyclic AMP- and rapamycin-induced growth arrest has been attributed to increased levels of p27 (45, 58). In our studies, p27 appeared to be the major player in negatively regulating human B cell growth. Cyclin E/cdk2 kinase activity inversely corresponded with the presence of p27 (Fig. 2*C*). The failure of IL-2 to significantly down-modulate p27, particularly in IgD^{high} B cells (Fig. 5), may explain why this cytokine is a poor inducer of B cell proliferation under the activation conditions used in the present study. This agrees with the observations that treatment of IL-4-stimulated human B cells with rapamycin effectively inhibited cell growth by restoring the protein levels of p27 (Fig. 6). These results suggest that down-modulation of p27 is necessary for normal human B cell cycle progression and that different stimuli exhibit varying capacities to modulate this kinase inhibitor.

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