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A

Cell Cycle Control in B Cell Terminal Differentiation Induced by IL-6: Repression of Epstein-Barr Virus LMP1 by CDK Inhibitor p18

by

Dongquan Chen

A dissertation submitted to the graduate faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1999

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THE CITY UNIVERSITY OF NEW YORK

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Abstracts

Cell Cycle Control in B Cell Terminal Differentiation Induced by IL-6: Repression of Epstein-Barr Virus LMP1 by CDK Inhibitor p18

by

Dongquan Chen

Adviser: Professor Selina Chen-Kiang

IL-6 induces orderly differentiation, cell cycle arrest and cell death in B cell terminal differentiation in an Epstein-Barr virus (EBV)-immortalized human IgG+ lymphoblastoid B cell line CESS. The mechanism underling this concerted process is not well understood. Here, we show that IL-6 activates the CDK inhibitor p18^{INK4C}, leading to sequestration of CDK6 by p18 and cell cycle arrest in IgG+ CESS cells. However, IL-6 induces differentiation without cell cycle arrest in IgM+ cells. Overexpression of p18 is sufficient to reconstitute coupled differentiation and cell cycle arrest in IgM+ cells. This suggests a role for p18 in IL-6-induced cell cycle control during B cell terminal differentiation.

It has been shown that concomitant with cell cycle arrest, the EBV transforming genes EBV nuclear antigen (EBNA) 1, EBNA2 and LMP1 are suppressed in IgG+ cells. Thus, there is an inverse relationship between the expressions of p18 and EBV transforming genes, and p18 may mediate the IL-6 signals for suppression of EBV expression. To address these possibilities, we first identified that suppression of LMP1, but not EBNA2, correlated with cell cycle arrest. We then showed that expression of p18 by an adenovirus recombinant virus led to cell cycle arrest in both IgM-bearing and IgG-

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bearing B cells. We further demonstrated that expression of p18 alone was sufficient to cause degradation of the LMP1. Together with the finding that IL-6 induces degradation of LMP1 by a mechanism independent of the proteosomes, our results suggest that p18 mediates the IL-6 signals to suppress the expression of LMP1 by proteolysis independent of the proteosomes.

Further investigation of EBV immortalization of primary human B lymphocytes reveals that IgM+, but not IgG+, cells are preferentially immortalized by EBV, correlating with elevated LMP1 expression and continuous cell cycling. This implies that transformation of human B cells by EBV and impairment of terminal differentiation may hinge on LMP1 expression, which in turn appears to be contingent on the B cell developmental stages. Therefore, we propose that IL-6 signals a critical interplay between p18 and the LMP1 protein that simultaneously reverses EBV transformation and induces cell cycle arrest, the hallmark of B cell terminal differentiation.

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Finally, I want to express my deepest gratitude to my parents, my wife and my lovely twin daughters. Their unconditional love, understanding and supports make all I have accomplished during the past five years possible.

I would like to dedicate this thesis to my parents.

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Format of the Thesis

This thesis was prepared in accordance with guidelines of the City University of New York. It was divided into six chapters. Chapter 1 is a general introduction. Chapter 2 is the Materials and Methods, which have been consolidated from all other related chapters. Chapter 3 "Induction of cell cycle arrest and B cell terminal differentiation by CDK inhibitor $p18^{INK4C}$ and IL-6. " has been published in Immunity. (1997). 6:47-56. Chapter 4. "Reversal of EBV-Immortalization by $p18^{INK4C}$-induced proteolysis of LMP1." is in preparation for submission. Chapter 5. "NF-IL6 regulation and function in B cell terminal differentiation." is a work in progress. Chapter 6 is the Summary and Future Studies. Figures and Tables are placed at the end of each chapter except in Chapter 1 and Chapter 6, in which they are put inside the chapters.

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Chapter 1.

Introduction

I. IL-6 and its function in B cells.

IL-6, a 20-30 kDa glycoprotein, is a pleiotropic cytokine that regulates immune responses, acute phase reactions, hematopoiesis and viral infections (Kishimoto et al., 1992; Chen-Kiang et al., 1993; Kishimoto et al., 1994; Chen-Kiang, 1995). The distinct functions of IL-6 in B cell proliferation and terminal differentiation have been prominently demonstrated in vivo and in vitro.

(1). IL-6 induces plasmacytoma and is requisite for secondary immune response.

It was reported in 1962 that mineral oil pristane can induce plasmacytoma (Potter, 1962). The mediator of the effect was then found to be a macrophage-derived factor (Nordan and Potter, 1986), and later purified as plasmacytoma growth factor (PCT-GF) (Nordan et al., 1987). From N-terminal sequence of PCT-GF, it was found that PCT-GF is IL-6 in human (Wong et al., 1988) and in mouse (Mock et al., 1989). That IL-6 mediates pristane-oil-mediated formation of plasmacytoma was further demonstrated in IL-6 transgenic mice and in IL-6 knockout mice. In IL-6 transgenic mice, massive plasmacytosis was found in thymus, lymph node and spleen. The plasma cells infiltrate to various tissues such as lung, liver, and kidney (Suematsu et al., 1989). However, IL-6 is also required for the secondary immune response. In IL-6 -/- mice, a profoundly impaired IgG and IgA secretion was found, although IgM-bearing cells that are earlier in the B cell lineage develop normally (Kopf et al., 1994). In addition, defective development of pristane-oil-induced plasmacytosis and plasmacytomas in IL-6 -/- mice was also reported (Hilbert et al., 1995).

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(2). IL-6 induces B cell terminal differentiation in vitro.

In our laboratory, the major hallmarks of terminal differentiation of B cells have been observed in vitro in IL-6-treated CESS cells, which are EBV-immortalized human clonal IgG1-bearing lymphoblastoid cells(Natkunam et al., 1994; Raynal et al., 1989). These hallmarks are: a markedly-enhanced synthesis and secretion of immunoglobulin (Ig); reduced surface expression of the major histocompatibility complex (MHC) class II (Halper et al., 1978; Natkunam et al., 1994); and the morphological maturation which is characteristic of plasma cells (Natkunam et al., 1994). The molecular basis for the increased Ig synthesis has been demonstrated to be an enhanced transcription of Igγ1 heavy chain and λ light chain genes and differential accumulation of secreted form-specific γ1 mRNA relative to alternatively-spliced membrane form-specific γ1 mRNA; This in vitro differentiation system represents the only in vitro system that can recapitulate all major hallmarks of B cell terminal differentiation.

II. IL-6 signaling

IL-6 belongs to a family of cytokines that share the same signal transducing receptor component gp130. This family includes ciliary neurotrophic factor (CNTF)(Davis et al., 1993), leukemia inhibitory factor (LIF) (Ip et al., 1992), oncostatin M (OSM) (Gearing et al., 1992), IL-11 (Kishimoto et al., 1994) and cardiotrophin-1(Ishikawa et al., 1996) in addition to IL-6.

Activation by the IL-6 family cytokines leads to either homodimerization of gp130 or heterodimerization of gp130 with a ligand-specific receptor and thus activation of gp130 by phosphorylation (Kishimoto et al., 1994; Stahl et al., 1994). The cytokine specific function is thought to be mediated by the ligand-specific receptor components in addition to gp130 (Kishimoto et al., 1994). The ligand-specific receptor subunit for the IL-6, IL-6Rα (gp80), is present in two forms: a membrane bound form and a soluble form (sIL-

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6R). The sIL-6R contains only the extracellular domain of the IL-6 receptor. The sIL-6R can be generated by either proteolysis after stimulation by phorbol esters (Mackiewicz et al., 1992; Mullberg et al., 1993) or by alternative mRNA splicing (Lust et al., 1992). Unlike other cytokine receptor, the sIL-6R exhibits, instead of antagonistic, an agonistic action on cells bearing gp130 (Mackiewicz et al., 1992).

(1). IL-6 activates early JAK-STAT signaling pathway.

Binding of IL-6 to its receptor rapidly activates the Janus kinase (JAK)- Signal Transducer and Activator of Transcription (STAT) pathway (Wegenka et al., 1993; Kishimoto et al., 1994; Stahl et al., 1994; Chen-Kiang, 1995; Zhang et al., 1995) by phosphorylation and activation of receptor-associated tyrosine kinases (JAK1 and JAK2), which in turn phosphorylates STAT3 and STAT1 (Wegenka et al., 1993; Akira et al., 1994; Zhang et al., 1995). Phosphorylated STATs homo or heterodimerize via interaction of reciprocal phosphotyrosine-Src homology-2 (SH2) domain (Shuai et al., 1994), translocate into the nucleus, and activate downstream gene transcription via binding to the cognate response sequences (Darnell et al., 1994; Zhang et al., 1995). Six members of STATs (STAT 1 to STAT5A, STAT5B and STAT6) have now been identified (Darnell, 1997). The knockout phenotypes of JAK/STAT family proteins (Table 1) suggest STATs have important roles in T cell activation, cytokine production and hence the host immune response (Darnell, 1997).

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Table 1. The phenotypes of disruptions in JAKs and STATs.

Targeted genes	Phenotypes	References
Jak1	Perinatal lethal.	(Liu et al., 1998)
Jak2	Embryonic lethal.	(Liu et al., 1998)
Jak3	Autosomal SCID.	(Nosaka et al., 1995; Park et al., 1995; Thomis et al., 1995)
Tyk2	Not reported.	
Stat1	Increased viral and bacterial susceptibility.	(Durbin et al., 1996; Meraz et al., 1996)
Stat2	Early embryonic lethal.	(Darnell, 1997)
Stat3	Early embryonic lethal.	(Takeda et al., 1997)
Stat4	Impaired IL-12-mediated effects.	(Kaplan et al., 1996; Thierfelder et al., 1996)
Stat5A	No breast development, lactation.	(Liu et al., 1997)
Stat5B	No breast development, lactation.	(Udy et al., 1997)
Stat6	Impaired IL-4-mediated effects.	(Kaplan et al., 1996; Shimoda et al., 1996)

(2). IL-6 induces a delayed Nuclear Factor (NF)-IL-6 pathway.

It is proposed that IL-6 activates two signaling pathways (Figure 1): the early and transient JAK-STAT pathway within minutes and also a delayed NF-IL6 pathway within hours (Chen-Kiang et al., 1993; Hsu and Chen-Kiang, 1993; Chen-Kiang, 1995; Zhang et al., 1995). The delayed NF-IL6 pathway is not well understood. The linkage between the two pathways is yet to be resolved. The promoter of NF-IL6 suggests that NF-IL6 may be one of the candidates which may link the two pathways. The functions

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and regulations of NF-IL6 will be discussed in the following section: NF-IL6, its potential role in cell cycle control and in transcriptional regulation.

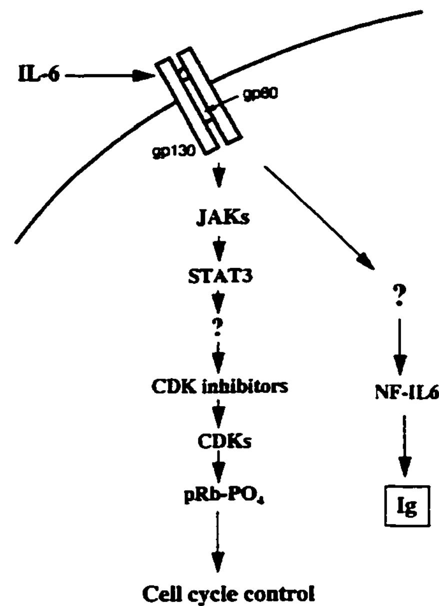

Figure 1. IL-6 induces early JAK-STAT and delayed NF-IL6 pathways.

III. Regulation of the cell cycle control by cyclin-dependent kinases (CDKs) and CDK inhibitors.

(1). G1/S progression.

In mammalian cells, cell cycle is regulated by a family of CDKs, which are associated with specific cyclins and CDK inhibitors (Xiong et al., 1993b; Meyerson and Harlow, 1994). CDK activity is enhanced by binding to cyclins, up or downregulated by phosphorylation, and is inhibited by binding of CDK inhibitors (Morgan, 1995; Elledge, 1996; Sherr, 1996). As shown in Figure 2, The G1/S checkpoint of cell cycle is mainly determined by the kinase activities of the cyclin-CDK complexes (cyclin D-CDK4, cyclin D-CDK6, and the cyclin E-CDK2) (Morgan, 1995; Elledge, 1996; Sherr, 1996). Retinoblastoma susceptibility gene product (pRB) is an important substrates of CDKs. It is generally believed that the hypophosphorylated pRB associates with the general

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transcription factor E2F family proteins. The phosphorylation of pRB (pRB-PO_4) by CDK-cyclin complexes release E2F, thus allowing E2F to activate transcription and the entry of S phase (Chellappan et al., 1991; Nevins, 1992; Morgan, 1995; Sherr, 1996).

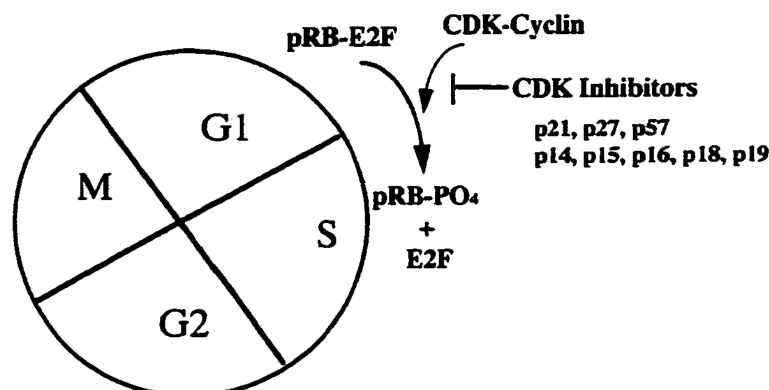

Figure 2. G1/S transition is controlled by CDK activity.

<u>(2). CDKs and cyclins promote cell cycle progression in B cells.</u>

It has been shown that CDK6 is the major CDK and cyclin expressed in lymphoid cell lines (Meyerson and Harlow, 1994; Palmero et al., 1997). CDK4 and CDK6 expressions were suppressed in apoptosis induced by crosslinking of the antigen receptor or by anti-IgM in a pre-B cell line (Ishida et al., 1995). These results suggest that CDKs and CDK inhibitors may play a role in antigen receptor signaling and thus may regulate lymphocyte cell cycle progression.

It has been shown that cyclin D2 is the major cyclins expressed in lymphoid cell lines (Meyerson and Harlow, 1994; Palmero et al., 1997). Cyclin D2, but not D1 or D3, is expressed in primary mature B lymphocytes (Tanguay and Chiles, 1996). Overexpression of cyclin D2 has also been observed in chronic B cell malignancy (Delmer et al., 1995), suggesting that cyclin D2 may promote cell cycle progression in B cells.

(3). Two families of CDK inhibitors and their functions.

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There are two families of CDK inhibitors: p21^{Cip1} and p16^{INK4a} families, as shown in Table 2. The p21^{Cip1} family of CDK inhibitors is thought to be general inhibitors of CDKs whereas the p16^{INK4a} family is more specific for CDK4 and CDK6 (Xiong et al., 1993a; Bates et al., 1994; Guan et al., 1994).

Table 2. The two families of CDK inhibitors.

p21 family	
p21$^{Cip1/WAF1}$	(El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993a)
p27^{Kip1}	(Polyak et al., 1994; Toyoshima and Hunter, 1994)
p57^{Kip2}	(Lee et al., 1995)
p16 family	
p15^{INK4b}	(Guan et al., 1994)
p16^{INK4a}	(Serrano et al., 1996)
p18^{INK4c}	(Guan et al., 1996; Hirai et al., 1995)
p19^{INK4d}	(Xiong et al., 1993b; Chan et al., 1995; Guan et al., 1996; Hirai et al., 1995)

Both p16 and p21 families of cyclin-dependent kinase (CDK) inhibitors block the phosphorylation of cyclin-dependent kinases by the CDK-activating kinase (CAK) (Aprelikova et al., 1995). As shown in Table 3, the study using gene-targeting strategy also shows the important roles of these CDK inhibitors in cell cycle control and their functions in B cell lineage. The disruption of INK4a locus, which encodes p16^{INK4a} and p19^{ARF}, cause B cell lymphoma. The difference between p16^{INK4a} and p19^{ARF} will be discussed in later section. Most recently, p18^{INK4c} deficient mice has been generated. They show an increase in T and B cells.

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Table 3. The phenotypes of disruptions in CDK inhibitors.

Targeted genes	Phenotypes	References
p21 family		
p21	Lack of G1 checkpoint control.	(Deng et al., 1995)
p27	Decreased fibroblasts in G0.	(Rivard et al., 1996)
p57	Increased apoptosis and delayed differentiation.	(Yan et al., 1997)
p16 family		
p15	Not reported.	
p16	Not reported.	
p16+p19 ^{ARF}	B lymphoma, fibrosarcoma et al.	(Serrano et al., 1996)
p18	Gigantism, organomegaly. Increased T, B cells. Pituitary hyperplasia Adenomas.	(Franklin et al., 1998)
p18+p27	Pituitary hyperplasia/Adenomas. Die early from adenomas.	(Franklin et al., 1998)
p19 ^{INK4d}	Not reported.	
p19 ^{ARF}	Lymphoma, fibrosarcoma et al.	(Kamijo et al., 1997)

(4). p21 family proteins and their functions in cell cycle progression.

p21 family CDK inhibitors include p21, p27 and p57. p21 was cloned as a cyclin D1-associated protein and functions at the G1 check-point (Harper et al., 1993; Xiong et al., 1993b). Mice lacking p21$^{CIP1/WAF1}$ undergo normal development, but are defective in G1 checkpoint control (Deng et al., 1995). Primary embryonic fibroblasts derived from p21-/- mice have significantly reduced numbers of premitotic cells (Dulic et al., 1998). These data suggest that in addition to G1/S transition, p21 may contribute to the

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implementation of late cell cycle checkpoint controls (Dulic et al., 1998). After the cloning of p27 (Polyak et al., 1994; Polyak et al., 1994; Toyoshima and Hunter, 1994), it is found that p21 and p27 have a broad specificity of inhibition of CDKs and can cause cell cycle arrest when overexpressed (Harper et al., 1993; Polyak et al., 1994; Polyak et al., 1994). In contrast to the widespread expression of p21 and p27 in tissues, the expression of p57, the third member of p21family, is tissue-specific, high in placenta and lower in various other tissues. Overexpression of p57 causes complete G1 arrest (Lee et al., 1995). Mice lacking p27 or p57 show a lack of quiescence (G0) in fibroblast (Rivard et al., 1996) and delayed differentiation during mice development (Yan et al., 1997).

p21 family proteins can be directly regulated by STATs. For example, STAT1 mediates cell cycle arrest via the induction of $p21^{CIP1/WAF1}$ (Chin et al., 1996), and $p27^{kip1}$ (Kaplan et al., 1998), suggesting that STAT family proteins may also actively participate in cell cycle control through regulations of CDK inhibitors.

Co-crystallization of cyclin A/CDK2/$p27^{Kip1}$ shows that binding of $p27^{Kip1}$ to both cyclin A and CDK2 complex mimics ATP-binding into the catalytic cleft of CDK2, thus preventing CDK2 from binding ATP and from being activated (Russo et al., 1996). These results also confirm the hypothesis that the p21 family of CDK inhibitors (e.g., p21, p27) forms a ternary complex with CDK and cyclin, while p16 family proteins forms a binary complex with CDK only (Harper et al., 1993; Xiong et al., 1993b; Polyak et al., 1994; Zhang et al., 1994), as discussed later.

<u>(5). p16 family proteins and their functions in cell cycle progression.</u>

The p16 family of CDK inhibitors includes many small molecular weight proteins such as p14, p15, p16, p18, p19 and p20 (Guan et al., 1994; Chan et al., 1995; Hirai et al., 1995 ; Guan et al., 1996; Serrano et al., 1996). They are relatively specific inhibitors for CDK4 and/or CDK6 (Bates et al., 1994; Guan et al., 1994; Guan et al., 1996; Hirai et al., 1995).

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Human p18^{INK4c} was cloned as a CDK6-interacting protein by yeast two-hybrid system for (Guan et al., 1994). This suggests a strong in vivo interaction between p18 and CDK6. Overexpression of p18 by transfection causes growth suppression in a human sarcoma cell line. In addition, the growth suppression by p18 correlates with wild-type pRB function (Guan et al., 1994), suggesting that p18-mediated cell cycle control depends on pRB, an important CDK substrate.

The regulation of p18 is not well understood. However, p18-deficient mice suggests a close relationship between p18 expression and the cell cycle control of B cells. In p18 deficient mice, T and B lymphocytes develop normally. However, as compared with wild-type mice, both T and B cells are increased in numbers and proliferate faster upon mitogen stimulation. This indicates the potential role of p18 in inhibiting cell cycle progression (Franklin et al., 1998). In addition, single knockout of either p18 or p27 shows a gradual progression from pituitary hyperplasia to an adenoma by 10 months of age. Double knockout of both p18 and p27 invariably died from pituitary adenomas by 3 months. These results suggest that p18 and p27 mediate two separate pathways to collaboratively suppress pituitary tumorigenesis (Franklin et al., 1998).

INK4a locus encodes two proteins through alternative reading frames: p16^{INK4a} and p19^{ARF}. It should be clarified that p19^{ARF} and p19^{INK4d} are distinct proteins encoded by different genes. p16^{INK4a} and p19^{ARF} double deficient mice showed various malignancies including B cell lymphoma (Serrano et al., 1996). Later, it was found that the single deficiency of p19^{ARF} leads to similar phenotypes including lymphomas while p16^{INK4a} expression is not affected (Kamijo et al., 1997). This suggests p19^{ARF} may be the main mediator for the loss of the function of the INK4a locus.

The co-crystallization of p19 and CDK6, p16 and CDK6 reveal that the bindings of p19^{INK4d} to CDK6 (Brotherton et al., 1998) and p16^{INK4a} to CDK6 (Russo et al., 1998) cause conformational changes of CDK6 thus preventing CDK6 from binding to ATP and cyclins. These results suggest that CDKs possess an inherent structural flexibility that is

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central to their regulation (Russo et al., 1998). These results also confirm the hypothesis that p16 family proteins (i.e., p15, p16, p18, p19) forms a binary complex with CDK (Guan et al., 1994).

(6). pRB-E2F interaction in G1/S progression.

As a physiological substrate of CDKs, the activity of pRB in cell cycle control depends on the activity of CDKs, which is in turn regulated by phosphorylation and by the binding of cyclins and CDK inhibitors(Morgan, 1995). It appears that only hypophosphorylated pRB can suppress cell proliferation (Chen et al., 1989). The formation of the complex containing pRB and E2F coincides with an inhibition of E2F-dependent transcriptional activity. A mutant pRB protein that does not associate with E2F does not inhibit transcription (Hiebert et al., 1992). The hypophosphorylated-pRB that predominates in the G1 and S phases of cell cycle is thought to regulate progression past a restriction point, thus allowing commitment to S phase and subsequent phases of cell division (Riley et al., 1994). A recent study shows that pRB represses transcription by recruiting a histone deacetylase (HDAC1) which physically interacts and cooperates with pRB, thus masking the E2F1 transactivation domain (Magnaghi-Jaulin et al., 1998), suggesting that pRB may also be regulated by protein-protein interaction.

IV. Transformation of human B cells by Epstein-Barr virus.

(1). Human B cells are main targets of EBV transformation.

EBV is prominently associated with human malignancies of lymphoid and epithelial organs such as B cell lymphomas, nasopharyngeal carcinoma, and non-Hodgkin's diseases (Kieff, 1998). Primary EBV infection may manifest itself as a benign lymphoproliferative disorder or infectious mononucleosis (IM) (Niedobitek et al., 1997). EBV infection can be either latent or cytolytic. During latent infection, EBV exists as episomes and replicates

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using oriP replication origin and host polymerase (Kieff, 1996; Robertson et al., 1996; Kieff, 1998). Lytic infection, on the other hand, results in cell death of the host (Kawanishi, 1993). The mechanism for the transition between latent and lytic infection is not well understood.

Among the eleven EBV genes that are expressed in latently infected B cells, five of them are required for B cell transformation (Kieff, 1996). These five genes are EBV Nuclear Antigen (EBNA)1, EBNA2, EBNA-3A, EBNA-3C and Latent Membrane Protein (LMP)1 (Cohen et al., 1989; Hammershmidt and Sugden, 1989; Kaye et al., 1993; Tomkinson et al., 1993). The structure and function of LMP1 are well studied. LMP1 is believed to be one of the most important EBV transforming genes during B cell immortalization (Kieff, 1998).

(2). LMP1 mimics CD40-CD40 ligand (CD40L) signaling pathway thus promotes cell cycle progression.

LMP1 is a 45-50 kDa transmembrane glycoprotein. It has six hydrophobic transmembrane domains that enable it to constitutively aggregate in the plasma membrane (Figure 3). The aggregation of LMP1 on cell surface is essential for transformation since diffuse expression resulting from a mutation abolish the transforming phenotype (Kieff, 1998). LMP1 and CD40 share a common PXQXT core TRAF (tumor necrosis factor receptor associated factor)-binding motif (Eliopoulos et al., 1997). In deed, LMP1 binds to TRAF1, TRAF3, and to a less extent TRAF2 in EBV-immortalized B lymphocytes (Devergne et al., 1996). These results suggest that the function of LMP1 in B cell transformation is closely related to the activation of CD40 signaling, which triggers the activation of downstream kinases and NF-κB.

The c-terminus of LMP1 contains two functional sites that may be responsible for CD40 signaling: transformation effector site (TES) 1 and TES2 (Izumi et al., 1997a; Izumi

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and Kieff, 1997b) (Figure 3 and Figure 4, panel C). TES1 binds TRAF and TES2 mediates high level of NF-κB activation (Eliopoulos et al., 1997). TES2 also constitutively associates with TRADD (TNF receptor death domain protein) in EBV immortalized B cells(Izumi and Kieff, 1997b), implicating a role of TES2 and TRADD in antiapoptosis, instead of inducing cell death (Kieff, 1998).

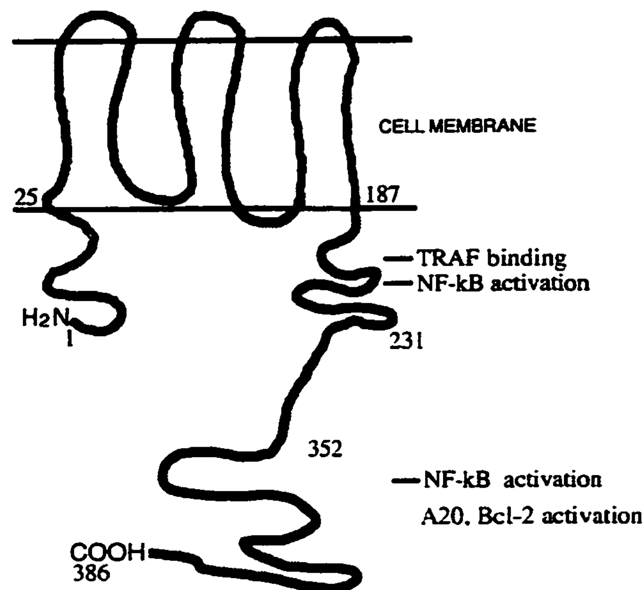

Figure 3. LMP1 is a transmembrane protein that binds to TRAF.

CD40 is expressed on B-lymphocytes and epithelial cells. The ligand for CD40 (CD40L) was first identified as a T-cell B-cell Activating Molecule (T-BAM) (Lederman et al., 1992;Lederman et al., 1994) and cloned as gp39 (Hollenbaugh et al., 1992; Noelle et al., 1992), a TNF-related activation protein (TRAP) (Graf et al., 1992) and T-BAM (Covey et al., 1994).

As shown in Table 4, the gene-targeting of molecules involved in CD40-CD40L signaling pathway shows that they have important functions in the activation of both T and B cells. CD40-CD40L interaction is thus considered important in B cell activation, proliferation and differentiation (Banchereau et al., 1991; Farrah and Smith, 1992; Kieff, 1998; Lederman et al., 1994). LMP1 mimics the CD40-CD40L signaling, thus leads to the

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activation of NF-κB, the antiapoptotic pathway and cell proliferation (Devergne et al., 1996; Gires et al., 1997; Kieff, 1998), as shown in Figure 4.

Table 4. The phenotypes of disruptions of CD40(L) and TRAFs.

Targeted gene	Phenotypes	References
CD40	Impaired Ig class switching and impaired germinal center formation.	(Kawabe et al., 1994).
	Absence of IgE and decreased IgG1, IgG2a	(Castigli et al., 1994).
CD40L	Impaired IgG1 response and impaired memory B cells.	(Whitmire et al., 1996).
	Impaired cellular immunity.	(Soong et al., 1996).
	Impaired Macrophage function.	(Stout et al., 1996).
	Impaired function of memory CD8+ CTL.	(Borrow et al., 1996).
	Impaired CD4+ T cell activation.	(Grewal et al., 1996).
TRAF2	Early death, atrophy of thymus and spleen and depletion of B cell precursors.	(Yeh et al., 1997).
TRAF3	Postnatal death and impaired T-dependent immunity.	(Xu et al., 1996).

Additional studies suggest an active role of LMP1 in regulating cell cycle progression by inducing cyclin D2 expression, pRB hyperphosphorylation, and loss of TGF-β1-mediated growth inhibition(Arvanitakis et al., 1995), and by inducing antiapoptotic molecules such as Bcl-2 (Wang et al., 1996) and A20 (Laherty et al., 1992; Miller et al., 1995).

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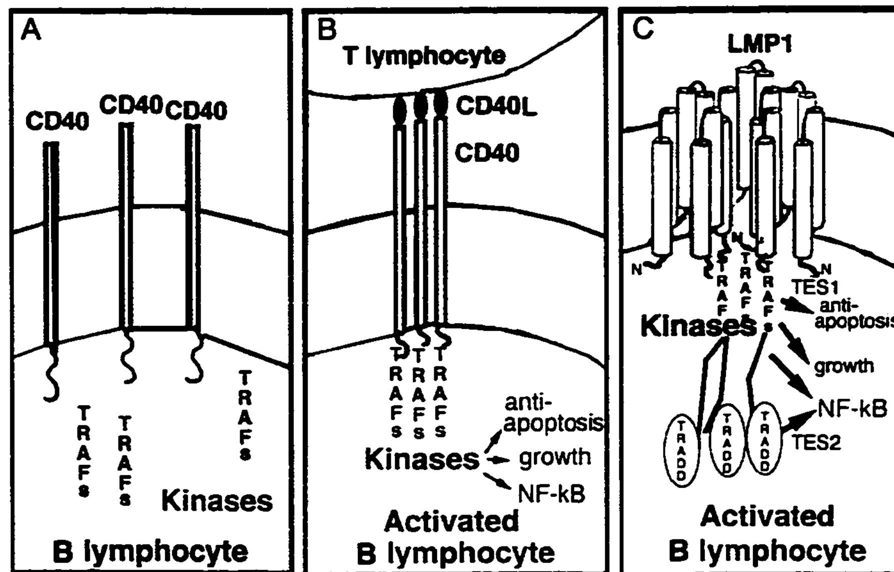

(Kieff E. 1998. J Natl Cancer Inst Monographys. 23:7)

Figure 4. LMP1 mimics a constitutively active CD40 pathway.

TRAFs: tumor necrosis factor receptor-associated factors.

TES: transformation effector site.

TRADD: TNF receptor death domain protein.

(3). The functions of other EBV transforming genes.

Two other EBV transforming genes, EBNA2 and EBNA-LP are the first EBV genes expressed after infection (Allday et al., 1989; Rooney et al., 1989) and are shown to be essential for both initiation and maintenance of B cell transformation (Kempkes et al., 1995; Wang et al., 1990).

EBNA2 is shown to repress immunoglobulin gene transcription (Jochner et al., 1996; Shore et al., 1989). These results suggest that EBV may evade host immune response by shutting off Ig production and use host antiapoptotic mechanism to promote survival of the infected cells.

It was shown in our lab that coincidental with IL-6-induced B cell differentiation and cell cycle arrest, EBNA1, EBNA2 and LMP1 are shut off in a human IgG+

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lymphoblastoid cell line CESS without activation of the viral lytic cycle (Altmeyer et al., 1997). This suggests that EBNA1, EBNA2 and LMP1 may play major roles in maintaining cell cycle progression in B cells.

V. NF-IL6, its potential role in cell cycle control and in transcriptional regulation.

(1). NF-IL6 is a basic leucine zipper transcription factor.

NF-IL6 is an IL-6-activated transcription factor of the CCAAT/enhancer binding protein (C/EBP) family in humans. Recombinant NF-IL6 binds to DNA through homodimerization or heterodimerization with recombinant Fos or Jun via its basic leucine zipper region (Hsu et al., 1994). The NF-IL6 gene has no intron; However, the NF-IL6 mRNA encodes three inframe isoforms: NF-IL6-1, NF-IL6-2 and NF-IL6-3, presumably due to alternative translation initiation from the same mRNA transcript (Descombes and Scheibler, 1991). NF-IL6-3 lacks the activating domain that is present in NF-IL6-1 and NF-IL6-2 and thus functions as a dominant negative inhibitor for transcription. This was first demonstrated in the activation of a synthetic promoter driven by NF-IL6 (LAP)-binding-site of the albumin gene (Descombes and Scheibler, 1991), subsequently confirmed with the synthetic promoter containing 4 tandem repeats of NF-IL6 responsive elements of the IL-6 gene (Hsu et al., 1994) and in the native TSG-6 gene promoter (Klampfer et al., 1994) in transient transfections.

(2). NF-IL6 is important for cell cycle control in B cell development.

NF-IL6 -/- mice showed an expansion of the B cell compartment, a phenotype similar to mice overexpressing IL-6 (Screpanti et al., 1995; Suematsu et al., 1992). By contrast to the function of IL-6 in promoting B cell proliferation and development, the combined function of three NF-IL6 isoforms appears to inhibit B cell proliferation and

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development. The inhibitor form NF-IL6-3, may thus play a more important role than the activator forms in the control of B cell proliferation. In addition, defective bactericidal and tumoricidal functions of macrophage was also reported in NF-IL6 -/- mice (Screpanti et al., 1995; Tanaka et al., 1995). That NF-IL6 has a role in cell cycle control is also supported by the report that NF-IL6 physically interacts with pRB(Chen et al., 1996), which control G1/S transition.

(3). NF-IL6 can be regulated by phosphorylation via multiple signaling pathways

NF-IL6 activity can be regulated by multiple signaling pathways including protein kinase C (PKC), Ras-MAP kinase and calcium-calmoldulin kinase (CamKII) (Figure 5) (Akira and Kishimoto, 1997). It has been shown that the Rat homologue of NF-IL6 (rNF-IL6) is found more in the cytoplasm but translocates into the nucleus within 30 minutes after c-AMP-mediated phosphorylation (Metz and Ziff, 1991). The transfection of p21ras induced the phosphorylation at position 235 (Thr-235) at the DNA-binding domain of NF-IL6. The amino acid sequence surrounding Thr-235 coincides with the consensus sequence of MAP kinase recognition (Trautwein et al., 1993). It was also reported that NF-IL6 is phosphorylated within the leucine zipper in response to increased intracellular calcium (Ca^{2+}) via activation of calcium-calmodulin-dependent kinase II (CaMKII) (Wegner et al., 1992).

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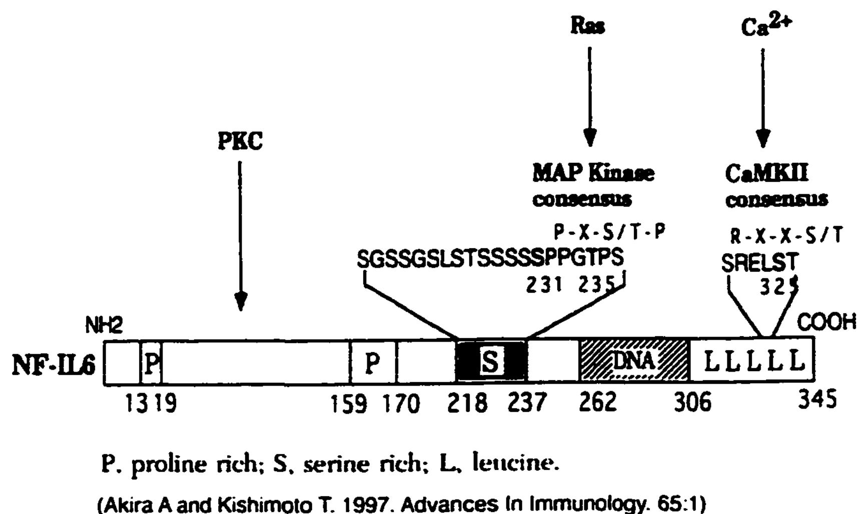

Figure 5. NF-IL6 is regulated by multiple signaling pathways.

P: proline rich region.

S: serine rich region.

L: leucine.

DNA: DNA binding domain.

PKC: protein kinase C.

MAP: mitogen-activated protein kinase.

CamKII: calcium-calmodulin-dependent kinase.

(4). NF-IL6 can be regulated by protein-protein interactions.

As mentioned before, in order to bind DNA, NF-IL-6 must form a homodimer or heterodimer with other leucine zipper proteins. This suggests that the NF-IL6 DNA-binding activity may be regulated by the other partners. It was shown that Fos and Jun repress transcription activation by NF-IL6 through interaction at the basic leucine zipper region (Hsu et al., 1994). NF-IL6 heterodimerizes with C/EBP-related ATF (C/ATF), a leucine zipper protein, before binding to a cAMP-response element (Vallejo et al., 1995). NF-IL6 is also shown to interact with pRB. Instead of inhibiting transcription, pRB enhances NF-IL6 binding to DNA (Chen et al., 1996). The interaction of NF-IL6 and

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pRB may therefore play a role in not only transcription regulation by NF-IL6 but also in cell cycle regulation by pRB.

(5). NF-IL6 can be regulated at transcriptional and translational levels by IL-6.

IL-6 enhances the synthesis of NF-IL6 mRNA in many cells, including M1 cells, a mouse myeloid leukemia cell line (Hsu et al., 1994) and 293 cells (Heiland and Knippers, 1995). IL-6 regulates NF-IL6-1 and NF-IL6-2 isoforms in ES cells and in T lymphoblastoid cells (Hsu and Chen-Kiang, 1993). These results suggest that NF-IL6 is regulated by multiple signaling pathways and at multiple levels.

The important functions of NF-IL6 in regulation of transcription and cell cycle progression lead us to ask how NF-IL6 promoter is regulated, which will be discussed in Chapter 5: NF-IL6 regulation and its function in B cell terminal differentiation.

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V. Aims of this study.

As discussed above, we have an excellent in vitro system to study the mechanisms of B cell terminal differentiation induced by IL-6. The specific aim of this study is to understand the mechanism that governs IL-6-medicated cell cycle control in B cell terminal differentiation. Since virtually nothing is known about cell cycle control and cellular differentiation during B cell terminal differentiation, the interaction between cell cycle control machinery of the host and EBV transforming genes of the virus may central to the cell cycle control in EBV-immortalized B cells, which are used as a tool. The main questions that we will address are: (1) how cell cycle is controlled in the terminal differentiation of B cells of different developmental stages, i.e., IgM+ and IgG+ cells; (2) how the host cell cycle control machinery interacts with EBV transforming genes in IgM+ and IgG+ cells; and (3) the possible mechanism that underlines the establishment of B cell immortalization, which may help explain the establishment of EBV-associated B cell lymphomas; (4) how NF-IL6 promoter is regulated? This will help understand of the function of NF-IL6 in B cell development.

We show in this study that the CDK inhibitor $p18^{INK4C}$ plays a major role in coupling cellular differentiation and cell cycle arrest via suppression of EBV transforming gene LMP1 by proteolysis. Therefore, we propose that the interplay between CDK inhibitors and EBV transforming genes controls the cell cycle progression in IL-6-mediated terminal differentiation of EBV-immortalized human B cells.

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Chapter 2

Materials and Methods

Cells and Cytokine

Cells of the EBV-immortalized human B lymphoblastoid cell line CESS (IgG+) and SKW (IgM+), and NJBC cells, derivatives of human Jurkat T cells expressing the high affinity human IL-6 receptor, have been described (Hsu et al., 1994; Natkunam et al., 1994). They were maintained in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% heat-inactivated fetal calf serum (Hyclone, Logan, Utah), 2mM L-glutamine, penicillin (100 U/ml), streptomycin (100 U/ml) and 2 mM nonessential amino acids. In some cultures, human IL-6 synthesized in a baculovirus vector (May et al., 1991) was added (40 Unit/ml) for time indicated, unless otherwise specified. The cell viability was determined by staining with trypan blue (Life Technologies, Inc.).

Separation of MHC Class II -Positive and -Negative Cells

MHC class II-negative and -positive cells were separated according to their interactions with anti-MHC class II antibodies conjugated to magnetic beads (HLA-beads) (Dynal, Lake Success, NY). At days 4 or 5 of culturing in the presence of IL-6 (40 U/ml), CESS cells (1×10^8) were collected and washed twice in the separation medium (RPMI supplemented with 2% FCS). The cell pellet was resuspended in 3 ml of separation medium containing 2×10^8 beads that had been washed 4 times in the same medium, and incubated with gentle rotation at 4 °C for 10 minutes. The MHC class II-negative cells were free from the beads and collected in the medium after removal of the beads by the use of a magnetic plate, and further enriched by repeating the procedure once more using freshly washed beads. The twice negatively selected cells were then transferred to a fresh flask and separated from contaminating beads by using a magnetic plate. This resulted in a

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population in which greater than 90% of the cells were MHC class II-negative. The MHC class II-positive cells (bound to beads) were resuspended in 30 ml of separation medium. After removal of contaminating MHC class II-negative cells present in the medium, nearly 95% of the cells that bound to the beads were MHC class II-positive.

Transfection and Selection of Transfected Cells.

Transfection of SKW cells by electroporation was essentially according to Hatada *et al* (E. Hatada, S. Chen-Kiang, and C. Scheidereit, unpublished). Briefly, SKW cells were maintained in the log phase of growth (2-5x10^5/ml) before transfection. After replacing the culture medium at 5 hours before transfection, the cells were collected and resuspended at 2x10^7 cells/ml in complete medium in room temperature. To each 0.4 ml of the cell suspension, 15 μg of DNA (2 mg/ml in 10mM Tris-HCl and 1 mM EDTA) was added and mixed gently. Following electroporation at 220 V, 750 μF with a Bio-Rad Gene Pulser II , the cells were immediately transferred to a small culture flask containing 10 ml of prewarmed medium. The culture medium was replaced at 24 hours and the transfected cells were analyzed at 48 hours after transfection when ~70% of the cells were viable. The expression plasmids used in the transfections were pCMV-p18, which contains a cDNA encoding the human p18 (Guan et al., 1994), pcDNA-3, the control pCMV vector; and pcDNA-3/T4, which encodes human CD4 (kindly provided by Dr. Dan Littman) as a surface marker for selection of transfected cells. Transfected cells expressing surface CD4 were selected by their interactions with anti-CD4 antibodies conjugated to magnetic beads (Dynal, Lake Success, NY). At 2 days after transfection, SKW cells (1x10^7) were collected by centrifugation and washed twice in separation medium (RPMI supplemented with 2% FCS). The cell pellet was resuspended in 2 ml of separation medium containing beads (1.5x10^6) that had been washed 4 times with the same medium, and incubated with gentle rotation at 4 °C for 15 minutes. The CD4-positive cells (bound to beads) were separated from the CD4-negative cells (free from beads) by the use of a

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magnetic plate. After removing the supernatant that contains the CD4-negative cells, the CD4-positive cells were washed once and collected.

HepG2 cells were transfected by calcium phosphate methods. Briefly, HepG2 cells were plated 1 day before transfection. Plasmid DNA is diluded by using 1.0 ml BES-buffered saline containing 50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na_2HPO_4. DNA-Calcium phosphate precipitates were made by adding 2 M $CaCl_2$ into the DNA solution to the final concentration of 125 mM. After 20-30 minutes at room temperature, the DNA-calcium phosphate precipitates were added to the top of the monolayer HepG2 cells after removing the medium in the plate. After incubation of 20 minutes, cells were supplemented with complete media and incubated in 37 °C for 40-48 hours before CAT assay.

M12 cells and Weri 27 cells were transfected by DEAE-Dextran methods. Briefly, 1.5 x 10^7 cells were fed 4 hours before transfection, washed in warm STBS which contains 25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4, 0.7 mM $CaCl_2$, 0.5 mM $MgCl_2$. The cells were collected and in which 1 ml of DEAE-Dextran (250 µg) / DNA in STBS was added. After mixing, cells were incubated at 37 °C for 1 hour. DMSO was added dropwise to 10 % final concentration and the mixture was incubated at room temperature for 2 minutes. The cells were washed twice with STBS, once with medium without serum and resuspend in complete medium with 10% fetal calf serum. Two days after transfection, cells were collected and CAT assay performed.

<u>chloramphenical acyteltransferase(CAT) assay</u>

Thin-layer chromatography (TLC) tank was equilibrated with chloroform: methanol = 19:1 two hours before the assay. The transfected cells were washed once with PBS, once with TEN solution containing 40 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 150 mM NaCl, resuspend in 50 µl (for up to 10^7 cells) of ice-cold 0.25 M Tris-HCl, pH7.5 in ependorf tube, frozen and thawed in dry ice-ethanol bath for five times. The lysate was

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cooled on ice, vertex for 30 seconds and microcentrifuge for 10 minutes at 4 °C. The supernatant was collected and protein measured by Bradford method. The protein concentration was adjusted to 10-50µg/ml in 0.25 M Tris-HCl buffer, pH 7.5 in 50 µl. The proteins were added into the reaction mixture containing 5 µCi / ml ^{14}C-labeled chroramphenicol, 10 mM Acetyl CoA, 0.25 M Tris-HCl, pH7.8. The mixture was incubated at 37 °C for 1 hour, extracted with ice-cold ethyl acetate, dried by speedvac for 40 minutes. The pellet was resuspend in 30 µl ethyl acetate, spotted onto the TLC plate, resolved by using 200 ml of chloroform: methanol in 19:1 in TLC tank. The plate was air dried and exposed to a film in -80 °C for 15 hours. The CAT activity was determined by measuring the radioactivity on the TLC plate by a scintillation counter.

Immunofluorescence Microscopy

Immunofluoresence microscopy was performed essentially as described before (Natkunam et al., 1994). Cells were collected and washed three times with the PBS/BSA buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4 , pH 7.4, 1 mM $MgCl_2$, 0.02% NaN_3 and 1% BSA). The PBS/BSA buffer was used in all subsequent steps unless otherwise indicated. For simultaneous detection of surface MHC class II and intracellular IgG, cells were incubated with a mouse Mab CA2.06 (1:400, kindly provided by Dr. M. Peterlin) on ice for 30 minutes and rinsed. The bound antibody was detected by incubation on ice for 30 minutes with polyclonal FITC-conjugated sheep anti-mouse antibodies (1:50, Cappel, Durham, NC), which had been preabsorbed against CESS cells to eliminate cross-reactivity with human IgG. After rinsing, the cells (10^5) were spun onto a slide by using a Cytospin centrifuge (Shandon, Pittsburgh, PA) at 200 rpm for 4 minutes, and permeablized in a freshly prepared and chilled solution of methanol and glacial acetic acid (95:5) at -20 °C for 20 minutes. The fixed cells were rinsed with the TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% Tween-20 and 0.02% NaN_3), and incubated with TBST containing 3% BSA (TBST/BSA) at room temperature

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for 10 minutes to block cross-reactivity. In all subsequent steps, TBST/BSA was used for antibody dilution and TBST was used for rinsing cells unless otherwise indicated. Intracellular IgG or IgM was detected by incubation with affinity purified polyclonal rhodamine-conjugated goat anti-human IgG or IgM antibodies (1:1000, Cappel) at 37 °C for 40 minutes. The cells were then rinsed twice with TBST, once with TBST (pH 8.4), and sealed under cover slip in 4-diazabicyclo (2.2.2) octane (DAPCO) (Sigma, St. Louis, MO) in glycerol (2.5% w/v). Visualization and photography were performed with the use of a Zeiss Axiophot microscope.

BrdU Labeling and Detection

For simultaneous detection of intracellular IgG and BrdU, CESS cells or EBV-immortalized freshly isolated human tonsillar B cells were cultured in complete medium containing BrdU (10 μM) for 24 hours, in the presence or absence of IL-6 (40 U/ml). The cells were then collected and stained with rhodamine-conjugated goat anti-human IgG antibodies (1:200, Cappel) at 37 °C for 1 hour as described above. The stained cells were rinsed in TBST, denatured in 2N HCl at room temperature for 10 minutes, and neutralized in freshly prepared 0.1 M sodium borate buffer (pH 8.5) at room temperature for 10 minutes as described (Hardy and Reynolds, 1991). The cells were then rinsed once with TBST, once with TBST/BSA, both at room temperature for 10 minutes, and incubated with a mouse anti-BrdU Mab (1:500 in TBST/BSA, DAKOPATTS, Denmark) at 37 °C for 40 minutes. The stained cells were rinsed by incubation in TBST/BSA at room temperature for 10 minutes. The bound mouse-anti-BrdU antibody was detected by incubation with FITC-conjugated sheep anti-mouse antibodies (1:500, Cappel) at 37 °C for 40 minutes. The cells were then rinsed once with TBST, counterstained with DAPI (4',6-diamino-2'-phenylindole dihydrochloride, 17 μM in TBST, Boehringer Mannheim, Germany), and rinsed with TBST (pH 8.4), all at room temperature for 10 minutes, before visualization and photography as described above.

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For simultaneous detection of intracellular IgM and BrdU, SKW cells or EBV-immortalized freshly isolated human tonsillar B cells were also cultured in the presence of BrdU for 24 hours before analysis (or from 24 to 48 hours after transfection), with or without IL-6 (50 U/ml). In some cultures, IL-6 was added immediately after transfection. After selection with anti-CD4 beads at 2 days after transfection, the CD4+ cells were rinsed in PBS/BSA supplemented with 5% goat serum to reduce the background, spun onto slides, permeabilized, and incubated with rhodamine-conjugated goat anti-human IgM antibodies (Southern Biotechnology Associates, Birmingham, Al, 1:50) at 37 °C for 1 hour. The stained cells were denatured and neutralized as described above for detection of BrdU in CESS cells. The cells were then rinsed once with TBST, once with TBST/BSA supplemented with 5% goat serum, both at room temperature for 10 minutes, and incubated with a FITC-conjugated mouse anti-BrdU Mab (1:10 in TBST/BSA/Goat Serum, Boehringer Mannheim) at 37 °C for 40 minutes. The cells were then rinsed, counterstained with DAPI and analyzed as described for CESS cells.

Immunoprecipitation and Immunoblotting

To detect pRB by immunoprecipitation and immunoblotting, cells ($5\text{-}10 \times 10^6$) were washed once with PBS and lysed by freezing and thawing 3 times in 0.5 ml of lysis buffer A containing 5 mM Tris-HCl (pH 7.4), 10 mM Hepes (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 4 mM PMSF, 3 µg/ml Aprotinin, 1 µg/ml Antipain, 0.3 µg/ml Leupeptin and 0.1% NP-40. The lysate was cleared by centrifugation in a microcentrifuge at 14,000 rpm for 15 minutes, and incubated with 5 µl of a rabbit anti-human pRB antiserum (0.47) (Chen et al., 1996) (kindly provided by Drs. P-L Chen and W-H Lee) at 4 °C for 30 minutes. Protein A-Sepharose CL-4B beads (75 µl of a 40 mg/ml suspension, Sigma) were added and incubation was continued at 4 °C for 30 minutes. The immune complexes were collected by centrifugation and washed 4 times with the lysis buffer A. The proteins in the immune complexes were resolved by SDS-PAGE on a 7%

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gel and transferred onto a nitrocellulose membrane by electrophoresis. After blocking overnight with PBS containing 0.1% Tween 20 and 5% non-fat milk, the membrane was incubated with a mouse anti-human pRB Mab (pmG245, 1:1000, kindly provided by Drs. P-L Chen and W-H Lee) (Chen et al., 1996), at room temperature for 30 minutes. The bound antibody was detected by using an epichemiluminescence immunoblotting system (ECL, Amersham, Arlington Heights, IL). The relative intensity of the autoradiographic signals was determined by scanning with an Arcus scanner.

To detect CDKs and CDK inhibitors, cells ($3\text{-}10 \times 10^6$) were lysed by incubation in 0.5 ml of lysis buffer B containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 0.1 mM Na_3VO_4, 4 mM PMSF, 3 µg/ml Aprotinin, 1 µg/ml Antipain, 0.3 µg/ml Leupeptin and 0.5 % NP-40 on ice for 15 minutes (Datto et al., 1995). The lysates were cleared by centrifugation at 14,000 rpm for 15 minutes. They were then incubated at 4 °C for 2 hours with affinity purified antibodies: a mouse monoclonal antibody against a GST-human p21 fusion protein (clone 6B6, PharMingen, San Diego), or rabbit polyclonal antibodies for the carboxyl terminal amino acid residues CLMQANGAGGATNLQ of human p18 (anti-p18C or 11256) (Guan et al., 1996), or CKENLDSHLPPSQNTSELNTA (306-326) of human CDK6 (C-21, Santa Cruz). The immune complexes were collected after incubation with Protein-A Sepharose CL-4B beads and washed four times with the lysis buffer B. To control for the specificity of immunoprecipitation, the CDK antibody was preincubated with a molar excess of antigenic peptides in 10-100 µl of lysis buffer B at room temperature for 2 hours before immunoprecipitation. The proteins were resolved by SDS-PAGE on a 10% gel and transferred onto a nitrocellulose membrane. After blocking non-specific binding with PBS containing 0.1% Tween 20 and 5% non-fat milk overnight, immunoblotting was performed by incubation with the indicated antibody at room temperature for 60 minutes and detected by ECL.

To detect LMP1 and p53 proteins, total cell lysate was made in 0.5 ml of lysis buffer containing 1% SDS and 20 mM Tris-HCl, pH 7.4, sonicated for two minutes, cleared by

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centrifugation. Proteins were quantified by the Bradford method. Approximately 40 μg of proteins were resolved by SDS-PAGE on a 10% gel and transferred onto a nitrocellulose membrane. Immunoblotting was performed as above.

Biosynthetic Labeling and Immunoprecipitation

For biosynthetic labeling of pRB and CDK6, 1×10^7 cells were washed once with RPMI medium free of methionine and serum at room temperature and incubated in 2 ml of methionine-free RPMI supplemented with 10% dialyzed fetal calf serum at 37 °C for 30 minutes. ^{35}S-methionine (specific activity 10 mCi/ml, DuPont-NEN) was added to 100 μCi/ml, and incubation was continued for 3-4 hours as indicated. pRB was immunoprecipitated from cell lysates and resolved by SDS-PAGE as described above. To detect CDKs and CDK inhibitors, cells were lysed by incubation on ice for 15 minutes in 0.5 ml of lysis buffer C which contains 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 20mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4, 1 mM PMSF, 25 μg/ml Aprotinin, 1 μg/ml Antipain, 25 μg/ml Leupeptin and 0.5% NP-40 (Xiong et al., 1992). Lysates were cleared by centrifugation at 14,000 rpm for 15 minutes and incubated with 1 μl of a preimmune rabbit serum at 4 °C for 60 minutes. Non-specific immune complexes were removed by incubation with Protein A-Sepharose CL-4B beads and centrifugation. p18 and CDK6 was immunoprecipitated by incubation at 4 °C for 4 hours with 1 μl of anti-p18C and CDK6 as described above. The immune complexes were collected and washed 4 times with lysis buffer C before resolving by SDS-PAGE on a 15% gel. ^{14}C-labeled molecular weight markers (DuPont-NEN) were used in electrophoresis and the gels were dried before autoradiography.

For biosynthetic labeling of LMP1 after infection of Ad5.GFP.p18 and Ad5.GFP, 1×10^7 CESS cells were washed once with RPMI medium free of both methionine and serum at room temperature and incubated in 2 ml of methionine-free RPMI supplemented with 10% dialyzed fetal calf serum at 37 °C for 30 minutes. ^{35}S-methionine and ^{35}S-

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cysteine (specific activity 10 mCi/ml, DuPont-NEN) were added to 100 μCi/ml, and incubation was continued for 30 minutes or 1 hour. Cells were lysed in 0.3 ml of lysis buffer containing 1% SDS and 20 mM Tris-HCl, pH 7.4, boiled for 5 minutes, sonicated for 2 minutes. The lysate was then diluted 1 to 10 with a buffer containing 20 mM Tris-HCl, pH 7.4, 150 mM NaCl. LMP1 was immunoprecipitated by anti-LMP1 monoclonal antibody from total cell lysates of $5x10^6$ and resolved by SDS-PAGE as described above. The immune complexes were collected and washed 4 times with lysis buffer before resolving by SDS-PAGE on a 10 % gel. The proteins were transferred onto nitrocellulose membrane before autoradiography and immunoblotting.

Flow cytometry analysis

Cell surface staining was performed as described before. Briefly, $2 x10^5$ cells were washed with PBS/BSA and incubated on ice for 30 minutes with either a mouse a fluorescein isothiocyanate (FITC)-conjugated anti-human-IgM polyclonal antibody (1:100, PanVera, Madison, WI), or an anti-human-IgG polyclonal antibody (1:200, Cappel/ICN), or a FITC-conjugated anti-CD19 antibody (1:50, Coulter, Hialeah, FL). The cells were then washed and resuspended in PBS / 0.1% BSA. 10^4 cells were analyzed by using a Becton-Dickinson FACSCalibur (Becton Dickinson, San Jose, CA).

The DNA contents in different stages of cell cycle were analyzed as described(Quelle et al., 1993) with the following modifications: $2x10^6$ cells with or without Adenovirus infection were washed once with PBS and incubated with a PBS buffer containing 50 μg/ml of propidium iodide (Sigma Chemical), 0.1% Triton X-100, 0.1% sodium citrate, and 1 mg/ml Rnase A for 30 minutes at 4 °C in the dark. The stained cells were washed twice with PBS and the DNA contents of 10,000 cells were analyzed with the FACSCalibur.

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RNA extraction and Northern hybridization

Total RNA was isolated by the guanidine thiocyanate procedure as previously described (Raynal et al., 1989). The CESS and SKW cells were either treated or untreated with IL-6, infected with Ad5.GFP or Ad5.GFP.p18 as described in the separate experiments. Total RNA from $6x10^6$ cells was run on a 1.2% agrose gel containing 2.2 M formaldehyde, transferred onto a nylon membrane for Northern hybridization. The membrane was hybridized separately with α-^{32}P-dCTP-labeled specific cDNA segments encoding LMP1, EBNA2, hγ1s or GAPDH.

Construction of recombinant adenoviruses containing p18 and LMP1.

The full length p18 cDNA was cloned into the HindIII and SalI sites of a pAd.GFP.CMV vector (Gall et al., 1996). The full length of LMP1 cDNA was cloned into HindIII and EcoRV sites of the pAd.GFP.CMV vector. The pAd.GFP.CMV vector contains the Ad5 origin and packaging elements, a GFP expression cassette driven by the CMV promoter, a second CMV expression cassette in the opposite orientation where the p18 or LMP1 genes were inserted, and a region of overlap recombination that corresponds to map unit (M.U.) 7.7-10.3 of Ad5. pAd.GFP.p18 or pAd.GFP.LMP1 was sequenced to confirm the identity of the insert. To generate recombinant virus, pAd.GFP.p18 or pAd.GFP.LMP1 was co-transfected with virus large fragment, which is generated by digesting purified dlAd5NCAT (Gall et al., 1996) viral DNA with XbaI and isolating using a sucrose gradient. This results in a 31Kb fragment, which corresponds to approximately 8-100 M.U. of DLAd5NCAT. Recombinant virus was plaqued and purified twice. Purified Ad5.GFP.p18 or pAd.GFP.LMP1 was grown up large scale by infecting 2.5 liters of suspension 293 cells with a multiplicity of infection of ~10 PFU. Virus was isolated using 5 freezes and thaws and further purified using one $CsCl_2$ cushion followed by a $CsCl_2$ gradient. $CsCl_2$ purified virus was then dialyzed in three changes of 3% Sucrose buffer containing 50 mM Tris-HCl, pH7.4, 10 mM $MgCl_2$. Dialyzed virus was

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aliquoted and stored at -80 °C. The resulting recombinant adenovirus vector is E1A-,E1B- and E3-. Control Ad5.GFP virus was also generated as above except no gene was cloned into the second CMV expression cassette.

The infections of CESS and SKW cells were performed as follows: 10^7 cells were washed with twice and resuspended in 0.2 ml of RPMI-1640 medium without serum. Adenovirus $2x10^4$ PFU were added and incubated at 37 °C for 1 hour. The cells were then supplemented with complete RPMI-1640 with 10 % FCS. The expression of GFP were monitored by FACS analysis.

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Chapter 3

Induction of Cell Cycle Arrest and B Cell Terminal Differentiation by CDK Inhibitor p18^{INK4c} and IL-6

running title: p18 activation in B cell terminal differentiation

key words: IL-6, plasma cells, cell cycle, CDK6, p21, pRB

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Acknowledgments for Chapter 3

Figure 6, Figure 7 and Figure 11 were done by Leslie Morse.

Figure 12 was completed with technical assistance from Leslie Morse.

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Abstracts

Cell cycle arrest and cell death are tightly coupled to terminal differentiation of B cells to plasma cells *in vivo.* This process was recapitulated *in vitro* by stimulation of IgG-bearing human B lymphoblastoid cells with interleukin-6 (IL-6), which led to orderly cell cycle arrest, differentiation, and apoptosis. In terminally differentiated plasmacytoid cells, phosphorylation of pRB was suppressed, correlating with the activation of D-type-cyclin-dependent kinase inhibitors, $p18^{INK4c}$ and $p21^{WAF1/Cip1}$. The expression of CDK6, however, remained unchanged. Activation of p18 by IL-6 was rapid, concomitant with marked enhancement of its association with CDK6 and cell cycle arrest. Overexpression of p18 in IgM-bearing lymphoblastoid cells, which differentiated in response to IL-6 but did not exit the cell cycle, reconstituted coupled differentiation and cell cycle arrest. Thus, CDK inhibitors, in particular p18, are likely to play a pivotal role in controlling cell cycle arrest and cell death in terminal differentiation of late stage B cells to plasma cells via inhibition of pRB phosphorylation by CDK6.

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Introduction

Terminal differentiation of mature B lymphocytes to plasma cells is essential for humoral immune response restricted to the B lineage. It is antigen-specific and requires costimulation by T cells and cytokines (Paul and Seder, 1994). *In vivo*, plasma cells synthesize and secrete large amounts of immunoglobulin (Ig), and then rapidly undergo cell death to effectively terminate the immune response. Thus, precise and timely withdrawal from the cell cycle is crucial for B cell terminal differentiation. Despite this understanding, little is known about the mechanism that governs differentiation-coupled cell cycle arrest and death in B cell terminal differentiation.

In other lineages, terminal differentiation is accompanied by cell cycle arrest in G1, which is largely controlled by the D-type cyclin-dependent kinases (CDKs). The activities of CDKs are in turn regulated by two families of inhibitors (Sherr and Roberts, 1995), of which $p21^{WAF1/Cip1}$(El-Deiry et al., 1993; Harper et al., 1993; Xiong et al., 1993a; Xiong et al., 1993b) is the most extensively studied. p21 is expressed in differentiated cells of nearly all lineages *in vivo* (Parker et al., 1995), and its activation correlates with MyoD mediated cell cycle arrest and terminal muscle differentiation (Halevy et al., 1995). However, p21 is not essential for development because mice appear normal in its absence (Deng et al., 1995). By comparison, ablation of the expression of p16 and p19 in the *INK4a* locus led to rapid development of soft-tissue tumors and B cell lymphoma in mice (Serrano et al., 1996). These findings support a role for CDK inhibitors of the p16 family in the control of cell proliferation *in vivo*, in particular in the B lineage. Negative regulation of cell cycle progression by CDK inhibitors is primarily mediated by inhibition of phosphorylation of pRB, the physiologic substrate for CDK4 and CDK6 (Kato et al., 1993; Matsushime et al., 1994; Meyerson and Harlow, 1994). In its hypophosphorylated form, pRB induces G1 arrest by sequestering the E2F transcription factors required for S

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phase entry (Goodrich et al., 1991; Nevins, 1992). Emerging evidence further suggests that pRB may also have a role in cellular differentiation by functioning as a coactivator for transcription regulators such as NF-IL-6 (Chen et al., 1996) and the glucocorticoid receptor (Singh et al., 1995). Accordingly, pRB is required for embryonic development (Lee et al., 1992).

The cytokine interleukin-6 (IL-6) is thought to play a major role in the differentiation and cell cycle control of late stage B cells. Ectopic expression of IL-6 in BALB/C mice results in c-myc translocation and plasma-cell tumors (plasmacytomas), as a result of accelerated terminal differentiation of B lymphocytes or deregulated proliferation of plasma cells (Nordan and Potter, 1986; Suematsu et al., 1989; Suematsu et al., 1992). Induction of plasmacytoma by overexpression of IL-6, however, is contingent on the genetic background, because in other strains of mice only IgG plasmacytosis was observed (Suematsu et al., 1989). The response of B cells to IL-6 appears to also depend on the stage of B cell development, because although IgM-bearing cells develop normally, IgG and IgA secretion are profoundly impaired in IL-6-deficient mice (Kopf et al., 1994). Together, these results suggest that IL-6 is critical for terminal differentiation of B lymphocytes *in vivo*, in particular late stage B cells after Ig class switching. Consistent with this hypothesis, major hallmarks of B cell terminal differentiation can be recapitulated *in vitro* by stimulation of activated IgG-bearing human B lymphocytes with IL-6 (Natkunam et al., 1994; Raynal et al., 1989). These include marked increases in Ig synthesis and secretion, extinction of surface major histocompatibility (MHC) class II expression and the development of the secretory machinery (Natkunam et al., 1994; Raynal et al., 1989). The molecular basis for enhanced Ig synthesis and secretion has been attributed to transcriptional activation of Ig genes and differential accumulation of Ig mRNAs (Raynal et al., 1989). Whether IL-6 also causes differentiation-coupled cell cycle arrest, however, is not known.

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Here we show that cell cycle arrest is coupled to cellular differentiation and cell death *in vitro* by IL-6 stimulation of IgG-, but not IgM-bearing human B lymphoblastoid cells. We present results to suggest that the CDK inhibitors $p18^{INK4c}$ may mediate cytokine signals for cell cycle arrest in B cell terminal differentiation by inhibiting CDK6 phosphorylation of pRB.

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Results

Terminal differentiation of B cells by IL-6 is coupled to cell death

Whether IL-6 signaling in B cells led to cell death in the context of terminal differentiation was investigated in the clonal, IgG1-bearing human lymphoblastoid CESS cells (Figure 6). IL-6 treatment simultaneously increased intracellular IgG and reduced surface MHC class II expression in 20-30% of the cells (Figure 6A), as observed previously (Natkunam et al., 1994). Of interest, cell proliferation was reduced in response to IL-6. By 6 days of IL-6 treatment, the number of viable cells was reduced to 10-30% of that of the untreated cells (data not shown). This appeared to result from apoptosis induced by IL-6 in a dose-dependent manner, as determined by trypan blue staining (data not shown). To confirm this possibility, the fate of the differentiated cells was analyzed after negative selection for the loss of surface MHC class II expression by using HLA-beads (anti-MHC class II antibody conjugated to magnetic beads) (Figure 6A and B). Except for the contaminating MHC class II-positive cell (5-10%), all differentiated cells (MHC class II-negative and free of HLA-beads) died within two days after separation (Figure 6B). The cell death was specific to IL-6-differentiated CESS cells, because the selection procedure did not inhibit the proliferation of either untreated CESS cells or those that remain undifferentiated after IL-6 treatment (MHC class II-positive, bound), or the MHC class II-negative NJBC T cells (free) (Figure 6B). Thus, IL-6 signaling in IgG-bearing B cells leads to coupled differentiation and cell death *in vitro*, as observed in terminal differentiation of B cells to plasma cells *in vivo*.

IL-6 signals lead to cell cycle arrest and differentiation

The relationship between cell cycle control and differentiation in B cell terminal differentiation was addressed by examining simultaneous DNA replication and Ig synthesis in response to IL-6 at the single cell level by immunocytochemistry (Figure 7). In the

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absence of IL-6, CESS cells cycled and synthesized a low level of Ig ($BrdU^+/Ig^{lo}$)(Figure 7A). Within one day of IL-6 stimulation, some cells ceased to incorporate 5-bromodeoxyuridine (BrdU) and increased Ig synthesis ($BrdU^-/Ig^{hi}$), suggesting coordinated cell cycle arrest and differentiation (Figure 7A). The proportion of these cells increased as a function of time of IL-6 treatment, inversely correlating with that of undifferentiated cells which continue to synthesize DNA ($BrdU^+/Ig^{lo}$) (Figure 7A and B). Consistent with apoptosis following cell cycle arrest and differentiation, the number of $BrdU^-/Ig^{hi}$ cells declined after maximum differentiation between 4-5 days of IL-6 treatment (Figure 7B). However, cell cycle arrest and differentiation were not obligatorily coupled, because similar increases and decreases in cells that ceased to synthesize DNA but failed to differentiate ($BrdU^-/Ig^{lo}$) were also observed in the course of IL-6 treatment. In addition, a very minor population of differentiated cells continued to incorporate BrdU($BrdU^+/Ig^{hi}$). No change in BrdU incorporation was detected in the control NJBC cells whose proliferation was not influenced by IL-6 (data not shown). Thus, IL-6 signaling in IgG-bearing B cells activates two pathways, one which leads to cell cycle arrest and the other differentiation, and only when the two are coupled does terminal differentiation proceed appropriately.

Phosphorylation of pRB is inhibited in differentiated B cells

Hypophosphorylated pRB causes G1 arrest (Goodrich et al., 1991) and may also function as a transcriptional regulator in cooperation with NF-IL6 (Chen et al., 1996), which is significantly regulated by IL-6 in B cells (D. Chen and S. Chen-Kiang, unpublished). Simultaneous cell cycle arrest and differentiation suggest a potential involvement of pRB in IL-6-induced B cell terminal differentiation. Analysis of pRB recovered in immune complexes showed that pRB phosphorylation was progressively suppressed in response to IL-6 (Figure 8A). Accordingly, while the phosphorylated form

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of pRB (ppRB) predominated in cycling B cells, the hypophosphorylated pRB (pRB) was favored by 4 days of IL-6 treatment (Figure 8D). However, considering 20-30% of cells respond to IL-6, hypophosphorylation of pRB in the differentiated B cells must have happened earlier. Consistent with a role for pRB in B cell terminal differentiation, the IL-6-differentiated cells (free) expressed only the hypophosphorylated pRB, whereas the undifferentiated cells (bound) and the IL-6 treated cells before separation (bound plus free) express all forms of pRB (Figure 8B). Phosphorylation of biosynthetically labeled pRB was significantly inhibited by 3 days of IL-6 treatment (Figure 8C), correlating with a change in the ratios between different forms of pRB to favor the hypophosphorylated form (Figure 8D). Hypophosphorylation of pRB therefore is the result of inhibition of phosphorylation and not dephosphorylation of phosphorylated pRB, and it correlates with IL-6-induced cell cycle arrest and differentiation in B cells.

p21 accumulates in terminally differentiated B cells

Inhibition of pRB phosphorylation may result from reduced synthesis of cyclins and CDKs, the physiologic kinases for pRB (Kato et al., 1993; Meyerson and Harlow, 1994) or activation of CDK inhibitors (Sherr and Roberts, 1995). The CDK inhibitor p21 has been shown to be activated by multiple differentiation agents including IL-6 (Steinman et al., 1994), and implicated in myocyte terminal cell cycle arrest (Halevy et al., 1995). In response to IL-6, the level of p21 protein rose in CESS cells, to two-fold of that of untreated cells by day 4, and then declined (Figure 9A and C). In addition, the increased expression of p21 as well as an immunoreactive 30 kDa protein occurred only in the differentiated, G1-arrested cells (20-30%) (free), and not in cells that failed to differentiate after IL-6 treatment (bound) (Figure 9B). While the molecular nature of the 30 kDa protein remains to be determined, these results suggest that p21 accumulates in B cells that are terminally differentiated.

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Activation of p21, however, did not temporally correlate with cell cycle arrest, which occurred within one day of IL-6 treatment (Figure 7A). Although changes in the p21 level in a small percentage of cells early in the course of IL-6 stimulation may elude biochemical detection, no association between p21 and CDKs was detected in differentiated cells (D. Chen and S. Chen-Kiang, unpublished). p21 therefore appears to accumulate in differentiated cells as a consequence, rather than a cause, of G1 arrest.

IL-6 rapidly activates the synthesis of p18 and its association with CDK6.

To address the potential role of other CDK inhibitors in cell cycle arrest induced by IL-6, we investigated the regulation of p18$^{INK4\ c}$ and its interaction with CDK6 in B cells in response to IL-6 (Figure 10). Both p18, a CDK inhibitor of the p16^{INK4} family, and CDK6 are preferentially expressed in haematopoietic lineage cells (Guan et al., 1994 ; Hirai et al., 1995; Meyerson and Harlow, 1994). *In vitro* and *in vivo*, p18 forms a stable binary complex with CDK6 and binds CDK4 weakly. p18 has also been shown to inhibit the kinase activity of CDK6 *in vitro* (Guan et al., 1994).

CDK6 was not regulated by IL-6 in B cells, because its level remained unchanged in CDK6 immune complexes recovered from all cells (Figure 10A), and CDK4 was not detectable in B cells (data not shown). However, a substantial fraction of CDK6 was present in the p18 immune complexes recovered from cells treated with IL-6 for 4 days, but not from untreated cells (A very low level of CDK6 was detectable in overexposures of the blot). The interaction between p18 and CDK6 was specific, because it was effectively competed by preincubation of either antibody used in immunoprecipitation with its corresponding antigenic peptide (Figure 10A). IL-6 therefore markedly enhances the stable interaction between p18 and CDK6 in B cells.

The association between p18 and CDK6 should precede or be coupled to cell cycle arrest, if p18 mediates IL-6 signals for the inhibition of pRB phosphorylation by CDK6 in B cells. We therefore examined the time course of p18 regulation and the interaction

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between p18 and CDK6 in response to IL-6 (Figure 10B). The p18 level rose significantly by 24 hours of IL-6 treatment, as determined by immunoblotting of p18 immune complexes with anti-p18. Reprobing the blot with anti-CDK6 showed a corresponding increase of CDK6 present in the p18 immune complexes. Analogous analysis of CDK6 immune complexes confirmed that the CDK6 level was constant, whereas the amounts of CDK6-associated p18 increased as a function of IL-6 treatment. (More p18 was detected in the CDK6 immune complexes than the p18 immune complexes because the anti-CDK6 antibody was more effective in immunoprecipitation). Analysis of biosynthetically labeled proteins recovered in CDK6 immune complexes further suggested that IL-6 induced the association between newly synthesized CDK6 and p18, whose identities were confirmed by immunoblotting (Figure 10C; data not shown). These results suggest that IL-6 increases the synthesis of p18, leading to enhanced association between p18 and CDK6, inhibition CDK6 phosphorylation of pRB in B cells, which is presumably the cause of cell cycle arrest, as illustrated in Figure 10b.

<u>Overexpression of p18 reconstitutes coupled cell cycle arrest and differentiation by IL-6</u>

To further investigate the contribution of p18 to B cell terminal differentiation, it is necessary to determine the functions of p18 in B cells in which differentiation by IL-6 is not coupled to cell cycle regulation. The clonal IgM-bearing human lymphoblastoid SKW cells, which represent B lymphocytes earlier in development than the IgG-bearing CESS cells, are ideal for this purpose. In response to IL-6, ~20% of SKW cells differentiated, as indicated by increases in intracellular IgM (Figure 11). DNA synthesis, however, continued in differentiated cells as determined by BrdU incorporation (Figure 11A), and cell proliferation and viability were unaffected by IL-6 treatment (Figure 11B). Thus, IL-6 signaling in the IgM-bearing SKW cells, unlike that in the IgG-bearing CESS cells, results in cellular differentiation that is not coupled to cell cycle arrest.

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The ability of p18 to induce cell cycle arrest was tested in SKW cells by transient transfections with an expression plasmid encoding the human p18 (Guan et al., 1994). The transfected cells, which also express surface CD4 from the cotransfected CD4-expression plasmid, were enriched by using anti-CD4 antibodies conjugated to magnetic beads at 48 hours posttransfection, when cell viability was ~70%. Analysis of simultaneous IgM synthesis and BrdU incorporation showed that 50% of the cells transfected with both p18 and CD4 ceased to incorporate BrdU, as compared to BrdU incorporation in all cells transfected with CD4 alone (Figure 12A, -IL-6). These results suggest that overexpression of p18, when reaching a critical concentration, was sufficient to cause G1 arrest of transfected cells.

Next, we examined if overexpression of p18 and IL-6 stimulation would lead to coupled cell cycle arrest and differentiation (Figure 12, +IL-6). IL-6 differentiation of SKW cells was not affected by the transfection procedure, because intracellular IgM levels were increased in ~20% of cells transfected with CD4 alone or p18 and CD4 combined, as in cells left untransfected (compare +IL-6 panels in Figure 12A with Figure 11A). Overexpression of p18 in conjunction with IL-6 stimulation, however, led to cessation of BrdU incorporation in 50% of the cells that were enhanced in IgM synthesis (panel CD4+p18, +IL-6). These cells die more rapidly than those that were differentiated and continued to synthesize DNA, or cells transfected with CD4 alone (panel CD4,+IL-6), presumably due to G 1 arrest resulting from overexpression of p18 (data not shown). Although the number of cells that are simultaneously G1-arrested and differentiated remains low (15-20% of the enriched population), it is significant and reproducible in four independent experiments. Taken together, these results suggest that overexpression of p18 and IL-6 stimulation can reconstitute coupled cell cycle arrest and differentiation of IgM-bearing cells. That p18 overexpression was the cause of cell cycle arrested was confirmed by the detection of p18 in immune complexes recovered from cells transfected with p18 and CD4, but not with CD4 alone or the control pCMV vector, or before transfection (Figure

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12B, upper panel). As anticipated, overexpression of p18 led to a marked increase in the formation of p18-CDK6 complexes, although a low level of p18-CDK6 was detected in cells before transfection, or transfected with CD4 or the combination of CD4 and the pCMV vector (Figure 12B, lower panel). Thus, p18 appears to be predominantly associated with CDK6 in B cells, and G1-arrest of transfected IgM-bearing cells was the result of overexpression of p18 and the subsequent increases in p18-CDK6 interaction.

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Discussion

An *in vitro* system to study cell cycle control in B cell terminal differentiation

The mechanism that underlies coordinated cell cycle control and differentiation in B cell terminal differentiation has remained elusive because *in vitro* systems that simultaneously recapitulate these two events were not available. In this study, we demonstrated that coupled differentiation and cell death could be reconstituted *in vitro* by IL-6 stimulation of human IgG-bearing lymphoblastoid cells. The clonal, Epstein Barr Virus (EBV)-immortalized human B cells mimic late stage mature B lymphocytes activated by antigen and T cell costimulation (Banchereau et al., 1994; Kempkes et al., 1995) and are thus poised for terminal differentiation. The coordinated differentiation and cell death in response to IL-6 suggest that IL-6 signaling reactivates the B cell terminal differentiation program and reverses immortalization of B cells by EBV. This *in vitro* differentiation system should therefore provide a valuable model for investigating the mechanisms that underlie B cell terminal differentiation, IL-6 signal transduction in B cells and the interaction between IL-6 and EBV. The ability to enrich differentiated plasma cells to near homogeneity further offers an opportunity to address the fate of the differentiated cells at the biochemical and molecular levels with confidence.

Cell cycle arrest represents one of the primary biological consequences of IL-6 signaling in late stage mature B cells, as revealed by single cell analysis of CESS cells (Figure 7). Of the cells exited from the cell cycle, 50% were increased in Ig synthesis and proceeded to programmed cell death, thus mimicking productive terminal differentiation of B cells *in vivo*. The fate of the remainder, which were cell cycle arrested but failed to differentiate, was less clear because selection for this population has not been possible. Since these two populations increased and decreased in synchrony during IL-6 treatment (Figure 7B), it is unlikely that one served as the precursor for the other. Rather, our data suggest that once B cells withdraw from the cell cycle in response to IL-6, they are committed to a pathway leading to death. If timely coupled to the differentiation pathway,

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productive differentiation ensues. Otherwise, terminal differentiation is non-productive (fails to activate Ig synthesis) but nonetheless also ends in cell death.

Role of CDK inhibitors in B cell terminal differentiation

Cell cycle arrest is thus linked to cell death as well as cellular differentiation in terminal differentiation of late stage B cells to plasma cells. As a first step toward understanding the mechanism that governs cell cycle arrest in B cell terminal differentiation, we investigated the regulation of three classes of genes that are crucial for the G1/S transition; pRB, CDKs and CDK inhibitors. On the basis that phosphorylation of pRB was inhibited in B cells in response to IL-6, and that differentiated, G1-arrested cells expressed only the hypophosphorylated form of pRB (Figure 8), pRB is likely to have an important role in mediating the IL-6 signals for cell cycle arrest in B cells.

The CDK inhibitors, in particular p18, are potentially the primary targets for IL-6 signals that lead to inhibition of pRB phosphorylation and cell cycle arrest. First, while CDK6 and CDK4 were not regulated, IL-6 activated the synthesis of p18 in IgG-bearing cells, concomitant with enhanced association of p18 with CDK6 and cell cycle arrest (Figure 10). Second, although other CDK inhibitors such as p21 was also increased in IL-6 differentiated cells, the timing of p21 activation and the lack of its interaction with CDKs do not support a causative role for p21 in IL-6-induced cell cycle arrest (Figure 9). Third, overexpression of p18 in IgM-bearing SKW cells led to enhanced p18-CDK6 association, G1 arrest, and in cooperation with IL-6 stimulation reconstituted coupled cell cycle arrest and differentiation (Figure 12). Initially identified as a CDK6-associated protein in the yeast two-hybrid system, p18 shares a significant protein sequence similarity with a conserved domain present in proteins of the Notch family, which are crucial for the determination of cell fate in Drosophila development (Guan et al., 1994; Hirai et al., 1995) p18 is markedly activated in myogenic differentiation, suggesting that it may also have a role in the terminal differentiation of other lineage (Franklin and Xiong, 1996). Thus,

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although our data do not exclude the potential contribution of other CDK inhibitors to IL-6 signaled cell cycle arrest, they strongly suggest that p18 has a pivotal role in regulating the cell cycle progression via inhibition of CDK6 phosphorylation of pRB in B cell terminal differentiation.

The IL-6 Responses in B cells.

Cell cycle arrest was not observed in IgM-bearing cells including the SKW cells, although they responded to IL-6 and increased in Ig synthesis as did the IgG-bearing CESS cells (Figure 11, Z. Liu and S. Chen-Kiang, unpublished). Integration of the two IL-6 signaling pathways, one which leads to differentiation and the other cell cycle control, therefore appears to be contingent on the developmental stage of the B cells. Whether IL-6 signaling in IgM-bearing cells also result in reduction of surface MHC class II expression, another hallmark of terminal B cell differentiation seen in CESS cells, is under investigation. Within each of the clonal IgG- and IgM-bearing cell populations, however, differentiation by IL-6 as determined by increases in Ig synthesis is limited to 20-30% of cells (Figure 6 and Figure 11). The molecular basis for this phenomenon is presently not known. Differentiation of B cells by IL-6 is not isotype or promoter-restricted because IL-6 enhances Ig synthesis in polyclonal tonsillar B lymphocytes after activation by EBV(Natkunam et al., 1994). It is, however, time-dependent and subject to negative feedback regulation, as prolonged exposure to IL-6 leads to unresponsiveness in both IgG- and IgM-bearing cells (Natkunam et al., 1994). Since the heterogeneity of IL-6 responsiveness and the feedback regulation can not be accounted for by differences in the density of the IL-6 receptor (Z. Liu and S. Chen-Kiang, unpublished), a major control for IL-6 responsiveness in B cells must lie further downstream in the IL-6 signaling pathways. The Jak-STAT pathway (Schindler et al., 1992; Schindler et al., 1992) is a potential candidate. It has been well established that IL-6 rapidly activates the latent transcription factors STATs (signal transducer and activator of transcription) in B cells (Zhang et al., 1995), as in cells of other lineages (Schindler and Darnell, 1995). While the B cell-specific

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target genes of STATs are not yet identified, serine phosphorylation of STATs, as we have shown, confers at least one level of regulation of STAT activity (Zhang et al., 1995). Elucidating the serine kinase pathway(s) that is coupled to the Jak-STAT pathway, and the developmental stage-specific target genes of STATs in B cells, should help to understand the molecular determinants for IL-6 response in B cells.

CDK inhibitors and B cells neoplasia

The molecular basis for plasma cell tumors, multiple myelomas in humans and plasmacytomas in mice is not understood despite the knowledge that IL-6 is necessary (Hilbert et al., 1995) and sufficient (Nordan and Potter, 1986; Suematsu et al., 1992) to induce plasmacytomas in mice of the appropriate genetic background. Accordingly, IL-6 promotes the growth of plasmacytoma and myeloma cells *in vitro* (Nordan and Potter, 1986). Within the context of B cell terminal differentiation, however, IL-6 signaling leads to cell death following cell cycle arrest in IgG-, but not IgM,-bearing human B cells (Figure 6 and Figure 7). What then determines the IL-6-induced proliferation of tumor-derived plasmacytoid cells and the death of terminally differentiated plasma cells? Mutations and deletions of at least one CDK inhibitor, p16, have been observed in primary tumors (Spruck et al., 1994). Deletion of both p16 and p19 in mice led to spontaneous tumors, in particular B cell lymphoma (Serrano et al., 1996). Activation of p18 synthesis and association between p18 and CDK6 in differentiation-coupled cell cycle arrest (Figure 7 and Figure 10), and induction of cell cycle arrest by overexpression of p18 (Figure 12) suggest that CDK inhibitors play a central role in controlling the progression of cell cycle in mature B cells. The *in vitro* B cell terminal differentiation system should provide a useful reference for studying plasma cell tumors. Further investigations of the interplay between CDKs and their inhibitors and the biological consequence in B cells should also help to elucidate the molecular mechanism that underlies deregulated proliferation in plasmacytomas.

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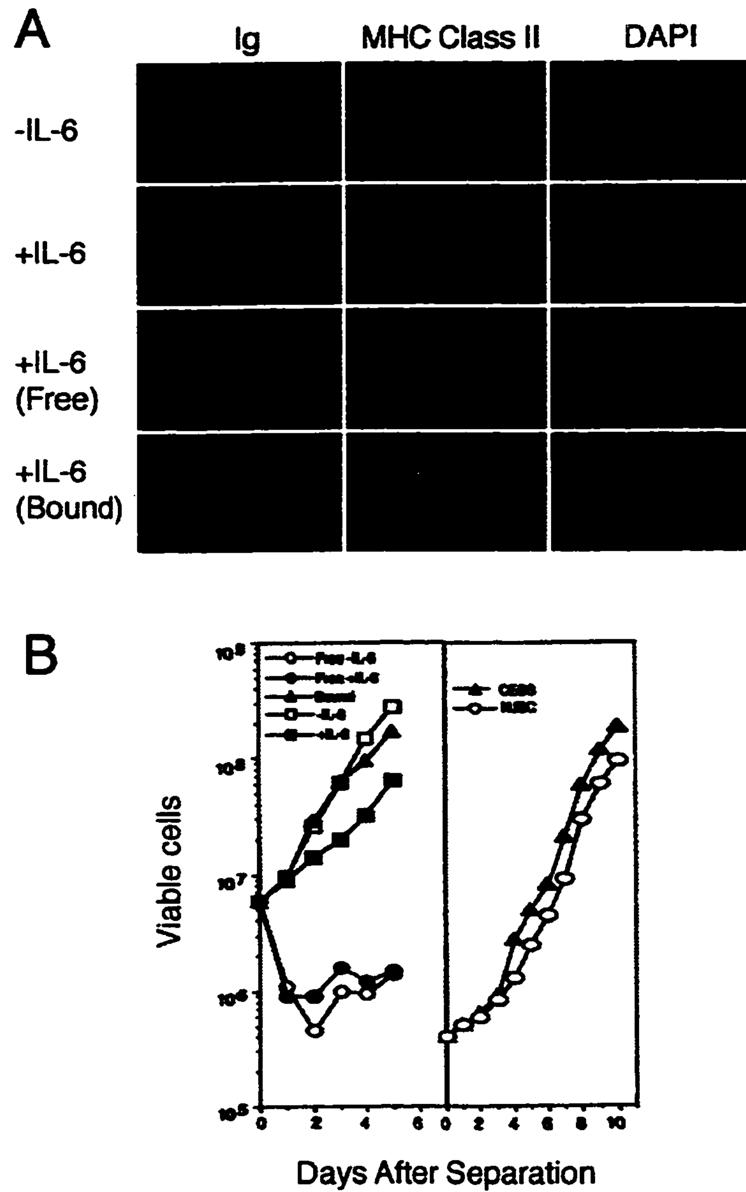

Figure 6. IL-6 induces cell death in B cell terminal differentiation.

(A) Selection of terminally differentiated B cells. CESS cells were cultured in the presence (+IL-6) or absence (-IL-6) of IL-6 for 5 days. MHC class II-negative (Free) and -positive (Bound) cells were separated by the use of HLA-beads, stained for intracellular IgG (red), surface MHC class II (green), and DAPI (blue) to localize the cell nucleus, and analyzed by

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immunofluorescence microscopy. The small spheres present in the "Bound" fraction represent HLA-beads that remained associated with cells after the staining procedure . (B) IL-6 induces the death of differentiated B cells. Left panel: CESS cells were cultured with (+IL-6) or without (-IL-6) IL-6 for 6 days. After separation by the use of HLA-beads, the MHC-positive cells (Bound) were cultured in the absence of IL-6. The MHC class II-negative cells were cultured in the presence (Free+ IL-6) or absence of IL-6 (Free-IL-6), as were the control cells without separation (+IL-6, -IL-6). Right panel: CESS (MHC class II-positive) and NJBC (MHC class II-negative) were subject to the HLA-bead separation procedure and cultured in the absence of IL-6. The viable cells were determined by trypan blue staining (in duplicates) on days after separation indicated.

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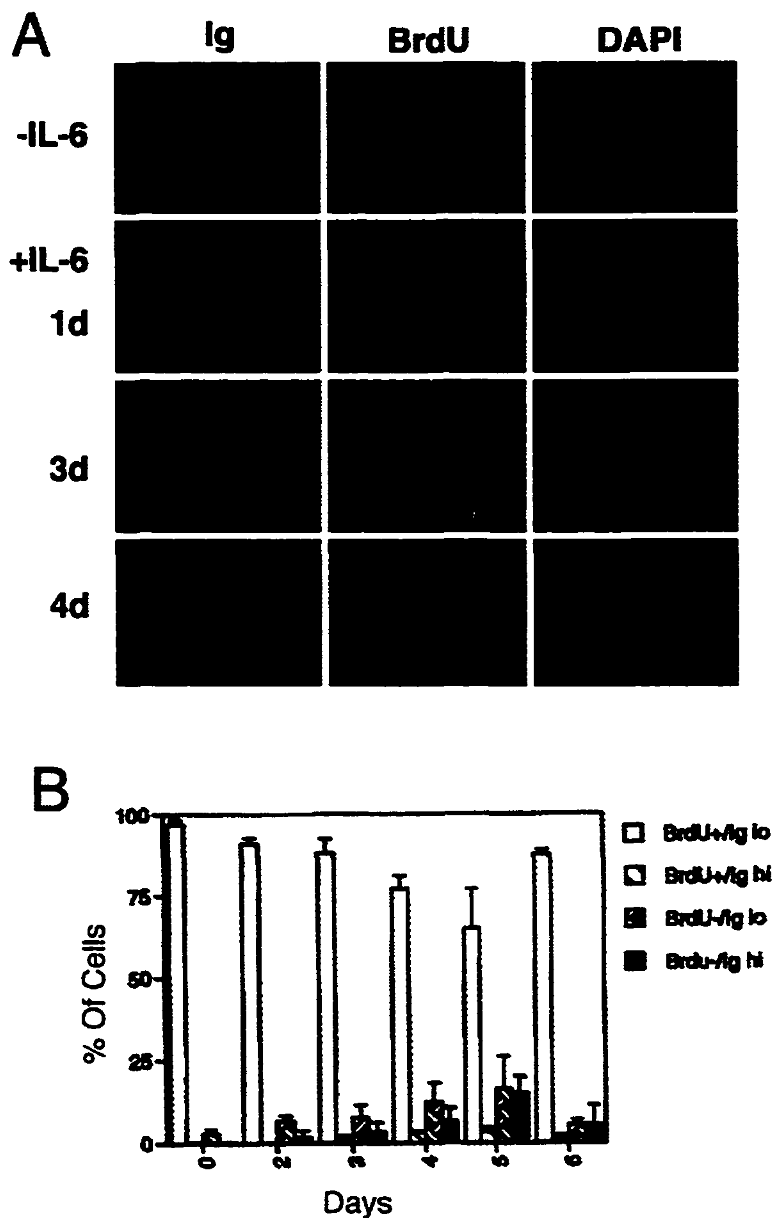

Figure 7. IL-6 induces cell cycle arrest.

(A) Simultaneous cell cycle arrest and differentiation. CESS cells were incubated in the presence (+IL-6) or absence (-IL-6) of IL-6 for days indicated, labeled with BrdU and stained for intracellular IgG (red), BrdU (green) and DAPI (blue) to localize the nucleus. (B) Time course of cell cycle arrest and differentiation. The presence (Ig^{hi}) or absence

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(Ig^{lo}) of enhanced intracellular IgG, and the presence (BrdU+) or absence (BrdU-) of BrdU incorporation were determined on days of IL-6 treatment as in (A). At least 500 stained cells were characterized for each time point shown in the histogram, and the error bars were derived from 5 samplings in a typical experiment. The experiment has been performed six times.

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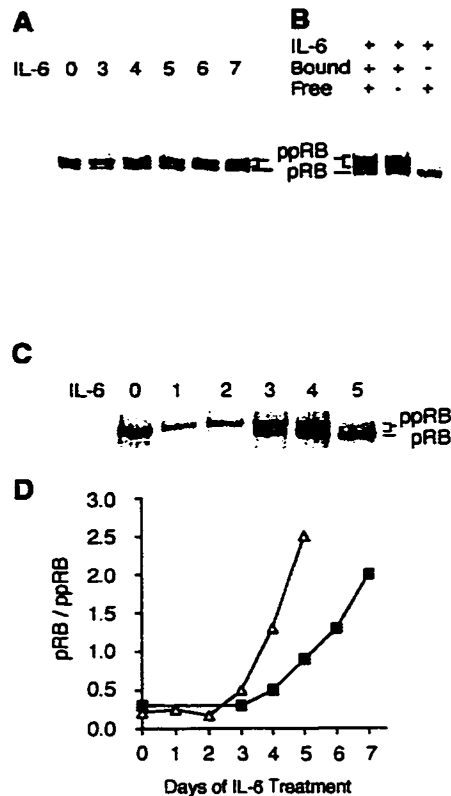

Figure 8. Inhibition of pRB phosphorylation by IL-6.

(A) IL-6 suppresses pRB phosphorylation. pRB in CESS cells ($5x10^6$) left untreated (0) or treated with IL-6 (30 U/ml) for days indicated was analyzed by immunoprecipitation and immunoblotting. The phosphorylated forms (ppRB) and hypophosphorylated pRB (pRB) were indicated. (B) pRB is hypophosphorylated in differentiated cells. pRB in CESS cells treated with IL-6 for 6 days and unseparated (+IL-6), or separated into differentiated (Free) and undifferentiated (Bound) populations by the use of HLA-beads, was analyzed by immunoprecipitation and immunoblotting. Each lane represents $5x10^6$ cells of each population. (C) Phosphorylation of newly synthesized pRB is inhibited by IL-6. pRB in CESS cells ($1x10^7$) was labeled with ^{35}S-methionine for 3 hours on days of IL-6 treatment indicated and analyzed by immunoprecipitation. (D) IL-6 regulates the ratios of pRB to ppRB. The relative intensities of steady state pRB and ppRB as shown in (A) (solid squares), and biosynthetically labeled pRB and ppRB as shown in (C) (open triangles) were determined by scanning, and expressed as ratios of pRB to ppRB.

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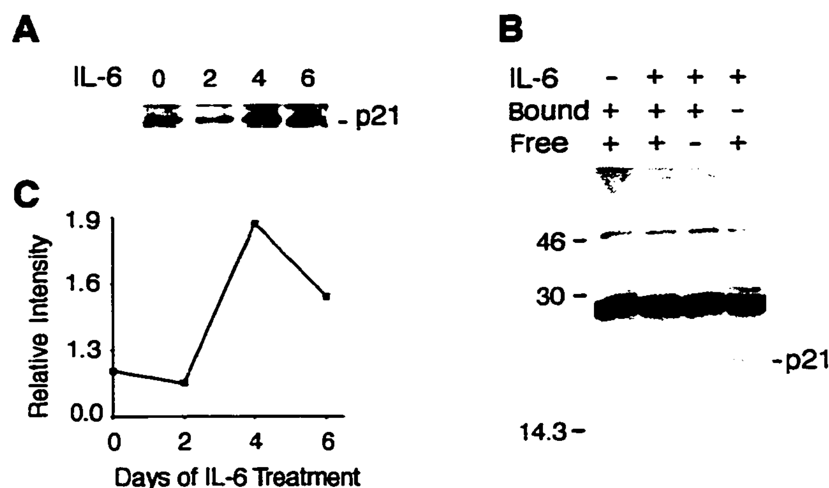

Figure 9. p21 is activated in IL-6-differentiated cells.

(A) Time course of p21 activation. p21 in CESS cells (1×10^7), left untreated (0) or treated with IL-6 for days indicated, was analyzed by immunoprecipitation and immunoblotting. (B) p21 is activated in differentiated cells. The p21 levels in CESS cells left untreated (0) or treated with IL-6 for 5 days and unseparated (+IL-6), or separated into differentiated (Free) and undifferentiated (Bound) populations, were analyzed by immunoprecipitation and immunoblotting. Each lane represents 3×10^6 cells of each population. The ^{14}C-labeled molecular weight markers were shown on the left (in kDa). (C) The relative intensity of p21 shown in (A) was determined by densitometry scanning, with the p21 level in untreated cells arbitrarily set as 1.

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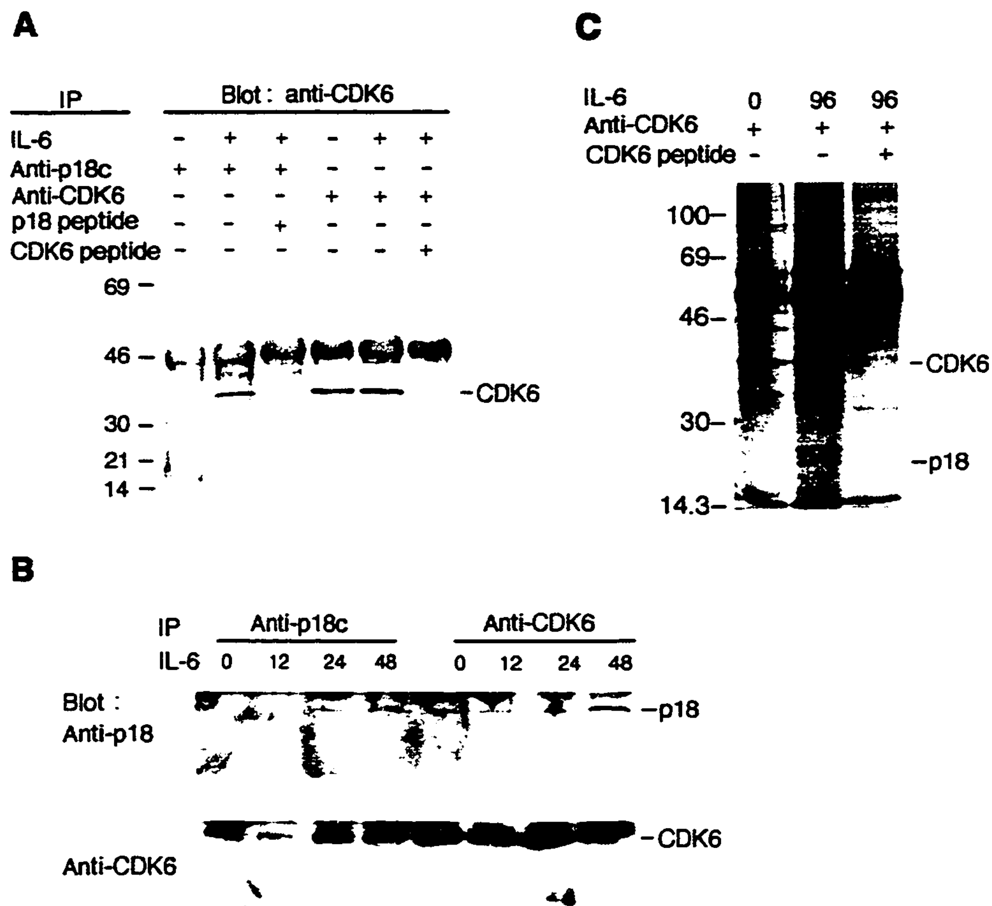

Figure 10. IL-6 activates p18 synthesis and its association with CDK6.

(A) IL-6 enhances the association of p18 with CDK6. p18 and CDK6 were immunoprecipitated with anti-p18-C or anti-CDK6, with or without preincubation with antigenic peptides, from $1x10^7$ CESS cells treated with or without IL-6 for 5 days. The immune complexes were analyzed by immunoblotting with anti-CDK6. (B) Time course of IL-6-induced p18 activation and p18-CDK6 association . p18 and CDK6 were immunoprecipitated with anti-p18-C or anti-CDK6 from $4x10^7$ CESS cells at hours after IL-6 treatment indicated. The immune complexes were analyzed by sequential blotting with anti-p18 (directed against recombinant p18) and anti-CDK6. (C) IL-6 activates the association of newly synthesized p18 and CDK6. ^{35}S-methionine-labeled CDK6 and

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CDK6-associated proteins were immunoprecipitated from CESS cells (1×10^7) left untreated (0) or treated with IL-6 for 96 hours, with or without preincubation with the CDK6 antigenic peptide as indicated. The proteins in the immune complexes were resolved by SDS-PAGE. The migrations of CDK6, p18 and the ^{14}C-labeled protein markers (in kDa) were marked.

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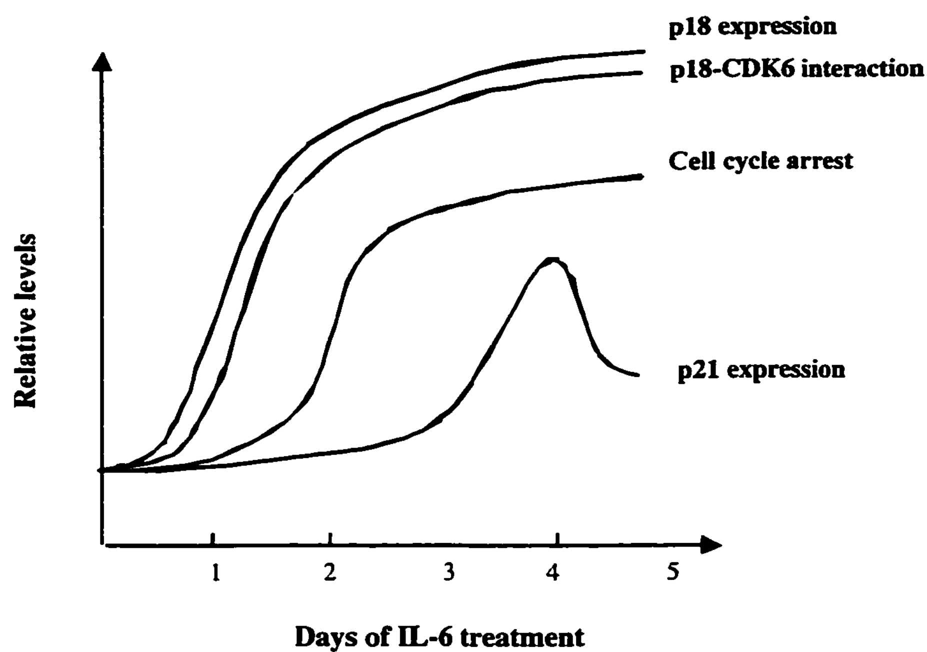

Figure 10b. The cell cycle control in IgG+ CESS cells in response to IL-6.

The relative levels of p18 expression, p18-CDK6 interaction, p21 expression, and cell cycle arrest, were indicated after IL-6 treatment (days).

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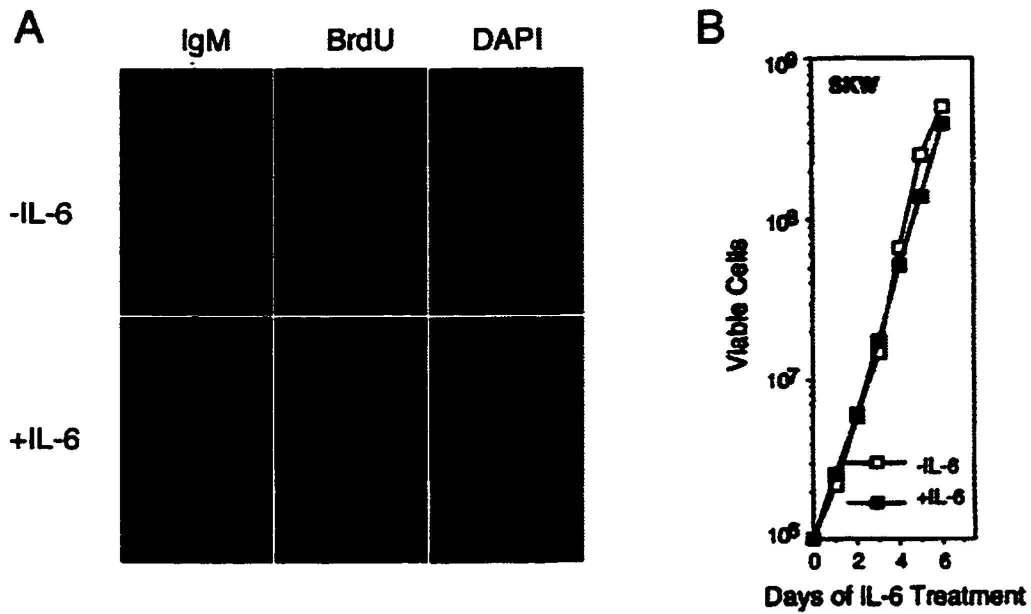

Figure 11. IL-6 activates IgM synthesis but not cell cycle arrest.

(A) IL-6 activates IgM synthesis but not cell cycle arrest. SKW cells were incubated in the presence (+IL-6) or absence (-IL-6) of IL-6 (50 U/ml) for 2 days, labeled with BrdU and stained for intracellular IgM (red), BrdU (green) and DAPI. (B) IL-6 does not affect the growth or viability of IgM-bearing cells. SKW cells were incubated in the presence or absence of IL-6 as indicated, stained with trypan blue and counted to determine cell growth and viability.

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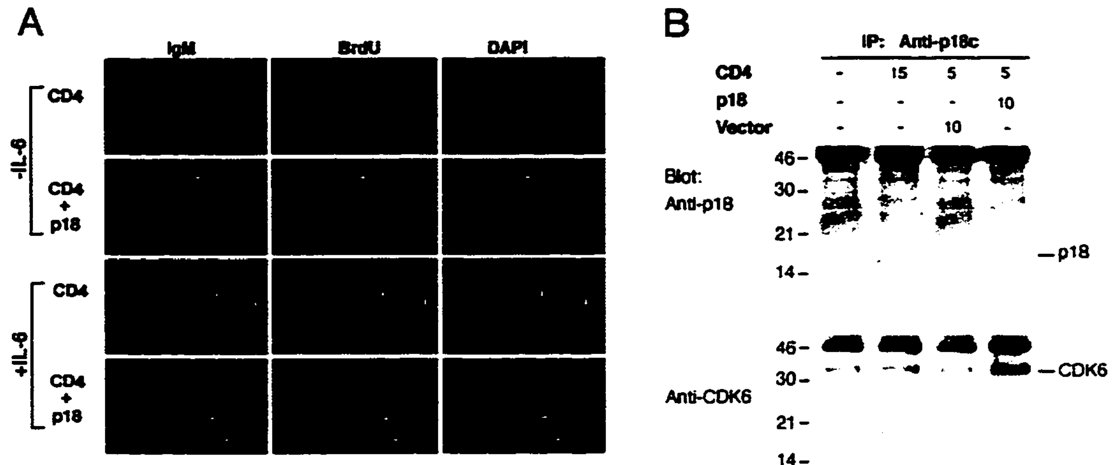

Figure 12. Overexpression of p18 and IL-6 stimulation reconstitutes coupled cell cycle arrest and differentiation.

(A) Overexpression of p18 causes cell cycle arrest. SKW cells were transfected with expression plasmids encoding CD4, or CD4 and p18 together, and cultured in the presence (+IL-6) or absence of IL-6 (-IL-6) for 48 hours as indicated. BrdU was added to the medium for the last 24 hours before staining for intracellular IgM (red), BrdU (green) and DAPI (blue). The small red spheres represent the anti-CD4 magnetic beads used for selecting transfected cells that remained after the staining procedure. The white arrows mark representative $CD4^{+}BrdU^{-}IgM^{Lo}$ cells transfected with CD4 and p18; $CD4^{+}BrdU^{+}IgM^{hi}$ cells transfected with CD4 and treated with IL-6; and $CD4^{+}BrdU^{-}IgM^{hi}$ and $CD4^{+}BrdU^{+}IgM^{hi}$ cells transfected with both CD4 and p18 and treated with IL-6. At least 500 cells were characterized for each panel shown. This experiment has been performed four times. (B) Enhanced expression of p18 and p18-CDK6 association in p18-transfected cells. SKW cells ($8x10^{6}$) were transfected with various amounts (μg) of CD4 and p18 expression plasmids, as well as the vector for p18 as indicated. Immunoprecipitation with anti-p18 peptide antibody and sequential blotting with anti-p18 and anti-CDK6 antibodies were performed as described in Figure 10, panel B.

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Chapter 4.

Reversal of EBV Immortalization of Human B Cells by p18 ^{INK4C}-Induced Proteolysis of LMP1

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Acknowledgments

The experiments presented in

Figure 13 were performed by Dr. Anne Altmeyer and Pey-Jen Yu.

Figure 17 were performed by Dr. Dos Sarbassov except for the

hγ1s probing in panel A.

Figure 18 were performed with advice from Jose Trevejo.

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Abstracts

Activation of the cyclin-dependent kinase (CDK) inhibitor p18^{INK4C} during IL-6-induced human B cell terminal differentiation is coupled to cell cycle arrest and reversal of B cell immortalization by the Epstein-Barr Virus (EBV). These results suggest that p18^{INK4C} may, in addition to inhibiting cell cycle progression, repress the expression of EBV transforming genes that are required for the maintenance of B cell transformation. We now show that IL-6-induced cell cycle arrest specifically correlates with repression of the one of the EBV transforming genes, LMP1. IL-6 repression of LMP1 occurs in late stage B cells after class switching which, compared with IgM-bearing cells before class switching, express a lower level of LMP1. EBV preferentially immortalizes primary IgM-bearing human B lymphocytes presumably because they support higher LMP1 expression. The expression of LMP1 therefore appears to be contingent on the differentiation stage of B cells and crucial for both the establishment and maintenance of B cell transformation by EBV. IL-6-signaled repression of LMP1 in B cells is apparently mediated by proteolysis independent of proteosome. The expression of p18^{INK4C} protein alone is sufficient to reconstitute proteolysis of the LMP1 and override immortalization by EBV regardless of the differentiation stage of B cells. Thus, p18^{INK4C} is likely to be a key mediator of IL-6 signals for simultaneous proteolysis of LMP1 and cell cycle arrest. To the best of our knowledge, this is the first demonstration of reversal of EBV immortalization by CDK inhibitor-mediated proteolysis of a viral transforming protein.

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Introduction

IL-6 induces coupled differentiation and cell cycle arrest in CESS cells (Morse et al., 1997). Altmeyer *et al* in our laboratory have shown that coincidental with cell cycle arrest, the EBV transforming genes including EBNA1, EBNA2 and LMP1 are shut off in CESS cells in response to IL-6 (Altmeyer et al., 1997). These results suggest a correlation between IL-6-induced cell cycle arrest and repression of EBV transforming genes. Among EBV transforming genes, EBNA2 has been shown to activate cell cycle by upregulating the positive cell cycle regulator such as cyclin D2 and CDKs (Sinclair et al., 1994; Kempkes et al., 1995; Sinclair et al., 1995). In addition, EBNA2 has been shown to repress Ig transcription (Jochner et al., 1996). These results suggest that EBNA2 promotes cell cycle progression and at the same time suppresses differentiation of B cells. Another EBV transforming gene, LMP1 is necessary for B cell transformation (Kieff, 1996). LMP1 and CD40 share a common PxQxT core TRAF binding motif and constitutively engages TRAFs (Devergne et al., 1996). Therefore, LMP1 mimics CD40 signaling and constitutively activates the NF-κB pathway, leading to cell cycle progression and cell survival (Eliopoulos et al., 1997). However, LMP1 alone is not sufficient to maintain B cell proliferation without CD40 signaling (Zimber-Strobl et al., 1996). These results suggest that even though the viral transforming genes usurp the cellular pathways, the cellular pathway can influence the function of the viral transforming genes.

We previously showed that the CDK inhibitor $p18^{INK4c}$ plays an important role in IL-6-induced cell cycle arrest in IgG+ CESS (Morse et al., 1997). The key supporting data are as follows: first, both the p21 and p18 families of CDK inhibitors can be activated by IL-6 during B cell terminal differentiation. p21 is activated late during this process (2 days). Therefore, p21 is likely to be downstream of IL-6-induced cell cycle arrest in CESS cells. By contrast, activation of p18 expression is more rapid, at one day of IL-6

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treatment, and correlates with cell cycle arrest in IgG+ CESS cells. Secondly, although IL-6 can not induce cell cycle arrest in IgM+ SKW cells, overexpression of p18 by transfection is sufficient to reconstitute coupled cell cycle arrest and cellular differentiation in response to IL-6 (Morse et al., 1997).

Together, these results suggest an inverse relationship between CDK inhibitor p18 and EBV transformation genes. The relative levels of expression of p18 and EBV transforming genes in response to IL-6 may control cell cycle progression in human B lymphoblastoid cells. The interplay between p18 and EBV transforming genes may also be crucial for the establishment and maintenance of EBV immortalization of human B cells. Our observation that cellular differentiation is coupled to cell cycle arrest in IgG+ cells, but not in IgM+ cells further implies a differentiation stage determinant in cell cycle control by p18 and EBV transforming genes. The fact that B cell lymphomas are primarily IgM+ is consistent with this notion, raising an important question as to how EBV immortalization is established.

We chose to address these questions using two strategies. One strategy was to determine the expressions of p18 and EBV transforming genes during IL-6-induced terminal differentiation and during immortalization of polyclonal tonsil B cells by EBV. The second strategy was to study the effect of p18 on EBV transforming genes by overexpression using adenoviral vectors, which allow us to efficiently transduce B cell lines that are normally resistant to transfection.

We found that the expression LMP1,but not EBNA2 correlates with sustained cell cycle progression in B cell differentiation by IL-6. The expression of LMP1 correlates with the efficiency of differentiation stage-dependent immortalization of EBV infected B lymphocytes. The expression of $p18^{INK4C}$ protein alone is sufficient to reconstitute proteolysis of the LMP1 and override immortalization by EBV regardless of the differentiation stage of B cells. Thus, $p18^{INK4C}$ is likely to be a key mediator of IL-6 signals for simultaneous proteolysis of LMP1 and cell cycle arrest.

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Results

<u>The inverse relationship between LMP1 expression and cell cycle progression.</u>

To address whether EBNA2 or LMP1 expression, or both, contribute to the uncoupling of differentiation and cell cycle arrest in IgM+ SKW cells in response to IL-6 (Morse et al., 1997), we stained for LMP1 or EBNA2 expression in SKW cells after IL-6 treatment. Unlike what we observed in IgG+ CESS cells, LMP1 expression did not change in IgM+ SKW cells after 4 days of IL-6 treatment (Figure 13, panel A). By contrast, EBNA2 expression was downregulated in IgM+ SKW cells after 4 days of IL-6 treatment (Figure 13, panel B), similar to that in IgG+ CESS cells (Altmeyer et al., 1997). Consistent with single cell analysis, immunoblotting analysis showed that LMP1 was expressed at a higher level in SKW cells than in CESS cells (Figure 13, panel C). No change in LMP1 level was observed in SKW cells after IL-6 treatment, although IL-6-differentiated CESS cells express much less LMP1 expression as previously shown(Altmeyer et al., 1997). Therefore, the lack of cell cycle arrest correlates with persistent expression of LMP1, but not EBNA2.

<u>The establishment of EBV-immortalized B cells correlates with LMP1 expression.</u>

To determine whether the difference of cell cycle control between IgG+ CESS cells and IgM+ SKW cells is relevant to polyclonal primary B cells, we examined the kinetics of expressions of surface IgM and IgG during EBV immortalization as described (Altmeyer et al., 1997). The expressions of surface IgM, IgG and CD19, a co-Ig receptor, were determined by FACS analysis. The IgM+ and IgG+ populations are equally represented (40-50%) in the freshly-isolated tonsil B cell population (Figure 14, panel A), although not all freshly isolated tonsil cells express CD19, presumably because of the presence of T cells and plasma cells that are thought to be CD19 negative. However, as a function of time after EBV immortalization, IgM+ cells gradually dominate the population.

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To determine whether LMP1 plays a role in the preferential establishment of IgM+ B cell, we determined the level of LMP1 expression by immunoblotting in IgG+ and IgM+ cells immediately after enrichment by expression of IgG and IgM at four weeks after EBV immortalization. The top 20% (IgG^{hi}) and lower 20% (IgG^{lo}) of IgG-bearing cells were collected for immunoblotting (Figure 14, panel B). LMP1 is expressed 2-3 times higher in the IgG^{lo} than in the IgG^{hi} population (Figure 14, panel C). Since the IgG^{lo} population represents IgM+ cells, LMP1 expression correlates to their ability to proliferation during EBV immortalization. Since crosslinking of the surface Ig may lead to cell death, we chose to analyze the kinetics of cell proliferation in viable IgM^{lo} (IgG^{hi}) and IgG^{lo} (IgM^{hi}) populations by trypan blue staining (Figure 14, panel D). We found that IgG^{lo} (IgM^{hi}) cells overgrow IgM^{lo} (IgG^{hi}). Thus, the level of LMP1 expression correlates with the preferential establishment of IgM+ population.

To further address the relationship between LMP1 expression and the establishment of IgM+ cells, we determined the kinetics of EBV immortalization (Figure 15, panel A). After EBV infection, the majority of cells died within two weeks; However, a small percentage of cells grow and become immortalized. Among the viable cells, the ratio of IgM+ to IgG+ cells increased over time, as shown in two independent experiments (Figure 15, panel B). Both IgM+ and IgG+ B cells can be induced by IL-6 into Ig-secreting cells, to similar extent after 4 days of treatment as determined by immunocytochemisty (Figure 15, panel B and panel D). This result is consistent with prior observations in this lab (Natkunam et al., 1994).

IgM+, but not IgG+ cells, cycle after EBV infection.

To address whether preferential establishment of IgM+ population is due to continuous cell cycling, we analyzed DNA replication in EBV-immortalized tonsil B cells by BrdU incorporation with or without IL-6 treatment. We chose 4 weeks after EBV infection to do the experiment, since the IgM+ and IgG+ populations are about equal

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(Figure 14, panel A and Figure 15, panel B). All differentiated IgG+ B cells are cell cycle arrested, whereas nearly 60% of the differentiated IgM+ B cells are cycling as indicated by BrdU incorporation (Figure 16, panel A). The differentiated cells that are replicating (BrdU+ / Ig^{hi} ratio) were determined after counting 300-500 cells (Figure 16, panel B). IL-6 treatment does not appear to reduce the percentage of replicating IgM+ cells. These results are consistent with our previous observations that IL-6 does not induce cell cycle arrest in IgM+ cells (Morse et al., 1997). The ability of polyclonal IgM+ cells to maintain cell cycle progression after EBV infection suggests that the ability to continue cycling is intrinsic to IgM+ cells and is the basis for the preferential establishment of immortalization of IgM+ cells.

Proteolysis of LMP1 in IL-6-induced terminal differentiation of IgG+ cells.

We next investigated the mechanism by which IL-6 shuts off LMP1 gene expression in IgG+ cells. To determine whether IL-6 inhibits transcription of LMP1 gene, the levels of LMP1 and EBNA2 mRNAs in IL-6-differentiated CESS cells as well as untreated control were determined by Northern hybridization. LMP1 and EBNA2 mRNA levels were found to remain unchanged in IL-6 differentiated CESS cells (Figure 17, panel A). As controls, the IL-6-induced cells showed a marked increase in the hγ1s mRNA encoding the secreted form of the IgG1 heavy chain and the GAPDH mRNA levels were constant. Thus, IL-6 signals do not repress the expression of LMP1 at the level of RNA transcription or stability.

The possibility that IL-6 may induce degradation of LMP1 protein, and if so, whether this was mediated by proteosome, was investigated by the use of the proteosome inhibitor lactacyctin. We found that whereas inhibition of the proteosome leads to a marked increase of the p53 levels as anticipated, no change in the levels of LMP1 was observed (Figure 17, panel B). Of interest, corresponding to the reduction of LMP1, the level of TRAF1 was also reduced in IL-6-differentiated cells (Figure 17, panel B). Together, these

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results suggest that IL-6 inhibits the expression of LMP1 through posttranscriptional mechanism independent of the proteosome.

Construction of a recombinant p18-Adenovirus.

IL-6 induces activation of p18 (Morse et al., 1997) and suppression of LMP1 in B cell terminal differentiation (Altmeyer et al., 1997). These findings raise the possibility that p18 and LMP1 may regulated the synthesis of each other. To express the p18 and LMP1 genes efficiently, the recombinant virus expressing p18 (Ad5.GFP.p18) and another virus expressing LMP1 (Ad5.GFP.LMP1), were constructed. Both viruses contain a CMV-driven cassette for the expression of green fluorescence protein (GFP) as an indicator of transgene expression (Figure 18). Theoretically, we expect equally molar transcripts of GFP and p18 genes since they are in separate CMV expression cassettes in the same construct. CESS, SKW and human tonsil B cells freshly-infected with EBV for 4 weeks were infected with Ad5.GFP.p18. The efficiency of the adenovirus transduction in CESS, SKW and human tonsil B cells freshly-infected with EBV are different. The CESS cells were more readily transduced (80% to 90%) than human tonsil B cells freshly immortalized with EBV (almost none). The mechanism that underlies the difference is not yet known.

Overexpression of p18 suppresses LMP1 expression by protein degradation.

The possibility that p18 represses the expression of LMP1 was investigated in CESS cells infected with Ad5.GFP.p18. Nearly all of CESS cells (95%) were infected, as determined by FACS analysis of GFP expression (Figure 19, panel A). p18 was expressed at a very high level after infection as shown by immunoblotting (Figure 19, panel B). Reprobing of the same membrane reveals a nearly complete disappearance of the LMP1 protein. The reduction of LMP1 was specific to the expression of p18, as it was not observed in cells infected with the control Ad5.GFP virus or uninfected cells. Therefore, the expression of p18 alone is sufficient to shut off LMP1 expression.

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Whether p18 represses the expression of LMP1 at the RNA level was determined by Northern Blotting. The same cell population was used for immunoblotting. Clearly, there was no change in the LMP1 mRNA level in cells expressing p18 (Figure 19, panel C). The equal RNA loading was indicated by the constant 18S and 28S RNA and by GAPDH probing. Therefore, the expression of p18 alone is sufficient to repress LMP1 expression at the post-transcriptional level.

To further determine whether suppression of LMP1 expression by p18 is due to inhibition of translation or protein degradation, newly translated LMP1 protein was analyzed by autoradiography. After ^{35}S-methionine labeling and immunoprecipitation, the labeled proteins were transferred onto a nitrocellulose membrane, which is used for both autoradiography and immunoblotting. As in previous experiment, CESS cells were efficiently transduced by virus Ad5.GFP and Ad5.GFP.p18 (Figure 20, panel A). The LMP1 protein was indeed translated in cells expressing p18, as indicated by the incorporation of ^{35}S-methionine into p18 protein after a labeling time of 30 minutes (Figure 20, panel B). Analysis of LMP1 protein by immunoblotting of the same membrane used in panel B reveals the absence of LMP1 at the steady-state in cells expressing p18. The suppression of LMP1 is specific to p18, since control virus containing GFP only did not suppress the expression of LMP1. These results corroborate the previous results shown in Figure 19, and demonstrate that p18 expression leads to LMP1 repression by proteolysis.

To verify p18 function in B cells infected by the recombinant virus, we analyzed the cell cycle distribution by FACS analysis of the cellular DNA content after propidium iodine (PI) staining. Almost 40% SKW cells and above 80% CESS cells were infected (Figure 21, panel A). In both cell lines, more cells were accumulated in G1 phase, as indicated by the increase of cells containing 2N DNA and reduction of cells containing 4 N DNA, which represent cells in G2/M (Figure 21, panel B). The p18 protein expressed by the recombinant virus is functional in causing cell cycle arrest. Collectively, these results

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provide compelling evidence that p18 induces simultaneous degradation of LMP1 protein and cell cycle arrest in B cells.

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Discussion

The interaction of LMP1 and p18 in IL-6 signaling.

It was shown in this laboratory that IL-6 shuts off EBV transforming genes such as LMP1 in B cell terminal differentiation (Altmeyer et al., 1997). In this study, we show that IL-6 activates p18 (Morse et al., 1997) and p18 activation causes proteolysis of LMP1 (Figures 19, 20). This suggests that LMP1 is downstream of p18 in IL-6 signaling. p18 is likely to mediate the IL-6 signals for repression of LMP1, thereby reversing LMP1-induced cell cycle progression. This hypothesis also explained our finding that IgM+ cells support a higher level of LMP1 expression (Figure 14, panel C), and proliferation in response to IL-6 (Figure 11 and Figure 16).

Can LMP1 regulate the expression p18, and thus promote cell cycle progression in IgM+ B cells? To address this question, a recombinant adenovirus containing LMP1 was also generated in the laboratory. However, for unknown reasons, CESS, SKW or freshly-immortalized human tonsil B cells did not express the LMP1 transgene after Ad5.GFP.LMP1 infection. It was reported that primary B lymphocyte is more difficult to transduce than immortalized cell line (Halbert et al., 1995). However, this does not explain why CESS cell is not transduce by Ad5.GFP.LMP1, since CESS cells were efficiently transduced by Ad5.GFP.p18. The functional differences between LMP1 and p18 must have contributed to this result.

CDK inhibitor p18 links IL-6 signaling pathway to the CD40-CD40L signaling pathway.

IgG+ and IgM+ differ not only in the developmental stages, but also in the expressions of LMP1 and p18 (Figure 22). High level of LMP1 expression may be the reason for the uncoupled differentiation and growth arrest in IgM+ B cells, since p18

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expression is not detectable in IgM+ SKW cells in response to IL-6 (data not shown). Since the activation of p18 suppresses the expression of LMP1 (Figure 19, panel B and Figure 20 panel C), p18 may inhibit the constitutive activation of NF-κB signaling by LMP1 in EBV-immortalized B cells (Figure 23). p18 is thus a link between the cell cycle regulatory pathway induced by IL-6 and the CD40-CD40L pathway induced by aggregation of LMP1 on cell surface. Therefore, the relative expression of p18 and LMP1 may determine whether cell will undergo proliferation or apoptosis.

Suppression of LMP1 by p18 suggests that p18 functions more than just a CDK inhibitor. This work also shed light on the mechanism of EBV-immortalization and EBV-associated B cell lymphomas. Recent studies show that the p18 may function as a tissue specific tumor suppresser: (1) The disruption of p18 or p27 leads to adenoma in the late stage of development (10 months), but disruptions of both p18 and p27 show a early occurrence of adenoma (Franklin et al., 1998). (2). The mutations or deletions of p18 and $p19^{INK4d}$ are rarely detected, while the mutations and deletions of p15 and p16 frequently occur in immortalized human cell lines or human primary tumors (Gemma et al., 1996; Koduru et al., 1995; Zariwala et al., 1996 a; Zariwala and Xiong, 1996 b). Therefore, the function of p18 as a tumor suppressor in immune system needs to be further analyzed. The analysis of lymphoid tissues from p18 -/- mice will help reveal the function of p18 in immune response and in tumor suppression.

This study showed the function of p18 in inducing cell arrest in both IgG+ and IgM+ B cells. This implies a possible use of p18 in gene therapy of IgG+ and IgM+ B cell lymphomas. Since IgM+ B cell lymphomas are more common than IgG+ B cell lymphomas, the efficient transduction of p18-containing adenovirus in IgM+ B cells, especially in primary IgM+ B cell, will pave the way for clinical applications of p18.

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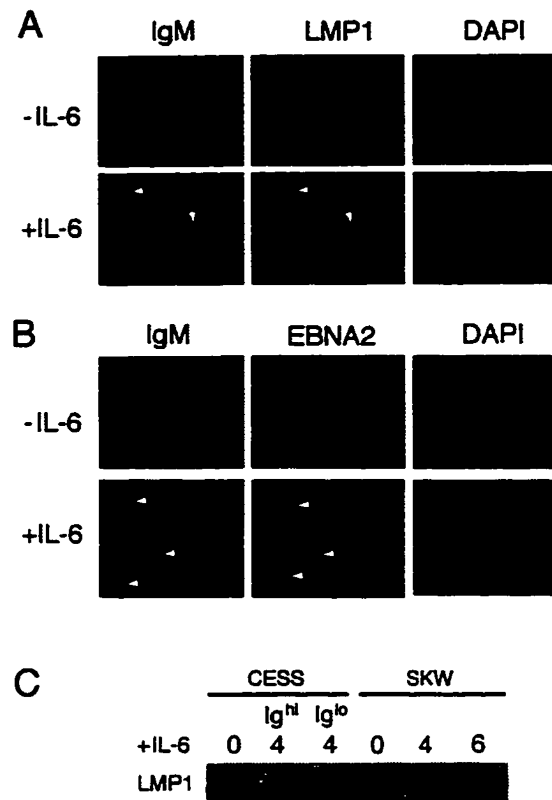

Figure 13. Cell cycle progression correlates with the expression of LMP1, but not EBNA2.

IgM+ SKW cells cultured without IL-6 (-IL-6) or with IL-6 (+IL-6) for 4 days were: (A) simultaneously stained for intracellular IgM (red) and cell surface expression of LMP1 (green); (B) simultaneously stained for IgM (green) and EBNA2 (red). DAPI staining (blue) is to localize the nucleus. Arrowheads indicate the corresponding cells. (C). CESS cells and SKW cells treated with IL-6 for time indicated (days) were analyzed by immunoblotting for LMP1 expressions. Ig^{hi} and Ig^{lo} represent the populations of differentiated and undifferentiated cells after separation as described before. Each lane represents $5x10^6$ cells of each population. (Dr. Anne Altmeyer and Pey-Jen Yu did the work shown in this figure.)

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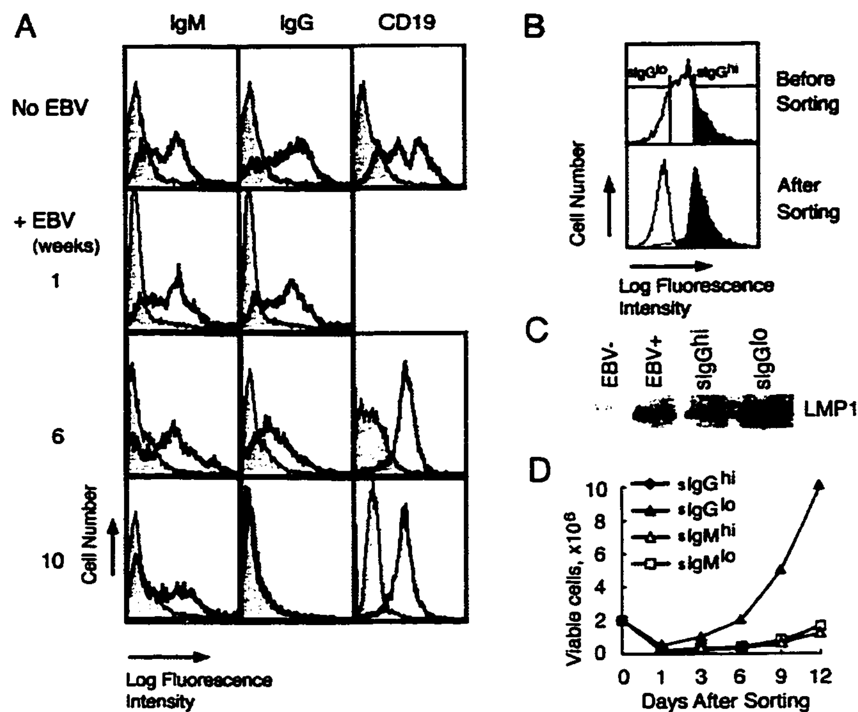

Figure 14. Differential establishment of EBV immortalization of IgM+ and IgG+ B cells.

(A) Kinetics of surface expressions of IgM, IgG and CD19. Freshly isolated human tonsil lymphocytes were separated and infected with EBV from B95.8 cells (Altmeyer et al., 1997). Cell surface expressions of IgM, IgG, CD19 on EBV-infected cells were analyzed by Flow Cytometry after staining the cells with FITC-labeled antibodies at different time (weeks) after infection. The staining by a FITC-labeled isotype control antibody was shown in the shaded histogram. (B) Four weeks after EBV-infection, the freshly-immortalized human tonsil B cells were separated into upper 20% and lower 20%, according to their surface expressions of IgG (sIg) by a cell sorter after staining with an anti-IgG-FITC. (C) The two populations represented as sIg^{hi} (upper 20%) and sIg^{lo} (lower 20%) were analyzed for LMP1 expressions by immunoblotting. Each lane represents $5x10^6$ cells. (D) Viable cells were counted after sorting by trypan blue exclusion.

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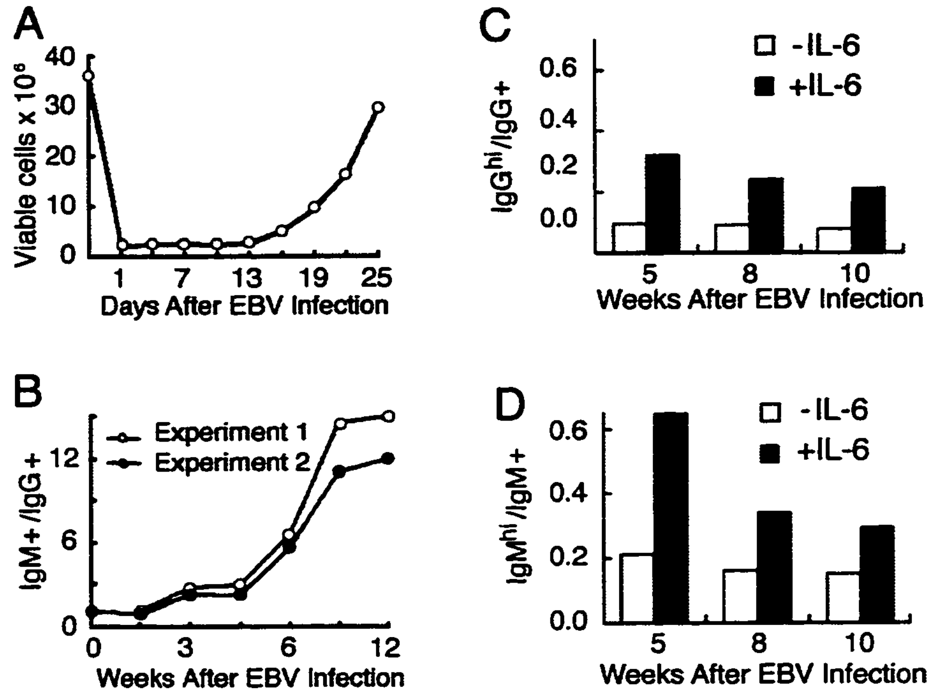

Figure 15. Preferential proliferation of IgM+ B cells after EBV infection. (A) Kinetics of viable cells after EBV infection. Freshly isolated human tonsil lymphocytes were separated by ficoll-paque and infected with EBV. Viable cells were counted after trypan blue staining. (B) Kinetics of IgM+ / IgG+ ratio after EBV infection. EBV-infection of fresh human tonsil B cells were stained anti-IgG-FITC and analyzed by Flow Cytometry. (C) and (D) Kinetics of IL-6 responses in IgG+ and IgM+ population. EBV-infected fresh human tonsil B cells were treated with IL-6 for 4 days, stained with anti-IgG (or IgM)-FITC for intracellular Ig. The Ig^{hi} population was determined by counting using microscopy. The Ig+ population were determined by FACS, and the ratio of IgG^{hi} / IgG+, IgM^{hi} / IgM+ were calculated. The staining by a FITC-labeled isotype control antibody was used to determine the negative population in the determinations of either Ig^{hi} population in microscopy or Ig+ population in FACS. Around 500 cells are represented in each group.

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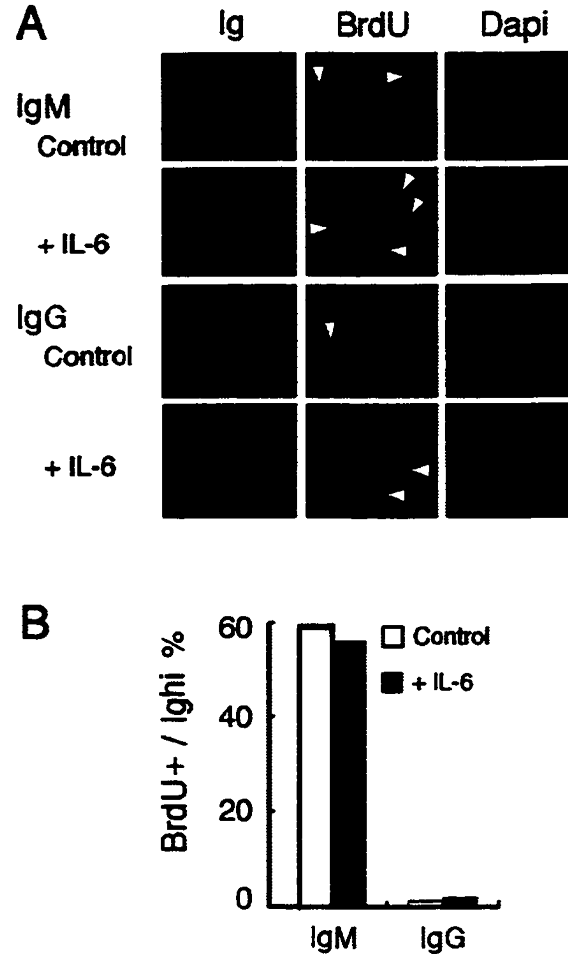

Figure 16. IL-6 induces strict coupling of differentiation and cell cycle arrest in IgG^{hi} cells but not in IgM^{lo} cells.

(A) Four weeks after EBV infection, tonsil B cells were incubated in the presence (+IL-6) or absence (Control) of IL-6 for 3 days, labeled with BrdU for 24 hours and stained for intracellular IgM (green) or IgG (green), BrdU (red) and DAPI (blue) to localize the nucleus. Arrowheads indicate BrdU staining corresponding to the differentiated cells (Ig^{hi}) in intracellular Ig staining. (B) The ratio of and BrdU(+)/Ig^{hi} was calculated after counting 300-500 cells.

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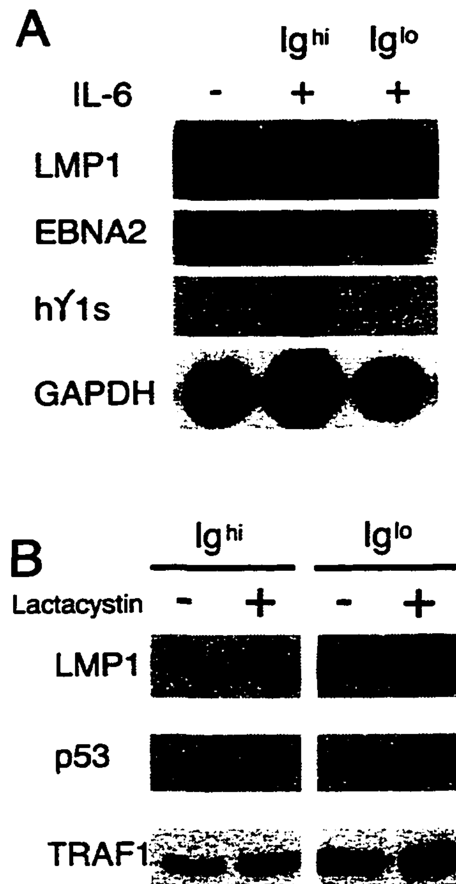

Figure 17. IL-6 suppresses LMP1 expression by proteosome-independent posttranscriptional mechanisms.

(A) CESS cells cultured with or without IL-6 for 4 days were separated with MHC II antibody-conjugated magnetic beads. Total RNA was extracted and Northern hybridization performed. cDNA encoding specific segments of LMP1, EBNA2, IgG1 and GAPDH were labeled with α-^{32}P-dCTP and used in the hybridization. (B) The expressions of LMP1, p53 and TRAF1 in the differentiated B cells (Ig^{hi}) and non-differentiated B cells (Ig^{lo}) were analyzed by immunoblotting. Lactacystin was added 2 hours before separation.

(Dr. Dos Sarbassov did the work shown in this figure, except for hγ1s probing in panel A.)

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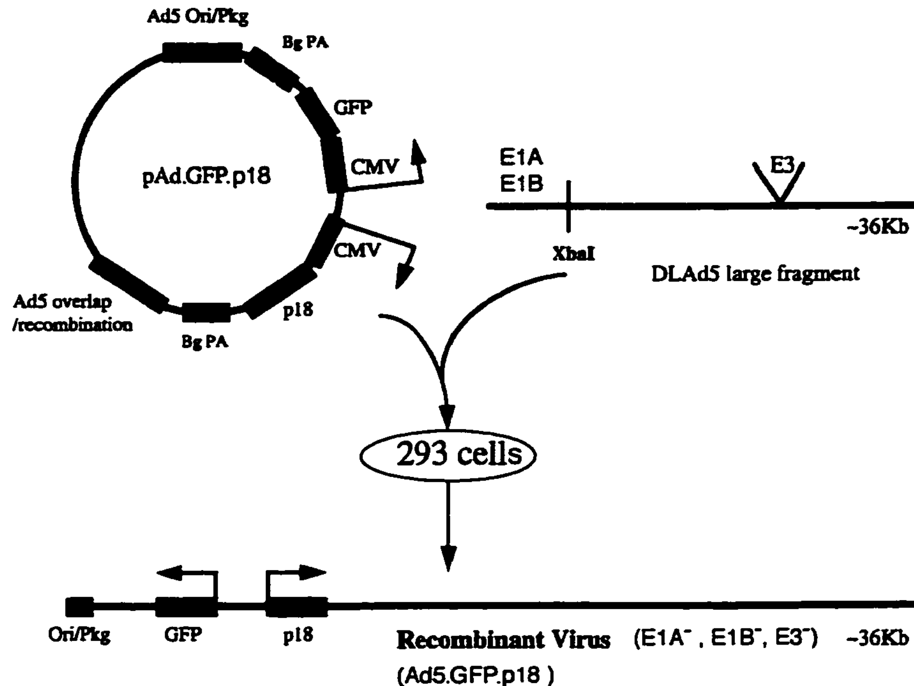

Figure 18. Construction of a recombinant adenovirus containing both GFP and p18 transgenes.

A full-length p18 cDNA was cloned into pAd.GFP vector. Two CMV expression cassettes in opposite directions drive the expression of either GFP or p18. pAd.GFP.p18 was sequenced to confirm the identity of the insert. The recombinant virus Ad5.GFP.p18 was generate by co-transfection of pAd.GFP.p18 with the large fragment of the dlAd5NCAT viral backbone which has deletions of E1A, E1B and E3. Ad5.GFP.LMP1 and a control virus containing only GFP (Ad5.GFP) were also generated as above.
(This figure is done with advice from Jose Trevejo.)

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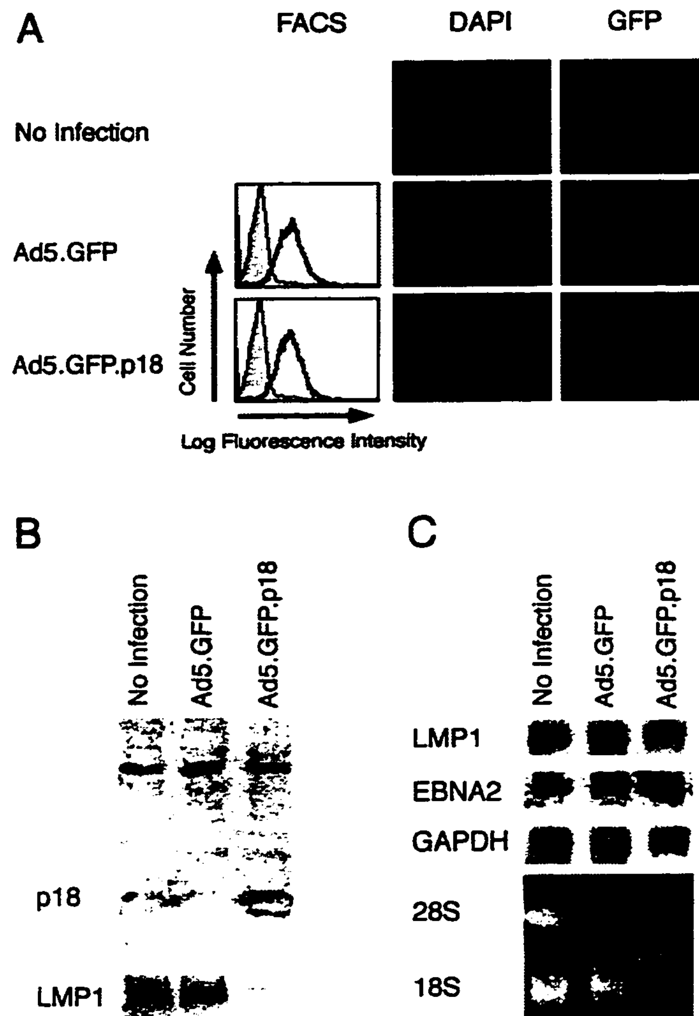

Figure 19. Overexpression of p18 suppresses LMP1 expression by posttranscriptional mechanisms.

(A) CESS cells, with or without infection of Ad5.GFP or Ad5.GFP.p18 for 4 day, were analyzed directly by Flow Cytometry for GFP expression (green) or by immunofluorescence microscopy after staining with DAPI (blue) to localize the cell nucleus. The fluorescence intensity obtained with uninfected cells is represented by the shaded histogram. (B) The expressions of p18 and LMP1 in CESS cells with or without

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infection of Ad5.GFP or Ad5.GFP.p18 for 4 day, were analyzed by immunoblotting. The same membrane was reprobed with an anti LMP1-antibody. (C) Total RNA from CESS cells was analyzed by Northern hybridization. The membrane was hybridized separately with α-^{32}P-dCTP-labeled cDNA encoding specific segments of LMP1, EBNA2 or GAPDH.

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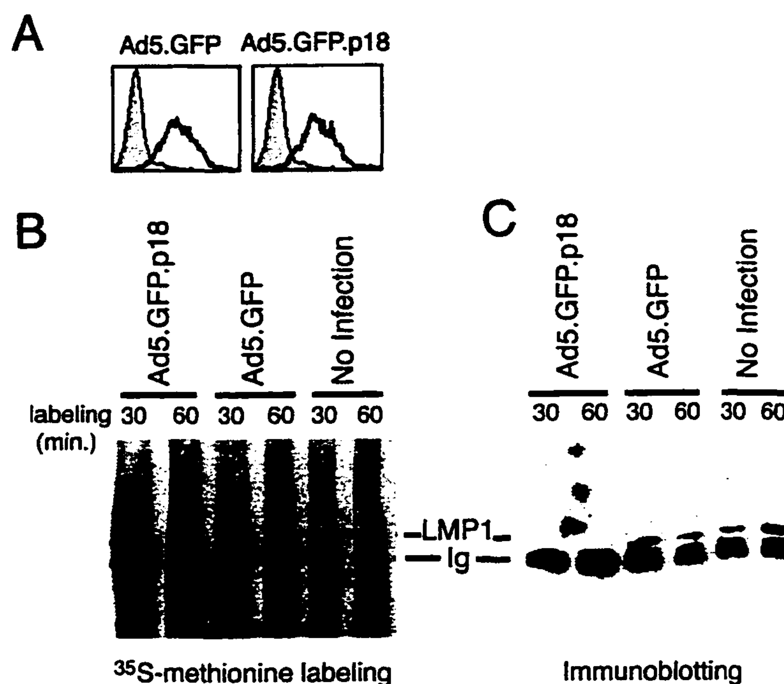

Figure 20. Overexpression of p18 induces proteolysis of LMP1.

CESS cells with or without infection of Ad5.GFP or Ad5.GFP.p18 for 4 day, were analyzed: (A) directly by Flow Cytometry for GFP expression. The fluorescence intensity obtained with uninfected cells is represented by the shaded histogram; (B) by biosynthetic labeling: cells were labeled by ^{35}S-methionine +^{35}S-cysteine for time indicated (minutes). Cell lysates were made according to the Materials and Methods, immunoprecipitated with an mouse anti-LMP1 antibody, resolved by SDS-PAGE. The resolved proteins were transferred onto a nitrocellulose membrane and exposed to a film after drying the membrane; (C) by immunoblotting: The same membrane from (B) was probed with anti LMP1-antibody. 40 μg of total proteins were analyzed in SDS-PAGE.

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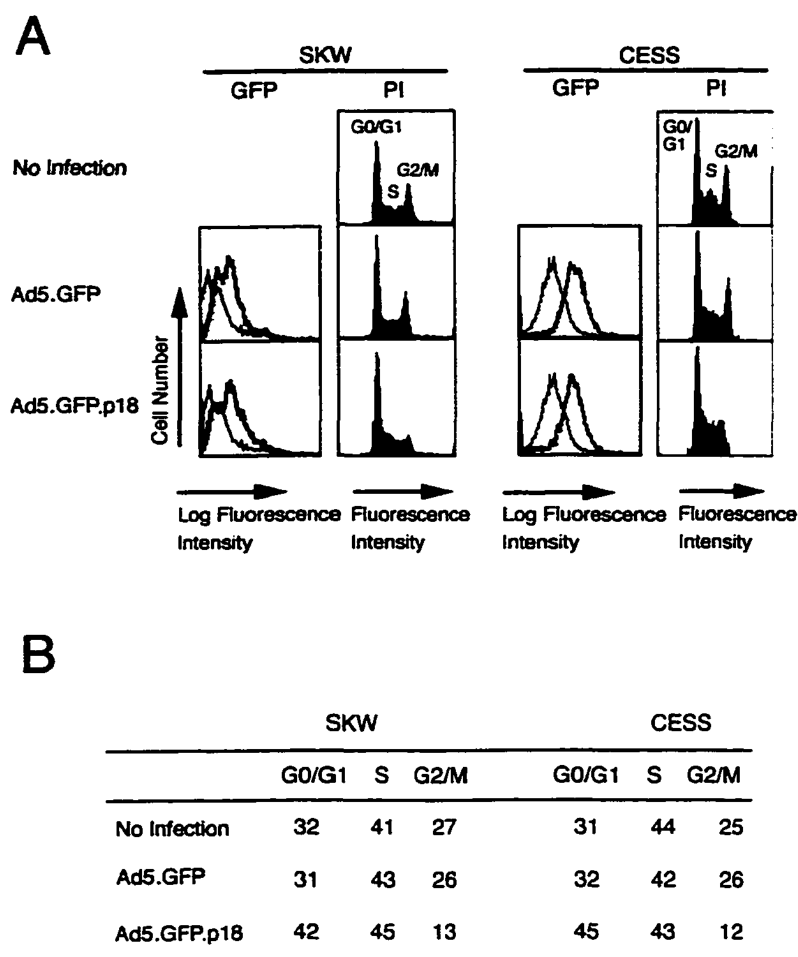

	SKW			CESS		
	G0/G1	S	G2/M	G0/G1	S	G2/M
No Infection	32	41	27	31	44	25
Ad5.GFP	31	43	26	32	42	26
Ad5.GFP.p18	42	45	13	45	43	12

Figure 21. Overexpression of p18 induced cell cycle arrest in both IgM+ SKW cells and IgG+ CESS cells.

(A). The relative DNA contents in different phases of cell cycle of IgM+ SKW cells and IgG+ CESS cells with or without infection of either Ad5.GFP or Ad5.GFP.p18 for 4 day were analyzed by flow cytometry after propidium iodine (PI) staining. GFP expressions were used to determine the efficiency of the infections. G_0/G_1, S and G_2/M phases are indicated. The fluorescence intensity obtained with uninfected cells is represented by the shaded histogram. (B). The percentage of cells in G_0/G_1, S and G_2/M phases were determined by FACS analysis as indicated (%).

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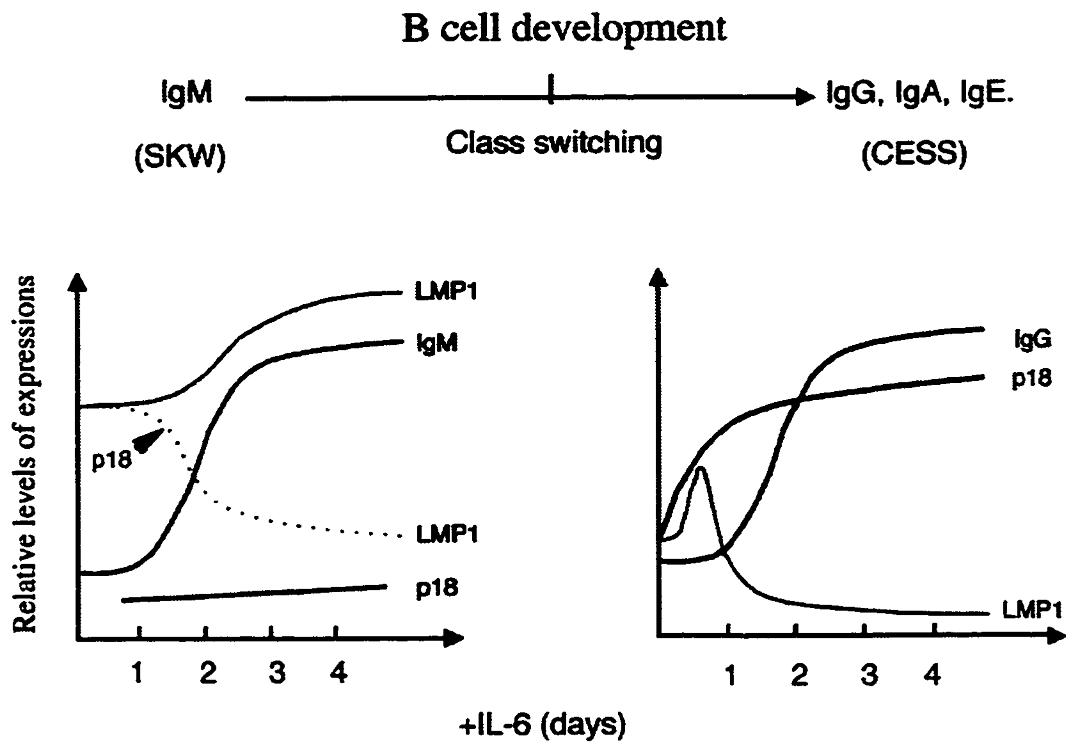

Figure 22. The expressions of p18 and LMP1 in IL-6-induced B cell terminal differentiation.

The relative levels of expressions of LMP1, IgM, IgG and p18 were determined by immunoblotting after IL-6 treatments. Overexpression of p18 induces degradation of LMP1, as indicated by a dotted line.

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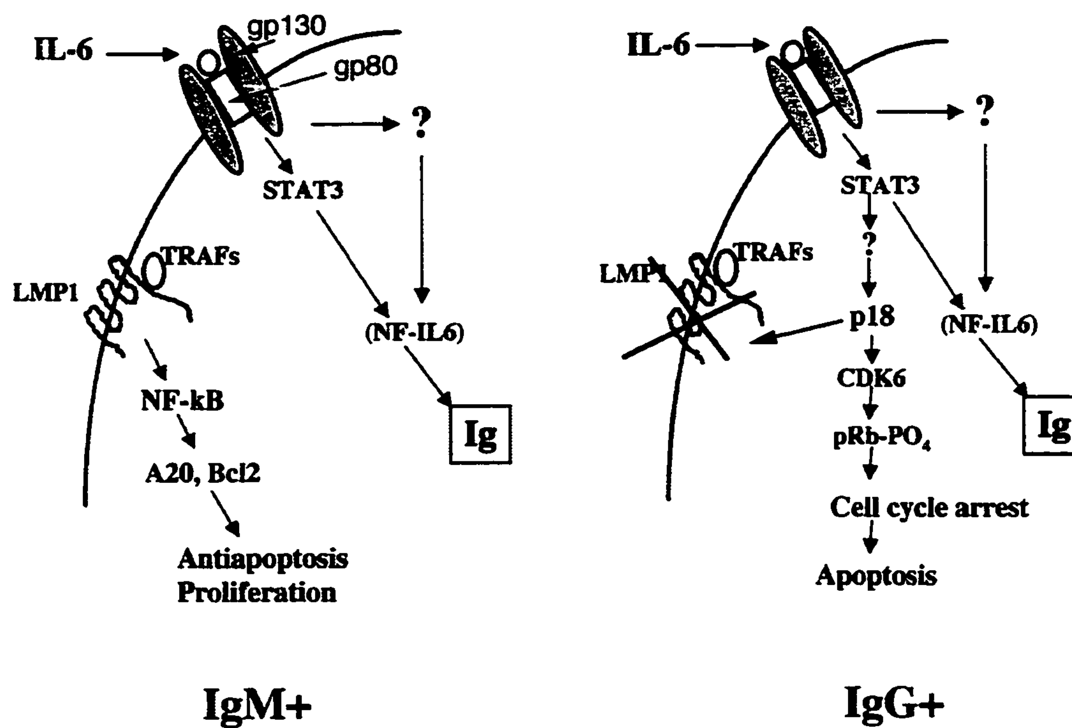

Figure 23. IL-6 signaling in IgM+ and IgG+ cells.

IL-6 binds to its receptor and triggers the activation of early JAK-STAT pathway and delayed NF-IL-6 pathway. LMP1 mimics a constitutive activation of CD40-CD40L pathway, which is responsible for the uncoupled differentiation and cell cycle arrest in IgM+ B cells. The activation of $p18^{INK4c}$ shuts off LMP1 expression and thus leads to the coupled differentiation and cell cycle arrest in IgG+ B cells.

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Chapter 5

NF-IL6 Regulation and Function in B Cell Terminal Differentiation.

Introduction

NF-IL6 is a basic leucine zipper (bZip) transcription factor of CCAAT / enhancer binding protein (C/EBP) family. The homologues of human NF-IL6 are named IL-6DBP (IL-6-induced DNA binding protein) in rat (Poli et al., 1990); LAP (liver-activating protein) in rat (Descombes et al., 1990); rNF-IL6 in rat (Metz and Ziff, 1991); alpha 1-acid glycoprotein / enhancer binding protein (AGP/EBP) in rat (Chang et al., 1990); CRP2 in mouse (Williams et al., 1991); C/EBPβ in mouse (Akira et al., 1990; Cao et al., 1991); and NF-M in chicken (Katz et al., 1993).

There are three isoforms of NF-IL6, which are translated in-frame from the same NF-IL6 mRNA transcript (Ossipow et al., 1993). NF-IL6-1 and NF-IL6-2 translated from the first and second start codon, contain the transactivation region, whereas NF-IL6-3 that are translated from the third start codon does not. The transcriptional regulatory function of NF-IL6 has been shown to depend on the ratio of the activator form (NF-IL6-2/LAP) to the inhibitor form (NF-IL6-3/LIP) (Descombes et al., 1990; Descombes and Scheibler, 1991; Ossipow et al., 1993; Hsu et al., 1994; Klampfer et al., 1994). Homodimerization or heterodimerization with other leucine zipper proteins is a requisite for the DNA-binding of NF-IL6. NF-IL6 -/- mice exhibit enlarged spleen and an expansion of B cell compartment (Screpanti et al., 1995), suggesting that NF-IL6 may also play an important role in B cell

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development by regulating B cell proliferation and differentiation. Therefore, how NF-IL6 promoter is regulated becomes an important question.

The NF-IL6 consensus binding sites, TT(G)NNGNAAT(G), are present in the promoters of many divers genes and believed to be important for their regulation (Akira and Kishimoto, 1992). These genes include IL-6 (Akira et al., 1992; Zhang et al., 1994), the LTR of HIV type1 (Henderson et al., 1996), IgH and Igκ enhancer (Akira and Kishimoto, 1992), human DNA topoisomerase I (hTOP1) (Heiland and Knippers, 1995) and human P-450 (Toda et al., 1995).

The activity of NF-IL6 is regulated by dimerization and protein-protein interactions. For example, c-Fos and c-Jun interact with NF-IL6 and suppress the DNA-binding activity of NF-IL6 (Hsu et al., 1994). pRB interacts with NF-IL6 both in vivo and in vitro. However, the interaction of pRB and NF-IL6 increases the binding of NF-IL6 to DNA (Chen et al., 1996). No consensus sequences for direct binding of pRB have been found.

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Objective

Based on the unpublished promoter sequence of the rat homologue of NF-IL6 (LAP) provided by Dr. Ueli Scheibler, we have identified two NF-IL6-binding sites and one STAT-binding site in the upstream. This suggests that the expression of NF-IL6 may be regulated by both STAT family proteins and by NF-IL6 itself (Figure 24). IL-6 rapidly activate STAT3 and STAT1(Zhang et al., 1995). The activated STAT3 and/or STAT1 may bind to the STAT-binding site (STNF) and activate the transcription of NF-IL6. The NF-IL6 transcript will be translated into three isoforms, which in turn bind to their own promoter and regulate its own transcription according to the ratio of inhibitor to activator forms of NF-IL6. The interaction between NF-IL6 and pRB may also play a role in the regulation. NF-IL6 might therefore be one of the proteins that link the IL-6-induced early JAK-STAT pathway and delayed NF-IL6 signaling pathway.

In this study, we found that the expression of NF-IL6 isoforms can be differentially regulated by IL-6 and the regulation is dynamic. The ratio of inhibitor to activator form increases over time in response to IL-6. The expressions of c-Jun and c-Fos are also coordinately regulated by IL-6. We also found that NF-IL6 promoter can be activated by NF-IL6-2 and inhibited by NF-IL6-3, pRB. These results suggest a role of NF-IL6 in the regulation of NF-IL6 transcription and in IL-6-mediated B cell terminal differentiation.

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Results

<u>NF-IL6 isoforms were differentially regulated by IL-6.</u>

As a first step to study the function of NF-IL6 in IL-6-induced B cell terminal differentiation, we examined NF-IL6 expression by immunoblotting of nuclear extracts from cells with or without IL-6 treatment. Two antibodies were used in immunoblotting (Figure 25). One antibody is monoclonal 21A that recognizes only the activator isoform of NF-IL6 (NF-IL6-1 and NF-IL-6-2). Another antibody is a polyclonal 4-4 that recognizes all three isoforms of NF-IL6. Without IL-6 treatment, NF-IL6-1 and NF-IL6-2 are barely detectable, whereas the inhibitor form NF-IL6-3 is the predominant isoform expressed in human B cells (Figure 26, pane A). Both activator and inhibitor forms can be activated by IL-6 and the regulation is dynamic. NF-IL6-1 and NF-IL6-2 expressions are increased rapidly within 1 hour of IL-6 treatment, peaked at 6 hours, declined by 1 day, and returned to the background level by 2 days of IL-6 treatment (Figure 26, panel A and B). The expression of NF-IL6-3 is also increased within 1 hour but remained at a relatively high level at 2 days of IL-6 treatment. These results suggest that the activator forms may function at early stage of B cell differentiation, whereas the inhibitor form at late stage of B cell differentiation, since the ratio of activator to inhibitor changed over time. Since only 20-30 % cells respond to IL-6 (Natkunam et al., 1994), the changes of the ratio of inhibitor to activator may be more obvious in the differentiated population.

<u>IL-6 enhances the expression of c-Fos, c-Jun and Jun B.</u>

Since Fos/Jun family proteins may modulate function of NF-IL6 (Hsu et al., 1994), the expressions of Fos/Jun and Jun B were analyzed by reprobing the same membrane. IL-6 stimulated a rapid increase of c-Jun expression and its level decreased within 1 day (Figure 26, panel A and Figure 27), similarly to NF-IL6-1 and NF-IL6-2. c-Fos was also transiently activated in 3 hours of IL-6 treatment (Figure 27), although not as significant as c-Jun. Jun B expression is increased within an hour and decreased within

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two hours. The activation of c-Jun and NF-IL6 with a similar kinetics suggest a possibility that these proteins may coordinate in IL-6-induced B cell terminal differentiation.

NF-IL6-3 inhibits the activation of the NF-IL6 promoter by NF-IL6-2.

We analyzed the regulation of NF-IL6 promoter in transient transfections using the deletion mutants of the NF-IL6 promoter provided by Dr. Ueli Scheibler. HepG2 cells that are liver epithelium cell lines, and M12 cells that are mouse B lymphoid cell lines, are used in transient transfection. We use HepG2 since this cell line expresses a low level of endogenous NF-IL6 but can support the expression of transfected NF-IL6 (Descombes and Scheibler, 1991). Since IL-6 responsiveness is cell-type specific, we also use M12 cell line in order to determine the regulation of NF-IL6 promoter in B cells. Chloramphenical acetyl transferace (CAT) was used as a reporter. JYM-CAT, a CAT reporter construct containing only a minimum promoter, is used as a negative control. RSV-CAT, a CAT reporter construct containing a strong RSV promoter, is used as a positive control. As expected, NF-IL6-2 activates the NF-IL6 promoter and NF-IL6-3 inhibits the activation of NF-IL6 promoter by NF-IL6-2 in both HepG2 (Figure 29, panel A) and M12 cells (Figure 29, panel B). The M12 cells are found to be more difficult to transfect than HepG2 cells. 40-CAT maintained 30% of the activity conferred by NF-IL6-2 (Figure 29, panel A). This region (position +1 to +50) contains no NF-IL6-binding site, or any binding site of known transcription factors. It is likely that other transcription factors, together with NF-IL-6, maintain the basal expression of NF-IL6 through this region.

pRB inhibits the activation of the NF-IL6 promoter by NF-IL6-2 in Weri-27 cells.

As a dual functional regulator of transcription, pRB may inhibit (Nevins, 1992) or enhance gene transcription (Chen et al., 1996). To investigate the regulatory role of pRB in the NF-IL6 promoter, we co-transfect NF-IL6-2 and pRB together with the reporters of NF-IL6 promoter into Weri-27 cells. We chose Weri-27 cells since they are retinoblastoma

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cells and do not express functional pRB. pRB inhibits the activation of the NF-IL6 promoter by NF-IL6-2 in a dose-dependent manner (Figure 30). A pRB mutant (CMV-mRB) was also used in the transfection. CMV-mRB harbors a mutation (Cys to Phe mutation in position 706), which abolishes the interaction of pRB with SV40 T antigen and the interaction of pRB with NF-IL6 (Bignon et al., 1990; Chen et al., 1996). In our experiment, we found that CMV-mRB can only partially reverse the inhibitory function of wild-type pRB on 120-CAT (Figure 30). More experiments are needed to determine the regulatory role of pRB on the NF-IL6 promoter.

The basal expression of 120-CAT in pRB positive HepG2 cells is less than 4 % of the RSV-CAT (Figure 29, 120-CAT without NF-IL6-2) but it is about 30% of the (RSV-CAT) in pRB negative Weri-27 cells (Figure 30, 120-CAT without NF-IL6-2). These results suggest an inhibitory function of the endogenous pRB on the NF-IL6 promoter.

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Discussion

1. NF-IL6 in B cell terminal differentiation.

Consistent with previous results, NF-IL6-2 (and maybe NF-IL6-1 as well) activates transcription, while NF-IL6-3 inhibits transcription. The two NF-IL6-binding sites may act cooperatively, since the deletion of 5NF (in 65-CAT) reduced the reporter activity as compared with 120-CAT.

The similar kinetics of the expressions of c-Jun, NF-IL6-1 and NF-IL6-2 in response to IL-6 suggests that these proteins may coordinate transcription in B cell differentiation. The sequential activation of Jun B, c-Fos and then NF-IL6 as well as c-Jun suggests that these molecules may function at different stages of IL-6 signaling. The loss of function of NF-IL6 in NF-IL6 -/- mice may affect more the function of NF-IL6-3 than that of NF-IL6-1 and/or NF-IL6-2. This result explains the uncontrolled B cell proliferation and differentiation in NF-IL6 -/- mice (Screpanti et al., 1995).

2. The cross-talk between NF-IL6 and JAK-STAT pathways.

Both STAT3 and STAT1 are activated minutes after IL-6 treatment in CESS cells (Zhang et al., 1995). The presence of a STAT-binding site in NF-IL6 promoter suggest that JAK-STAT pathway may converge with NF-IL6 pathway by regulating the transcription of NF-IL6. NF-IL6 may be one of the linkages between the two pathways. The function of STAT family proteins on the regulation of NF-IL6 needs to be addressed.

3. NF-IL6 and pRB in cell cycle control.

pRB is an important substrate of major CDKs and suppresser of E2F family transcription factors. The interaction of pRB with E2F family proteins controls G1/S progression. The interaction of NF-IL6 and pRB suggests a regulatory role of NF-IL6 on

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pRB function, thus on the cell cycle control. NF-IL6 deficient mice also reveal an inhibitory function of NF-IL6 (Screpanti et al., 1995), especially NF-IL6-3, in the inhibition of B cell proliferation. From my study, the ratio of inhibitor / activator forms of NF-IL6 increases over time by IL-6 treatment, indicating that inhibitor NF-IL6-3 may be more important in IL-6-induced cell cycle control and later stage of B cell terminal differentiation.

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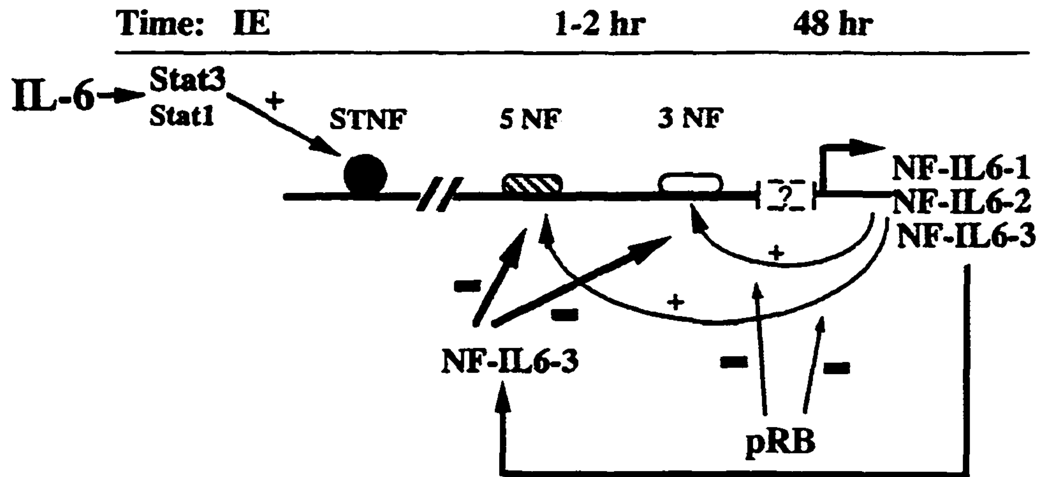

Figure 24. The proposed model for the regulation of NF-IL6 transcription.

IE: immediate early genes.

STNF: STAT binding site on NF-IL6 promoter.

5'-CTGTTTCCCAGAAG-3' -352 to -344

5NF: 5' NF-IL6-binding site on NF-IL6 promoter.

5'-CGGCGTGACGCAGCC-3' -106 to -92

3NF: 3' NF-IL6-binding site on NF-IL6 promoter.

5'-GCTCGGCGTGACGCAGCC-3' - 60 to -46

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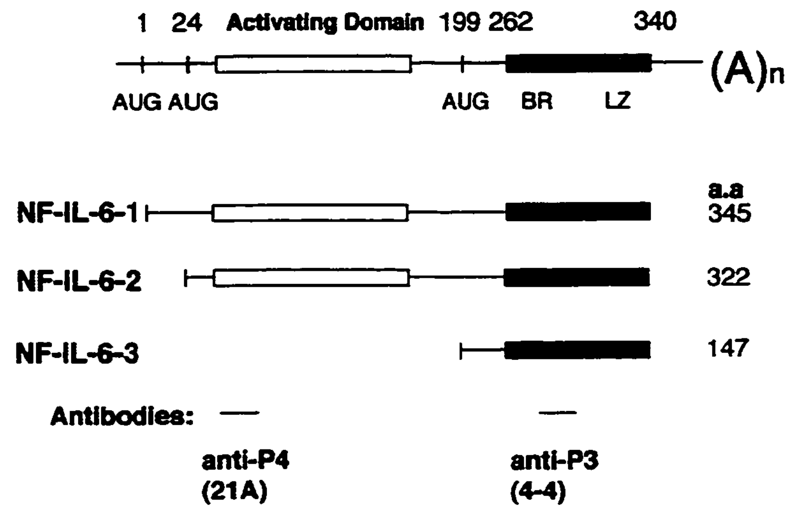

Figure 25. NF-IL6 isoforms and the antibodies against them.

Antibodies against the different regions of NF-IL6 were indicated.

anti-P4: monoclonal anti-peptide4 antibody, 21A, which recognizes NF-IL6-1 and NF-IL6-2.

anti-P3: polyclonal anti-peptide3 antibody, 4-4, which recognizes all three isoforms of NF-IL6.

BR: Basic region.

LZ: leucine zipper region.

a.a: amino acid.

(A)n: poly A tail.

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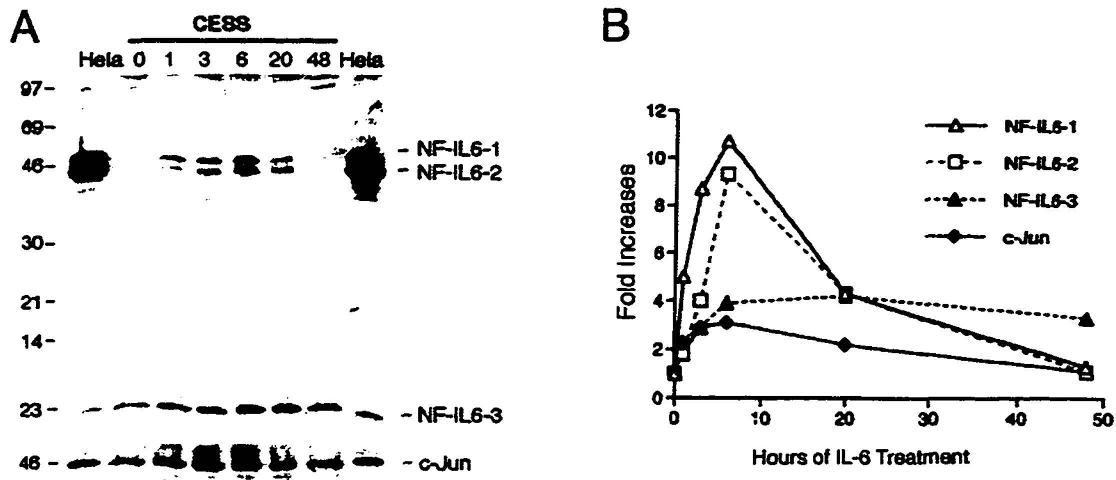

Figure 26. NF-IL6 isoforms are differentially regulated by IL-6.

(A) CESS cells were incubated in the presence or absence of IL-6 for the time indicated (hours). Nuclear extracts were made separately from 10^7 control and the induced CESS cells. The nuclear proteins (25 µg) were resolved by SDS-PAGE in a 10 % gel, transferred onto the nitrocellulose membrane and immunoblotted with antibodies to NF-IL-6 and c-Jun after stripping the same membrane. HeLa cell nuclear extracts were used as positive control. Molecular weight was indicated in kDa. (B) The relative intensities of NF-IL6 and c-Jun shown in (A) were determined by densitometry scanning, with the level in untreated cells arbitrarily set as 1.

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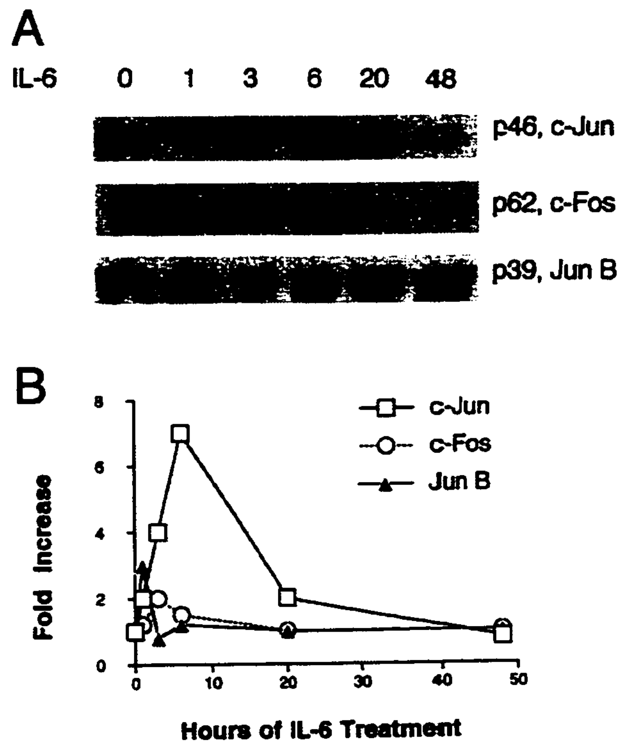

Figure 27. c-Jun, c-Fos and Jun B are activated by IL-6.

(A) CESS cells were incubated in the presence or absence of IL-6 for the time indicated (hours). Nuclear extracts were made separately from 10^7 CESS cells. The nuclear proteins (25 μg) were resolved in 10 % SDS-PAGE, transferred onto the nitrocellulose membrane and immunoblotted with antibody to c-Jun, c-Fos and Jun B after stripping the same membrane and reprobing with another antibody. Molecular weight was indicated in kDa. (B) The relative intensities of c-Jun, c-Fos and Jun B shown in (A) were determined by densitometry scanning, with the level in untreated cells arbitrarily set as 1.

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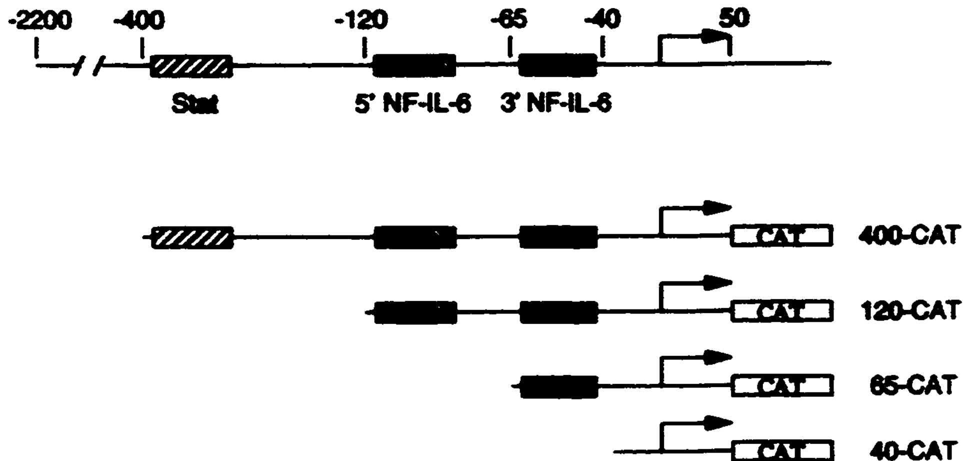

Figure 28. NF-IL-6 promoter and its CAT reporters of deletion mutants.

STNF: STAT binding site in NF-IL6 promoter.

5'-CTGTTTCCCAGAAG-3' -352 to -344

5 NF: 5' NF-IL6 binding site.

5'-CGGCGTGACGCAGCC-3' -106 to -92

3 NF: 3'NF-IL6 binding site.

5'-GCTCGGCGTGACGCAGCC-3' - 60 to -46

CAT: chloramphenical acetyl transferase.

Arrows indicate transcription initiation sites.

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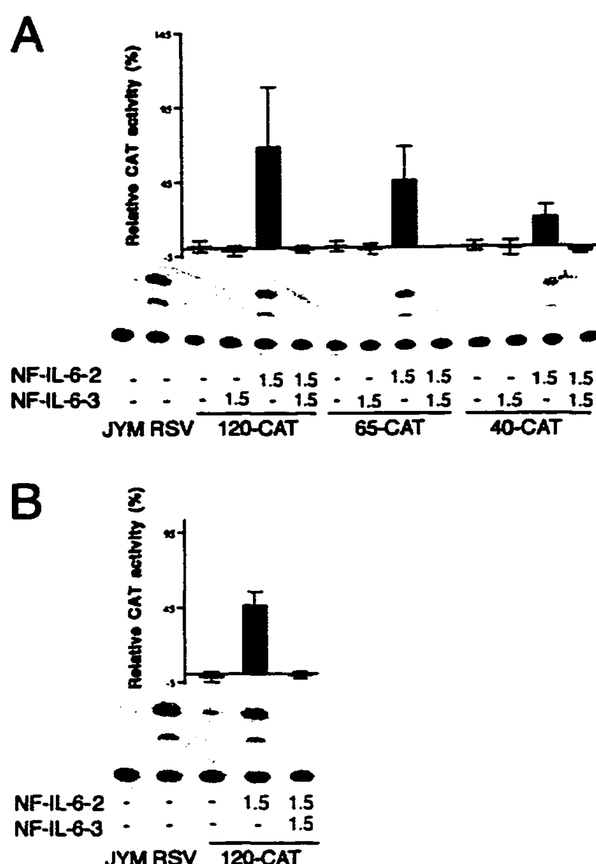

Figure 29. NF-IL6-3 inhibits the activation of NF-IL6 promoter by NF-IL6-2.

(A). HepG2 cells ($3x10^6$)were plated 1 day before transfection by the calcium phosphate methods. The reporter plasmid 120-CAT, 65-CAT and 40-CAT (each in 1.5 μg) were co-transfected together with NF-IL6-2 (we used rat homologue of human NF-IL6-2, pSCT-NF-IL6-2, 1.5 μg, 1X) and NF-IL6-3 into HepG2 cells. (B). M12 cells were transfected by DEAE-Dextran methods. NF-IL6-2 was cotransfected with NF-IL6-3. pBluescript vector was used as carrier DNA so that the total amount of DNA in each transfection is 7μg. The transfected cells in (A) and (B) were harvested at 40 h after transfection and assayed for CAT activity and exposed to a film. JYM-CAT (JYM) and RSV-CAT were used as negative and positive control. The histogram represents the radioactivity measured by a scintillation counter after autoradiography.

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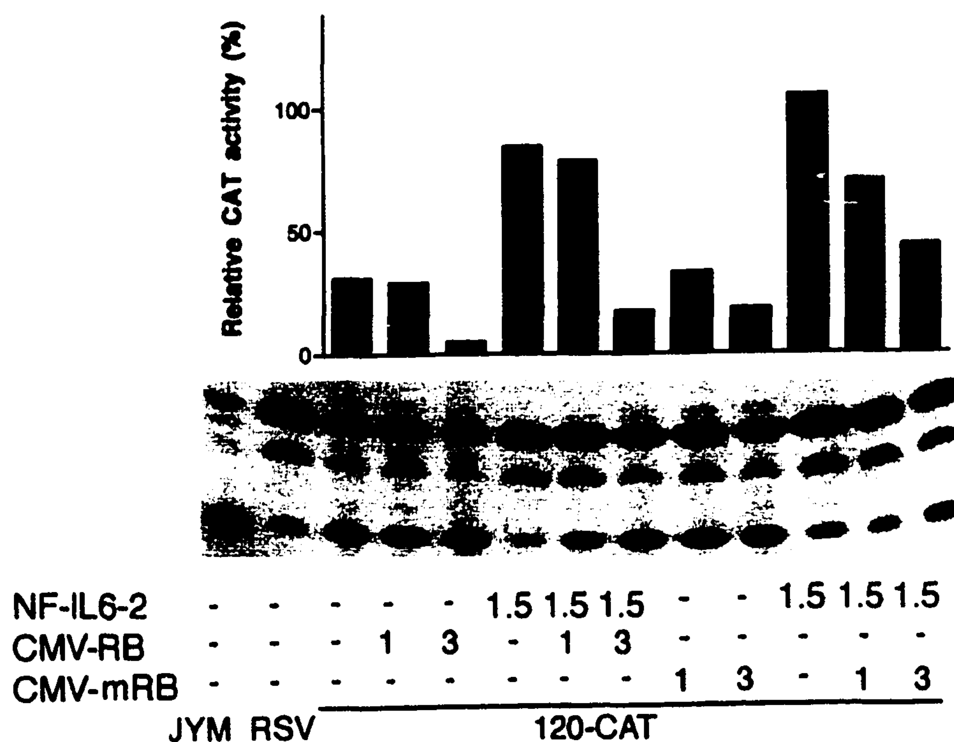

Figure 30. pRB inhibits the activation of NF-IL6 promoter by NF-IL6-2.

Weri-27 cells (3×10^6) were fed 4 hours before transfection by DEAE-Dextran methods. The reporter plasmid 120-CAT (1.5 μg) was co-transfected with NF-IL6-2 (pSCT-NF-IL6-2, 1.5 μg, 1X), CMV-RB and a pRB mutant (CMV-mRB). pBluescript vector was used as carrier DNA so that the total amount of DNA in each transfection is 8μg. The cells were harvested at 40 h after transfection and assayed for CAT activity and expose to a film. JYM-CAT (JYM) and RSV-CAT (RSV, each in 7 μg) were used as negative and positive control in transfections. The histogram represents the radioactivity measured by scintillation counter after autoradiography.

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Chapter 6

Summary and Future Studies.

1. The specificity of p18-induced proteolysis of LMP1.

We found that the relative expressions of p18 and LMP1 may be important in IL-6-induced B cell terminal differentiation and cell cycle control in Chapters 3 and 4. Our results do not preclude the possibility that other CDK inhibitors such as p15, p16 and p27 may function in a similar fashion. In support of this possibility, $p15^{INK4b}$ and $p16^{INK4a}$ have been found to be deleted or mutated in a variety of human tumor-derived cell lines and primary tumors (Zariwala et al., 1996 a; Zariwala and Xiong, 1996 b); p18 deficient mice are increased in cell numbers and exhibits hyperproliferation of T, B lymphocytes upon mitogen stimulation (Franklin et al., 1998). Overexpression of these CDK inhibitors of INK4 family may drive B cells into a similar pathway as p18. The specificity by which CDK inhibitor family proteins degrade LMP1 remains to be investigated.

Clearly, expression of p18 initiated a proteolytic cascade, which is independent of the proteosome and which does not degrade p53. Are there other EBV transforming proteins such as EBNAs that are subject to degradation by the p18-mediated proteolysis? There are all subjects of future studies.

2. Is there a mutual regulation between p18 and LMP1?

Conversely, can LMP1 promote cell cycle progression via suppression of CDK inhibitors such as p18? It is likely to be the case, since it has been shown that overexpression of LMP1 activates cyclin D2 expression and hypophosphorylation of pRB (Arvanitakis et al., 1995). We have not yet succeeded in infection of B cells with LMP1 adenovirus. More experiments are needed to optimize the condition for infections before

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we can test this possibility.

3. Gene therapy of IgM+ EBV-associated B cell lymphomas.

Since IgM+ B cell lymphomas are more common than IgG+ B cell lymphomas, more experiments are needed to optimize the infection of IgM+ SKW cells and primary B cells. After that, the regulation of LMP1 by p18 in SKW cells will be performed. Thorough understanding of the functions of CDK inhibitors in IgM+ cells and optimizing of infection condition may suggest new arenas of gene therapy of B cell lymphomas in the future.

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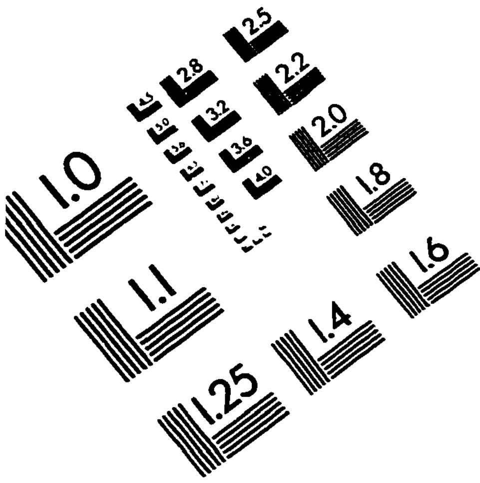
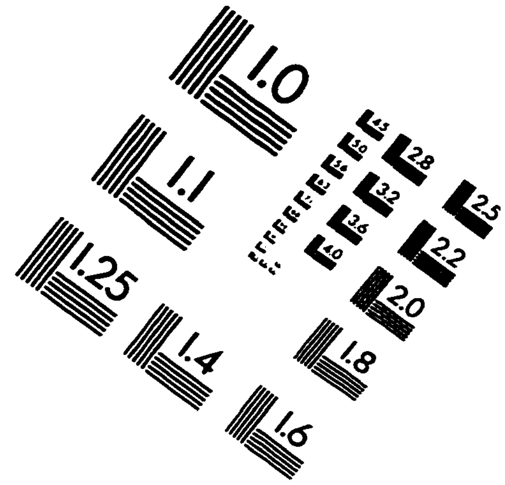
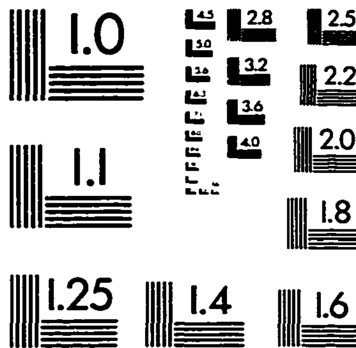
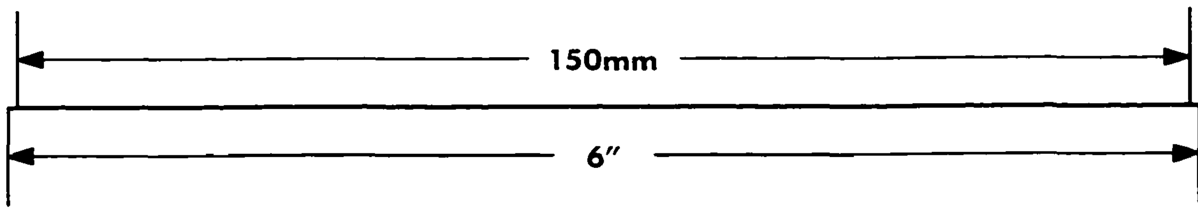
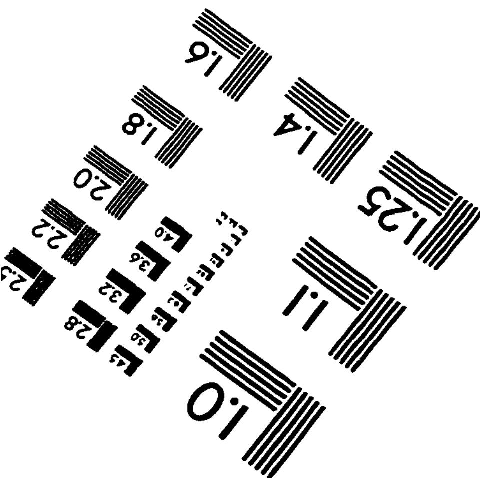
IMAGE EVALUATION TEST TARGET (QA-3)











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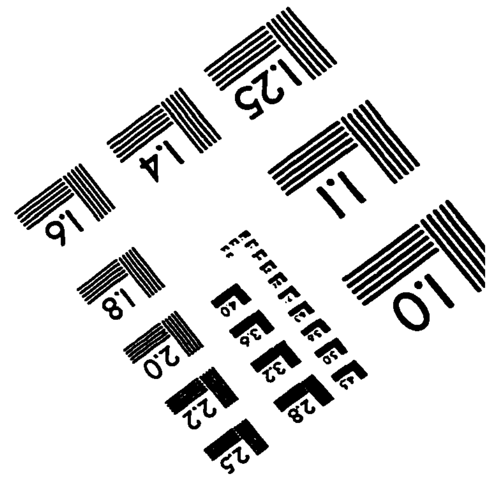

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