Regulation of Growth of B Lymphoma by CD40, CD54/ICAM-1, CD95 and CD95L

By

Qiaoke (Jack) Gong

Department of Experimental Medicine

McGill University

Montreal, Canada

March 2000

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment

for the degree of Master of Science

Abstract

The effect of CD40, CD54/ICAM-1 and CD95 (Fas/APO-1) ligation in murine B lymphomas was investigated. Crosslinking ligation of CD40 induced p53, the p53-regulated CDKI $p21^{Cip1/Waf1}$, and apoptosis in 3 lymphoma cell lines of a mature phenotype (A20, M12 and TA3), but not in 2 lymphomas of an immature phenotype (WEHI-279 and WEHI-231). Association of Mdm2 with p53 was reduced in A20. Expression of Bax and Bcl-2 was unaffected by CD40 ligation in all lines. Ligation of CD95 induced apoptosis in A20, not in M12, TA3 or WEHI-279. Crosslinking ICAM-1 on A20 had no effect on growth, but induced tyrosine phosphorylation of a 90-100KDa band in ICAM-1 immunoprecipitates, consistent with ICAM-1 itself. My results demonstrate that CD40 and CD95 differentially affect B cell lymphomas according to their developmental stage and show the potential for tumor suppression by signals that promote growth of non-transformed B cells.

Acknowledgments

I am especially grateful to my supervisor, Dr. Trevor Owens, for giving me the opportunity to do these experiments, for his guidance, advice, encouragement and understanding, and for giving me the opportunities to attend the scientific meetings and to present my work.

I would also like to thank to my colleagues , past and present, Elise Tran, Iris Doyle, Lyne Bourbonnière, Grace Chan, Micheal Gensen, Michelle Krakowski, Mina Hassan-Zahraee, Joanna Holland, Gail Verge, Maria Caruso, Annette Hollmann, for helping me in my work in this lab.

Most of all I am grateful to my parents, my brothers and my sister for their love, support and encouragement from remote China.

Thanks to all my friends for their support and encouragement.

Résumé

L'effet de liaison de CD40, CD54/ICAM-1 et CD95 sur les lymphomes à cellules B a été étudié. Le crosslinking de CD40 a induit l'expression de p53 et du CDKI $p21^{Cip1/Waf1}$, une molécule controlée par p53. De plus, ce crosslinking a induit l'apoptose dans trois lignées cellulaires de lymphomes B matures (A20, M12 et TA3), mais pas dans celles qui sont immatures (WEHI-279 et WEHI-231). L'association de mdm2 avec p53 a été réduite dans les cellules A20. L'expression de Bcl-2 et Bax n'a pas été affectée par la liaison de CD40 dans aucune lignée. La liaison de CD95 a induit l'apoptose dans la lignée cellulaire A20, mais pas dans les autres lignées cellulaires. Le crosslinking d'ICAM-1 sur les cellules A20 n'a pas affecté leur croissance, mais plutôt induit une bande de phosphotyrosine dans les immunoprécipités d'ICAM-1 au poids moléculaire de 90-100KDa, correspondant à celle de la molécule d'ICAM-1. Ces résultats démonstrent que le CD40 et le CD95 affectent différemment les lymphomes à cellules B selon leur stage de développement et suggèrent le potentiel pour supprimer les tumeurs par des signaux qui incitent la croissance des cellules B non-transformées.

Table of Contents

Contents.....Page

List of figures

Chapter 2

Chapter 3

Preface

According to the "Guideline for Thesis Preparation" of the Faculty of Graduate and Research at McGill University, a paper which has been submitted for publication has been incorporated into this thesis. Instead of connecting texts between sections, I would like to present an overview of the thesis format. Chapter 1 is a review of the literature pertinent to the results presented in chapters 2 and 3. Chapter 2 is a manuscript which has been submitted for publication. Some parts of the original paper were kept in the thesis, which were removed from the final version of the paper. It contain its own Summary, Introduction, Materials and Methods, Results, Discussion, References and Figures. Chapter 3 presents data of regulation of B lymphoma by CD54, CD95 and CD95L. It contains its own Introduction, Materials and Methods and Results. Chapter 4 presents a general discussion related to the data given in both chapter 2 and 3. References, listed alphabetically, are included at the end of each chapter and are not complied elsewhere in the thesis.

I am the first author of the included manuscript. Dr. Trevor Owens is my supervisor.

Chapter 1

Literature Review

Overview of B cell development and T cell-dependent B cell activation

The B lymphocyte life cycle includes development in the bone marrow (BM), maturation and differentiation in the periphery. B cell development in the bone marrow progresses through a sequence of defined stages (Burrow and Cooper, 1997). These stages may be differentiated by expression of a variety of cell surface markers and by the status of immunoglobulin gene rearrangements. Pro-B cells and pre-B cells reside in the bone marrow and depend partially on stimuli from the BM microenvironment for survival and developmental progression. Immature B cells expressing membrane IgM leave the bone marrow and migrate to the periphery where they traverse a transitional stage to become resting mature B cells characterized by expression of both IgM and IgD (Benschop and Cambier, 1999). The earliest marker that defines cells committed to the B cell lineage is B220, an isotype of CD45, a membrane tyrosine phosphatase. Other cell surface molecules defining early developmental stage of B cells and their progenitors include CD19 and CD43 (Benschop and Cambier, 1999). Expression of CD40 arises at the late pro-B cell stage (Duchosal 1997). CD40 has been implicated as a key regulator of the differentiation branch point between memory and plasma cells (Aspin et al., 1995; Lane et al., 1995; Callard et al., 1995). Continual exposure of activated germinal center B cells to CD40 ligand

maintains a B-cell-like morphology and favors memory cell generation, whereas CD40 ligand withdrawal initiates plasma cell differentiation.

B cell activation by antigen requires binding of the antigen by the B cell surface immunoglobulin receptor and interaction of the B cell with antigen-specific helper T cells. The B cell receptor binds and internalizes antigen, which is processed within the B cell. Peptides bound to MHC II are presented to T cells in the form of MHC-peptide complexes, which signal via the TCR to activate the T cells. As a result, T cells upregulate CD40 ligand (CD154) on their surface. Interaction between CD40-CD40L leads to upregulation of CD80 (B7.1) and CD86 (B7.2) on B cells. These costimulatory ligands induced on B cells then act to amplify the response of the T cells (Grewal and Flavell, 1996). Once activated, the T cells in turn promote B cell activation, both by releasing cytokines such as IL-2, 4, and 5 and by direct intercellular contact (Clark and Ledbetter, 1996). B cells consequently undergo isotype switching and proliferate and differentiate into plasma cells and memory B cells.

Intercellular Adhesion Molecule-1 (ICAM-1)

Intercellular adhesion molecule-1 (ICAM-1) is a member of the immunoglobulin family of homologous proteins. It is a cell adhesion molecule expressed by several cell types, including leukocyte and endothelial cells. The common form ICAM-1 protein consists of 505 amino acids, containing five extra cellular Ig-like domains (453 residues), a transmembrane spanning region (24 residues), and a cytoplasmic tail (28 residues) (Staunton et al., 1988). ICAM-1 participates in cellular interaction by binding to several ligands via different domains. It binds to two integrins belonging to the β2 subfamily, which are CD11a/CD18

(LFA-1) on T cells and CD11b/CD18 (MAC-1) on macrophages (Marlin et al., 1987; Diamond et al., 1990; Diamond et al., 1993). The basic function of ICAM-1 is the induction of specific and reversible cell-cell adhesion, resulting in intercellular communication. Interaction between ICAM-1 and its ligands plays a role in T-cell mediated host defense mechanism and in generating an inflammatory response (Springer, 1990), as well as in myeloid cell lineage differentiation (Lord et al., 1990). While the adhesive role is clearly established, increasing evidence suggests that ICAM-1 is a signaling molecule. Crosslinking ICAM-1 upregulated MHC II expression on resting B cells and co-crosslinking ICAM-1 and MHC II induced expression of a functional IL-2 receptor on B cells (Poudrier and Owens, 1994). Co-ligation of CD54 with IgM resulted in inhibition of IgM-mediated Ca^{++} mobilization in a B lymphoma line (van Horsen et al., 1995). In addition, it has been shown that CD-initiated signals which inhibit anti-IgM antibody-induced B cell apoptosis are transduced through LFA-1/ICAM-1 (Mayumi et al., 1995). Despite the evidence that CD54 transduces signals in several cell types, very little is known about mechanism. It was reported that tyrosine phosphorylation of several cellular proteins following ligation of ICAM-1 was observed in both peripheral blood human T cells and in a leukemic T cell line (Chirathaworn et al., 1995) and in a rat brain endothelial cell line (Durieu-Trautmant et al., 1994). Crosslinking of ICAM-1 on the B lymphoma cell line A20 induced an increase in tyrosine phosphorylation of several cellular proteins, resulting in activation of Raf-1 and mitogen-activated protein kinases (Holland and Owens, 1997). ICAM-1 crosslinking resulted in a reorganization of the endothelial actin cytoskeleton to form stress fibers and activation of the small GTP-binding

protein Rho (Etienne et al., 1998; Adamson et al., 1999), demonstrating the involvement of ICAM-1 in lymphocyte migration through brain endothelial cells. ICAM-1 crosslinking on endothelial cells caused activation of Erk-1 and AP-1 transcription factor complex which is implicated in ICAM-1-mediated VCAM-1 expression (Lawson et al., 1999). Incubation of B-lymphoid Raji cells with fibrinogen resulted in the increase tyrosine phosphorylation of the receptor-associated tyrosine kinase, pp60 (Src) and ERK-1 (Gardiner and D'Souza, 1999). ICAM-1 binding in rat astrocytes induced cAMP accumulation and activation of the mitogen-activated protein kinase extracellular signal-regulated kinase (Etienne-Manneville et al., 1999). These data suggest that ICAM-1 acts as a signal transducer, but the precise mechanism of how ICAM-1 delivers the signal still remains unclear.

CD40 and CD40 signaling

CD40, a 45-KDa to 50-KDa surface glycoprotein, is a member of TNFαR family of molecule that is expressed by B cells, myeloid cells, dendritic cells, follicular dendritic cells, endothelial cells, fibroblasts, epithelial cells, and some carcinomas (Nishioka and Lipsky, 1994; Van Kooten and Banchereau, 1996, Stamenkovic et al., 1989; Freudenthal and Steinman, 1990). The ligand for CD40 (CD40L or CD154) is a 33-KDa molecule expressed by activated T cells (Spriggs et al., 1992). Interaction between CD40 and CD40 ligand plays a critical role in a variety of B cell functions, including production of immunoglobulins and immunoglobulin class switching (Jabara et al., 1990), increased intercellular adhesion via LFA-1/ICAM-1 interaction (Barrett et al., 1991), proliferation (Paulie et al., 1989) and induction of B7-1 and B7-2 costimulatory molecules (Ranheim

and Kipps, 1993). Whereas CD40 signaling causes proliferation of B cells and rescues germinal center B cell from apoptosis it can actually promote programmed cell death in malignancies, including B lymphocytes and carcinomas (Funakoshi et al., 1994; Hess and Engelmann, 1996). Recombinant human CD40 ligand inhibited proliferation and induced apoptosis in human breast cancer cells (Hirano et al., 1999). CD40 ligation induced Fas expression on human B lymphocytes and facilitated Fas-mediated apoptosis (Schattner et al., 1995; Rothstein et al., 1995).

The intracellular signal transduction pathway utilized by CD40 to generate its diverse and potent effects on B cells are not yet clearly defined. There are no kinase domains or tyrosine residues in the cytoplasmic tail. However, ligation of CD40 has been shown to induce a number of intracellular signals, including phosphorylation of serine/threonine kinases in resting B cells from human tonsils (Uckun et al., 1991), activation of protein tyrosine kinases (PTK), which is essential to deliver its rescue signal (Knox and Gordon, 1993), activation of lyn, a src-family kinase, and the phosphorylation of phospholipase Cγ2 and PI3 kinase in Daudi Burkitt lymphoma line (Durie et al., 1994). Increased intracellular Ca^{++} levels have also been observed (Klaus et al., 1994). Engagement of CD40 has also been demonstrated to activate JAK and STAT in the A20 B lymphoma (Hanissian et al., 1997). In addition, CD40 signaling has been shown to induce intracellular cAMP (Knox et al., 1993; Kato et al., 1994), and to activate transcription factors NF-AT, NF-κB, and AP-1 (Choi et al., 1994; Berberich et al., 1994; Francis et al., 1995). CD40 ligation activated p38 MAP kinase and C-Jun N-terminal kinase in dendritic cells and tonsillar B cells (Aicher at al., 1999; Sakata

et al., 1999). Crosslinking CD40 stimulated the expression of A1, an anti-apoptotic protein of the Bcl-2 family, which rescues WEHI-231 B lymphoma cells from anti-IgM-induced apoptosis (Kuss et al., 1999). In addition, the transmembrane protein p23 has been shown to associate with CD40 (Morio et al., 1995). A recent study showed that CD40 directly interacts with Ku protein through the membrane-proximal region of cytoplasmic CD40 (Morio et al., 1999). Ku protein has been proved to be required for Ig class switching and rearrangement of Ig heavy and light chain (Casellas et al., 1998). Engagement of CD40 recruits to the CD40 cytoplasmic domain four members of the tumor necrosis receptor-associated factor (TRAF) family, TRAF2, TRAF3, TRAF5, and TRAF6 (Kehry 1994). Among these, TRAF2, TRAF5, and TRAF6 have been shown to mediate NF-κB production and TRAF3 was involved in CD23 induction by CD40 in RAMOS cells (Sato et al., 1995; Cheng et al., 1995; Mosiolos et al., 1995; Hu et al., 1994). TRAF3 was also implicated as a common mediator in the transduction of growth inhibitory signals generated via the CD40 and LMP1 pathway in human epithelial cells (Eliopoulos et al., 1996). Using cells isolated from TRAF2-deficient mice, Nguyen et al. (1999) demonstrated that TRAF2 is required for CD40-induced proliferation and NF-κB activation and that TRAF2 has a role in the T-dependent B cell response, specifically in Ig-isotype switching. CD40 signaling was impaired in splenocytes in the absence of TRAF2. TRAF2 was also implicated in the upregulation of intercellular adhesion molecule-1 (ICAM-1) induced by CD40 engagement (Lee et al., 1999). Gene-targeted disruption of TRAF6 completely abolished CD40-mediated NF-κB activation (Lomaga et al., 1999). These data suggest that TRAF2 and TRAF6 are both essential for CD40 signaling. It is clear

that CD40 signaling activates a host of signaling molecules and produces a variety of outcomes, but it is not yet clear which pathways lead to particular consequence, or how this segregation of function occurs.

Fas (CD95/APO-1) and Fas ligand (FasL, CD95L)

Fas (CD95/APO-1), a 48-KDa cell surface protein belonging to the tumor necrosis factor receptor family/nerve growth factor receptor family (Itoh et al., 1991; Oehm et al., 1992) is expressed in various tissues including thymus, heart, lung and liver (Watanabe-Fukunaga et al., 1992). Fas is characterized by the presence of a death domain (DD) within the cytoplasmic region and has been shown to trigger apoptosis upon ligation with anti-Fas antibodies (Trauth et al., 1989; McGahon et al., 1995) or Fas ligand (Suda et al., 1993). Ligation of CD95 results in aggregation of its intracellular death domain, leading to the recruitment of FADD (MORT-1) (Boldin et al., 1995; Chinnaiyan et al., 1995). FADD recruits caspase-8 through its death domain and activates caspase-8, inducing apoptosis (Boldin et al., 1996; Muzio et al., 1996). Such apoptosis occurs in many cell types of different hematopoietic origin, including T and B cells (Trauth et al., 1989) and a variety of hematopoietic and nonhematopoietic cell lines (McGahon et al., 1995).

Fas ligand (FasL, CD95L) is a cell surface molecule belonging to the tumor necrosis factor/nerve growth factor family (Suda et al., 1993). FasL is constitutively expressed in immune-privileged tissue such as eye, brain and testis, as well as on some tumors, and is inducibly expressed in T, B and NK cells during activation of the immune system (Suda 1995; Hahne et al., 1996; Arase et al., 1995). Genetic and immunological

analysis of Fas and FasL have indicated that Fas is involved in the clonal deletion of T cells in the periphery, down regulation of the immune response and cytotoxic T-lymphocyte-mediated cytotoxicity, by inducing T cell apoptosis (Ju et al., 1995; Dhein et al., 1995; Brunner et al., 1995). That FasL has been shown to be expressed by some non-lymphoid tissue cells raised the possibility that cancer cells might also be immune privileged, representing a novel mode of immune evasion (O'Connell et al., 1996; Hahne et al., 1996; Strand 1996; Saas 1997). Tumor cells can directly avoid T-cell recognition in at least two ways. Tumor cells can upregulate Fas ligand and induce T-cell apoptosis via Fas on the T cell. Tumor cells can also downregulate or genetically alter components of their antigen processing and presentation machinery including MHC I, the TAP transporter subunit, and subunits of the proteasome (Ferrone et al., 2000). The cytoplasmic enzyme complex can digest whole cellular proteins into the peptide fragments that bind to MHC class I antigens.

The absence of CD95 or its specific ligand is associated with lymphoproliferative disorders and autoimmunity, as is the case in lymphoproliferation *(lpr)* and generalized lymphoproliferative-disorder *(gld)* mutant mice. Autoreactive T and B cells are not eliminated in the periphery in these mice, and mice suffer from autoimmunity and lymphadenopathy (Watanabe-Fukunaga et al., 1992; Takahashi et al., 1994).

Overview of the biochemistry of Programmed Cell Death (PCD)

Programmed cell death or apoptosis is involved in the removal of superfluous and damaged cells in most organ systems (Kroemer et al

1995). It is an orderly process that proceeds through several morphological phases characterized by chromatin condensation, nuclear fragmentation, shrinkage of cells, and release of cytochrome c from mitochondria into cytoplasm (Kerr et al, 1972; Kluck et al., 1997; Yang et al; 1997). Apoptosis is distinct from necrosis, which is a disorderly mode of cell death (Duvall et al., 1986). The induction of PCD or apoptosis is characterized by a heterogeneity of potential PCD-triggering signal transduction pathways. The biochemical mechanisms involved in apoptosis have been extensively studied. Molecules that participate in the apoptotic process include a series of different proto-oncogenes and oncosuppressor genes as well as cell cycle regulators.

p53

The p53 protein was originally identified as a nuclear phosphoprotein bound to the large transforming antigen of the SV40 DNA virus (Linzer et al., 1979). p53 is a 393-amino acid protein with major domains characterized as 1) transcriptional activation, 2) proline-rich domain, 3) DNA-binding domain, 4) nuclear localization signal and 5) carboxy-terminal domain (Harris 1996). p53 participates in cellular responses to a number of pathological insults resulting in cell cycle arrest or apoptosis (Levine, 1997). These biological consequences are believed to represent the tumor suppressor function of p53 and are tightly linked to the ability of p53 to function as a DNA sequence-specific transcription factor. Activation of p53 as a transcription factor is correlated with cell proliferation (Ginsberg et al., 1991; Ragimov et al., 1993), cell differentiation (Weintraub et al., 1991) and DNA repair (Kastan et al., 1992). Deficiency of the p53 gene in mice significantly enhanced

proliferation in several types of cells (Tsukada et al., 1993) and such mice develop a high incidence of a variety of tumors. The most frequent tumors are lymphomas (Donehower et al., 1993).

The transcriptional activity of p53 appears to be regulated predominantly at the protein level. Two mechanisms can be envisaged to regulate p53 activity, one through p53-mdm2 association (see below), another through phosphorylation (Prives 1998). Phosphorylation of specific residues, such as serine 15, is known to be essential to the transcriptional activities of p53.

mdm2

The mouse double minute 2 (mdm2), a proto-oncogene, was originally discovered in a tumorigenic cell line-derived from non-transformed Balb/c cells. (Snyder et al., 1988). mdm2 was found to have a nuclear localization signal, an acidic domain characteristic of other transactivators and zinc finger motifs (Fakharzadeh et al., 1991). Overexpression of mdm2 protein has been detected in a number of human malignancies, indicating that this oncogene plays an important role in carcinogenesis. One mechanism by which mdm2 overexpression may lead to uncontrolled cellular proliferation is through its ability to complex with p53 and inhibit p53-mediated activity. Co-immunoprecipitation experiments showed that mdm2 physically binds to p53. Function for this mdm2/p53 interaction has been shown in experiments wherein overexpression of mdm2 could inhibit the ability of p53 to transactivate p53-responsive reporter genes (Momand et al., 1992; Oliner et al., 1992). It has also been shown that the mdm2 gene is transcriptionally regulated by p53, demonstrating that p53/mdm2 auto-

regulatory feedback exists in cells to maintain appropriate ratios of these proteins (Barak et al., 1993; Wu et al., 1993).

Cyclin-dependent kinase inhibitor (CDKI)

During recent years a number of proteins, known as cyclin-dependent kinase inhibitors, have been shown to play an important role in regulating cell cycle (Sherr and Robert., 1998). These proteins bind to and thereby inhibit the activity of cyclin/CDK complexes at appropriate time points in the cell cycle. Mammalian CDK-inhibitors described to date include $p21^{Cip1/Waf1}$, $p27^{Kip1}$, $P15^{Ink4B}$, p18, p19 and p57.

$p21^{Cip1/Waf1}$ and $p27^{Kip1}$ have been the mostly extensively studied CDKI. p21 acts downstream of numerous signaling pathways, and can be induced by p53, transforming growth factor β, and other antimitogenic stimuli (el-Deiry et al., 1993; Datto et al., 1995). In tissue culture systems, p21 is upregulated in proliferating cells and appears to play a role in governing progression through the cell cycle (Nakanishi et al., 1995; Missero et al., 1996). In addition, p21 is induced in some cell types during senescence and terminal differentiation, and it is thought to play a key role in downregulating CDK activity in these settings (Guo et al 1995). p21 is also involved in G1 arrest upon IgM cross-linking on Daudi cells (Marches et al., 1998). Furthermore, in vitro studies suggest that p21 inhibits stress-activated protein kinase (SAPK) activity and proliferating cell nuclear antigen (PCNA) expression (Shim et al.,1996; Waga et al., 1994; Luo et al., 1995). Finally, at low stoichimetric concentrations, p21 may serve as an assembly factor for active cyclin/CDK complexes (Zhang et al., 1994). p27 is constitutively expressed in many cells, and the level of this protein is elevated in Go phase and declines as cells enter the cell

cycle (Poon et al., 1995; Polyak et al., 1994). p27 has been implicated as a mediator of growth arrest induced by TGFß, cAMP, and other extracellular factors (Polyak et al., 1994; Kato et al., 1994). p27 has also been shown to be responsible for anti-IgM-mediated G1 arrest in the murine lymphoma cell line WEHI-231 (Ezhevsky et al., 1995).

Bcl-2 family proteins

Bcl-2 was discovered at the chromosomal translocation point t (14, 18) in human B lymphoma (Cleary and Sklar, 1985). The Bcl-2 family is now known to be divided into two groups of proteins, death antagonists (Bcl-2, Bcl-XL, Bcl-w) or death agonists (Bax, Bak, Bcl-Xs, Bad, Bid) (Atan et al., 1999). These proteins can function to inhibit or induce apoptosis in a variety of cell systems (Oltvai and Korsmeyer, 1994; Reed 1997). Bcl-2 family proteins have been proposed to regulate apoptosis through both homo- and heterodimerization (Oltvai et al., 1993; Yang et al., 1995). When Bax is preferentially expressed, Bax homodimers would be formed and apoptosis would be promoted. When Bcl-2 is preferentially expressed, Bcl-2 homodimers would be formed and apoptosis would be suppressed. These proteins may be regulated both transcriptionally and post-transcriptionally. A death stimulus induces the translocation of Bax to the mitochondria where it is integrated in the membrane and cross-linked as a homodimer. Bid is cleaved by caspase-8 upon activation of TNFα or Fas receptors (Li et al., 1998). Cleaved Bid then translocates to the mitochondria resulting in the release of cytochrome c. BAD was shown to be phosphorylated by Akt (Datta et al., 1997, Blume-Jensen et al., 1998). Bax can be transactivated and Bcl-2 is transrepressed by p53 (Miyashita et al., 1994).

Lymphomagenesis

Lymphomagenesis can be viewed as a multistep process involving various independent transforming events. Chromosomal translocations are the most frequently recognized alteration associated with B-cell lymphomagenesis. These genetic changes are responsible for the activation of a variety of genes contributing to B-cell transformation, including the proto-oncogenes c-myc, genes encoding for proteins involved in control of cell cycle, differentiation and apoptosis, such as cyclin D1, Bcl-2 and the newly identified PAX-5 (Amakawa et al., 1998; Gaidano et al., 1993; Cotter et al., 1989; Aisenberg 1995). In addition, inactivation of tumor suppressor genes, such as p53, pRb, and p16, has been implicated in lymphomagenesis (Gaidano et al., 1993 Cotter et al., 1989; Aisenberg 1995). Infectious agents may also play a role in the development of lymphoma. Epstein-Barr-virus can directly infect B lymphocytes and induce proliferation through the expression of virus-encoded transforming proteins (Klein 1994). Chronic antigenic stimulation including by EBV and hepatitis C virus is also a contributing factor. Increasing evidence indicates that various cytokines may promote the growth of B lymphoma cells through autocrine (IL-3, IL-10). or paracrine (IL-2, IL-4, IL-6) stimulation, thus constituting additional factors which may play a role at all stages of lymphomagenesis (Hsu et al., 1993).

B cell malignancies are considered to represent the maturation arrest of a cell at a specific time-point in normal B cell ontogeny. Therefore, B cell malignancies share phenotype (morphology and cell-surface molecules)

and some of their biology with their untransformed cellular counterparts (Silberstein and Rao, 1998).

Rationale of this study

Lymphomagenesis and autoimmunity result from dysregulation of lymphocyte proliferation. Signal transduction controls the differentiation, proliferation or apoptosis of cells. The apoptotic pathway is critical in the attenuation of immune responses and in the elimination of autoreactive cells. Understanding the mechanism of apoptosis induced by crosslinking CD40 in B lymphoma cells would allow us to better manipulate the growth of lymphocytes, to control tumor growth and to prevent autoimmune diseases.

References

Adamson, P, Etienne, S., Couraud, P. O., and Calder Vand Greenwood, J. (1999). Lymphocyte migration through brain endothelial cell monolayers involves signaling through endothelial ICAM-1 via a rho-dependent pathway. J. Immunol. *162*, 2964-2973.

Aicher, A., Shu, G. L., Magaletti, D., Mulvania, T., Pezzutto, A., Craxton, A., and Clark, E. A. (1999). Differential role for p38 mitogen-activated protein kinase in regulating CD40-induced gene expression in dendritic cells and B cells. J. Immunol. *163*, 5786-5795.

Aisenberg, A. C. (1995). Coherent view of non-Hodgkin's lymphoma. J. Clin. Oncol. *13*, 2656-2675.

Amakawa, R., Ohno, H., Fukuhara, S. (1999). t(9;14)(p13;q32) involving the PAX-5 gene: a unique subtype of 14q32 translocation in B cell non-Hodgkin's lymphoma. Int. J. Hematol. *69*, 65-69.

Arase, H., Arase, N., and Saito, T. (1995). Fas-mediated cytotoxicity by freshly isolated natural killer cells. J. Exp. Med. *181*, 1235-1238.

Arpin, C., Dechanet, J., Van Kooten, C., Merville, P., Grouard, G., Briere, F., Banchereau, J., and Liu, Y. J. (1995). Generation of memory B cells and plasma cells in vitro. Science *268*, 720-722.

Barak, Y., Juven, T., Haffner, R., and Oren, M. (1993). mdm2 expression is induced by wild type p53 activity. EMBO J. *12*, 461-468.

Barrett TB, Shu G., and Clark EA. (1991). CD40 signaling activates CD11a/CD18 (LFA-1)-mediated adhesion in B cells. J. Immunol. *146*, 1722-1729.

Benschop, R. J., and Cambier, J. C. (1999). B cell development: signal transduction by antigen receptors and their surrogates. Curr. Opin. Immunol. *1*, 143-151.

Berberich, I., Shu, G. L., Clark, and E. A. (1994). Cross-linking CD40 on B cells rapidly activates nuclear factor-kappa B. J. Immunol. *153*, 4357-4366.

Blume-Jensen, P., Janknecht, R., Hunter, T. (1998). The kit receptor promotes cell survival via activation of PI 3-kinase and subsequent Akt-mediated phosphorylation of Bad on Ser136. Curr. Biol. *8*, 779-782.

Boldin, M. P., Goncharov, T. M., Goltsev, Y. V., and Wallach, D. (1996). Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell *85*, 803-815.

Boldin, M. P., Varfolomeev, E. E., Pancer, Z., Mett, I. L., Camonis, J. H., and Wallach, D. (1995). A novel protein that interacts with the death domain of Fas/APO1 contains a sequence motif related to the death domain. J. Biol. Chem. *270*, 7795-7798.

Brunner T, Mogil RJ, LaFace D, Yoo NJ, Mahboubi A, Echeverri F, Martin SJ, Force WR, Lynch DH, Ware CF, et al. (1995). Cell-autonomous Fas (CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. Nature *373*, 441-444.

Burrows, P. D., and Cooper, M. D. (1997). B cell development and differentiation. Curr. Opin. Immunol. *9*, 239-244

Callard, R. E., Herbert, J., Smith, S. H., Armitage, R. J., and Costelloe, K. E. (1995). CD40 cross-linking inhibits specific antibody production by human B cells. Int. Immunol. *7*, 1809-1815

Casellas, R., Nussenzweig, A., Wuerffel, R., Pelanda, R., Reichlin, A., Suh, H., Qin, X. F., Besmer, E., Kenter, A., Rajewsky, K., and Nussenzweig, M. C. (1998). Ku80 is required for immunoglobulin isotype switching. EMBO J. *17*, 2404-2411.

Cheng, G., Cleary, A. M., Ye, Z. S., Hong, D. I., Lederman, S., and Baltimore, D. (1995). Involvement of CRAF1, a relative of TRAF, in CD40 signaling. Science *267*, 1494-1498.

Chinnaiyan, A. M., O'Rourke, K., Tewari, M., and Dixit, V. M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell *81*, 505-512.

Chirathaworn, C., Tibbetts, S. A., Chan, M. A., and Benedict, S. H. (1995). Cross-linking of ICAM-1 on T cells induces transient tyrosine phosphorylation and inactivation of cdc2 kinase. J. Immunol. *155*, 5479-5482.

Choi, M. S., Brines, R. D., Holman, M. J., and Klaus, G. G. (1994). Induction of NF-AT in normal B lymphocytes by anti-immunoglobulin or CD40 ligand in conjunction with IL-4. Immunity *1*, 179-187.

Clark, E. A., and Ledbetter, J. A. (1994). How B and T cells talk to each other. Nature *367*, 425-428.

Cleary, M. L, Sklar, J. (1985). Nucleotide sequence of a t(14;18) chromosomal breakpoint in follicular lymphoma and demonstration of a breakpoint-cluster region near a transcriptionally active locus on chromosome 18. Proc. Natl. Acad. Sci. U. S. A. *82*, 7439-7443.

Cotter, F. E. (1993). Molecular pathology of lymphomas. Cancer. Surv. *16*, 157-174.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., Greenberg, M. E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell *91*, 231-241.

Datto, M. B., Li, Y., Panus, J. F., Howe, D. J., Xiong, Y., and Wang, X. F. (1995). Transforming growth factor beta induces the cyclin-dependent

kinase inhibitor p21 through a p53-independent mechanism. Proc. Natl. Acad. Sci. U. S. A. *92*, 5545-5549.

Dhein, J., Walczak, H., Baumler, C., Debatin, K. M., and Krammer, P. H. (1995). Autocrine T-cell suicide mediated by APO-1. Nature *373*, 438-441.

Diamond, M. S., Garcia-Aguilar, J, Bickford, J. K, Corbi, A. L., and Springer, T. 1993. The I domain is a major recognition site on the leukocyte integrin Mac-1 (CD11b/CD18) for four distinct adhesion ligands. J. Cell. Biol. *120*, 1031-1043.

Diamond, M. S., Staunton, D. E., de Fougerolles, A. R., Stacker, S. A., Garcia-Aguilar, J, Hibbs, M. L., and Springer, T. A. 1990. ICAM-1 (CD54): a counter-receptor for Mac-1 (CD11b/CD18). J Cell Biol *111*, 3129-3139.

Donehower, L. A., and Bradley, A. (1993). The tumor suppressor p53. Biochim. Biophys. Acta. 1155, 181-205.

Duchosal, M. A. (1997). B-cell development and differentiation. Semin. Hematol. *34* (1 Suppl 1), 2-12.

Durie, F. H., Foy, T. M., Masters, S. R., Laman, J. D., and Noelle, R. (1994). The role of CD40 in the regulation of humoral and cell-mediated immunity. Immunol. Today *15*, 406-411.

Duvall, E., Wyllie, A. H., Morris, R. G. (1985). Macrophage recognition of cells undergoing programmed cell death (apoptosis). Immunology *56*, 351-358.

el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817-825.

Eliopoulos, A. G., Dawson, C. W., Mosialos, G., Floettmann, J. E., Rowe, M., Armitage, R. J., Dawson, J., Zapata, J. M., Kerr, D. J., Wakelam, M. J., Reed, J. C., Kieff, E., and Young, L. S. (1996). CD40 induced growth inhibition in epithelial cells is mimicked by Epstein-Barr Virus-encoded LMP1: involvement of TRAF3 as a common mediator. Oncogene *13*, 2243-2254.

Etienne, S., Adamson, P., Greenwood, J., Strosberg, A. D., Cazaubon, S., and Couraud, P. O. (1998). ICAM-1 signaling pathways associated with Rho activation in microvascular brain endothelial cells. J. Immunol. *161*, 5755-5761.

Etienne-Manneville, S., Chaverot, N., Strosberg, A. D., Couraud, P. O. (1999). ICAM-1-coupled signaling pathways in astrocytes converge to cyclic AMP response element-binding protein phosphorylation and TNF-alpha secretion. J. Immunol.*163*, 668-674.

Ezhevsky, S. A., Toyoshima, H., Hunter, T., and Scott, D. W. (1996). Role of cyclin A and p27 in anti-IgM induced G1 growth arrest of murine B-cell lymphomas. Mol. Biol. Cell *7*, 553-564.

Fakharzadeh, S. S., Trusko, S. P., and George, D. L. (1991). Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. EMBO J. *10*, 1565-1569.

Ferrone, S., Finerty, J. F., Jaffee, E. M., and Nabel, G. J. (2000). How much longer will tumour cells fool the immune system? Immunol. Today *21*, 70-72.

Francis, D. A., Karras, J. G., Ke, X. Y., Sen, R., and Rothstein TL. (1995). Induction of the transcription factors NF-kappa B, AP-1 and NF-AT during B cell stimulation through the CD40 receptor. Int. Immunol. *7*, 151-161.

Freudenthal, P. S., and Steinman, R. M. (1990). The distinct surface of human blood dendritic cells, as observed after an improved isolation method. Proc. Natl. Acad. Sci. U S A *87*, 7698-702.

Funakoshi, S., Longo, D. L., Beckwith, M., Conley, D. K, Tsarfaty, G., Tsarfaty, I., Armitage, R. J., Fanslow, W. C., Spriggs, M. K., and Murphy, W. J. (1994). Inhibition of human B-cell lymphoma growth by CD40 stimulation. Blood *83*, 2787-2794.

Gaidano, G., Dalla-Favera, R. (1993). Biologic and molecular characterization of non-Hodgkin's lymphoma. Curr. Opin. Oncol. *5*, 776-784.

Gardiner, E. E., and D'Souza, S. E. (1999). Sequences within fibrinogen and intercellular adhesion molecule-1 (ICAM-1) modulate signals required for mitogenesis. J. Biol. Chem. *274*, 11930-11936.

Ginsberg, D., Mechta, F., Yaniv, M., and Oren, M. (1991). Wild-type p53 can down-modulate the activity of various promoters. Proc. Natl. Acad . Sci . U . S. A. *88*, 9979-9983.

Grewal, I. S., and Flavell, R. A. (1996). A central role of CD40 ligand in the regulation of CD4+ T-cell responses. Immunol. Today *17*, 410-414.

Guo, K., Wang, J., Andres, V., Smith, R. C., and Walsh, K. (1995). MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. Mol. Cell. Biol. *15*, 3823-3829.

Hahne, M., Rimoldi, D., Schroter, M., Romero, P., Schreier, M., French, L. E., Schneider, P., Bornand, T., Fontana, A., Lienard, D., Cerottini, J., and Tschopp, J. (1996). Melanoma cell expression of Fas(Apo-1/CD95) ligand: implications for tumor immune escape. Science *274*, 1363-1366.

Hanissian, S. H., Geha, R. S. (1997). Jak3 is associated with CD40 and is critical for CD40 induction of gene expression in B cells. Immunity *6*, 379-387.

Harris, C. (1996). Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies. J. Natl. Cancer Inst. *88*, 1442-1455.

Hess, S., and Englemann, H. (1996). A novel function of CD40: induction of cell death in transformed cells. J.Exp.Med. *183*, 159-167.

Hirano, A., Longo, D. L., Taub, D. D., Ferris, D. K., Young, L. S., Eliopoulos, A. G., Agathanggelou, A., Cullen, N., Macartney, J., Fanslow, W. C., and Murphy, W. J. 1999. Inhibition of human breast carcinoma growth by a soluble recombinant human CD40 ligand. Blood *93*, 2999-3007.

Holland, J., and Owens, T. (1997). Signaling through intercellular adhesion molecule 1 (ICAM-1) in a B cell lymphoma line. The activation of Lyn tyrosine kinase and the mitogen-activated protein kinase pathway. J. Biol. Chem. *272*, 9108-9112.

Hsu, S. M., Xie, S.S., Waldron, J. A. Jr. (1992). Functional heterogeneity and pathogenic significance of interleukin-6 in B-cell lymphomas. Am. J. Pathol. *141*, 915-23.

Hu, H. M., O'Rourke, K., Boguski, M. S., and Dixit, V. M. 1994. A novel RING finger protein interacts with the cytoplasmic domain of CD40. J. Biol. Chem. *269*, 30069-30072.

Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y., and Nagata, S. (1991). The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell *66*, 233-243.

Jabara, H. H., Fu, S. M., Geha, R. S., and Vercelli, D. (1990). CD40 and IgE: synergism between anti-CD40 monoclonal antibody and interleukin 4 in the induction of IgE synthesis by highly purified human B cells. J. Exp. Med. *172*, 1861-1864.

Ju, S. T., Panka, D. J., Cui, H., Ettinger, R., el-Khatib, M., Sherr, D. H., Stanger, B. Z., Marshak-Rothstein,.A. (1995). Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature *373*, 444-448.

Kato, J. Y., Matsuoka, M., Polyak, K., Massague, J., and Sherr, C. J. (1994). Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. Cell *79*, 487-496.

Kato, T., Kokuho, T., Tamura, T., and Nariuchi, H. (1994). Mechanisms of T cell contact-dependent B cell activation. J. Immunol. *152*, 2130-2138.

Kehry, M. R. (1996). CD40-mediated signaling in B cells. Balancing cellsurvival, growth, and death. J. Immunol. *156*, 2345-2348.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br. J. Cancer *26*, 239-257.

Klein, G. (1994). Epstein-Barr virus strategy in normal and neoplastic B cells. Cell *77*, 791-793.

Kluck, R. M., Bossy-Wetzel, E., Green, D. R., and Newmeyer, D. D. (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. Science *275*, 1132-1136.

Knox, K. A., Johnson, G. D., and Gordon, J. 1993. Distribution of cAMP in secondary follicles and its expression in B cell apoptosis and CD40-mediated survival. Int. Immunol. *5*, :1085-1091.

Knox, K.A., and Gordon, J. (1993). Protein tyrosine phosphorylation is mandatory for CD40-mediated rescue of germinal center B cells from apoptosis. Eur. J. Immunol. *23*, 2578-2584.

Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J. L., and Mignotte, B. (1995). The biochemistry of programmed cell death. FASEB J. *9*, 1277-1287.

Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., and Kastan, M. B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. U. S. A. *89*, 7491-5

Kuss, A. W., Knodel, M., Berberich-Siebelt, F., Lindemann, D., Schimpl, A., and Berberich, I. (1999). A1 expression is stimulated by CD40 in B cells and rescues WEHI 231 cells from anti-IgM-induced cell death. Eur. J. Immunol. *29*, 3077-3088.

Lawson, C., Ainsworth, M., Yacoub, M., and Rose, M. (1999). Ligation of ICAM-1 on endothelial cells leads to expression of VCAM-1 via a nuclear factor-kappaB-independent mechanism. J Immunol. *162*, 2990-2996.

Lee, H. H., Dempsey, P. W., Parks, T. P., Zhu, X., Baltimore, D., and Cheng, G. (1999). Specificities of CD40 signaling: involvement of TRAF2 in CD40-induced NF-kappaB activation and intercellular adhesion molecule-1 up-regulation. Proc. Natl. Acad. Sci. U. S. A. *96*, 1421-426.

Levine AJ. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-31

Li, H., Zhu, H., Xu, C. J., Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell *94*, 491-501.

Linzer, D. I., and Levine, A. J. (1979). Characterization of a 54K dalton cellular SV40 tumor antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. Cell *17*, 43-52.

Lomaga, M. A., Yeh, W. C. Sarosi, I., Duncan, G. S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., van der Heiden, A., Itie,

A., Wakeham, A., Khoo, W., Sasaki, T. Cao, Z., Penninger, J. M., Paige, C. J., Lacey, D. L., Dunstan, C. R., Boyle, W. J., Goeddel, D. V., and Mak, T. W. (1999). TRAF6 deficiency results in osteopetrosis and defective interleukin-1, CD40, and LPS signaling. Genes Dev. *13*, 1015-1024.

Lord, K. A., Abdollahi, A., Hoffman-Liebermann, B., and Liebermann, D. A. (1990). Dissection of the immediate early response of myeloid leukemia cells to terminal differentiation and growth inhibitory stimuli. Cell Growth Differ. *1*, 637-645.

Luo, Y., Hurwitz, J., and Massague, J. 1995. Cell-cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. Nature *375*, 159-161.

Marches, R., Scheuermann, R. H., and Uhr, J. W. (1998). Cancer dormancy: role of cyclin-dependent kinase inhibitors in induction of cell cycle arrest mediated via membrane IgM. Cancer Res. *58*, 691-697.

Marlin, S. D., and Springer, T. A. (1987). Purified intercellular adhesion molecule-1 (ICAM-1) is a ligand for lymphocyte function-associated antigen 1 (LFA-1). Cell *51*, 813-819.

Mayumi, M., Sumimoto, S., Ohshima, Y., Katamura, K., Heike, T., Hata, D., Kanazashi, S., and Furusho, K. (1995). Role of LFA-1/ICAM-1-dependent cell adhesion in CD40-mediated inhibition of anti-IgM antibody-induced B-cell death. J. Allergy Clin. Immunol. *96*, 1136-1144.

McGahon, A. J., Martin, S. J., Bissonnette, R. P., Mahboubi, A., Shi, Y., Mogil, R. J., Nishioka, W. K., and Green, D. R. (1995). The end of the (cell) line: methods for the study of apoptosis in vitro. Methods Cell. Biol. *46*, 153-185.

Missero, C., Di Cunto, F., Kiyokawa, H., Koff, A., and Dotto, G. P. (1996). The absence of p21Cip1/WAF1 alters keratinocyte growth and differentiation and promotes ras-tumor progression. Genes Dev. *10*, 3065-3075.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A,, Hoffman, B., Reed, J. C. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene *9*, 1799-1805.

Momand, J., Zambetti, G. P., Olson, D. C., George, D., Levine, A. J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell *69*, 1237-1245.

Morio, T., Hanissian, S. H., Bacharier, L. B., Teraoka, H., Nonoyama, S., Seki, M., Kondo, J., Nakano, H., Lee, S. K., Geha, R. S., and Yata, J. (1999). Ku in the cytoplasm associates with CD40 in human B cells and translocates into the nucleus following incubationwith IL-4 and anti-CD40 mAb. Immunity *11*; 339-348.

Morio, T., Hanissian, S., and Geha, R. S. (1995). Characterization of a 23-kDa protein associated with CD40. Proc. Natl. Acad. Sci. U. S. A. *92*, 11633-11636.

Mosialos, G., Birkenbach, M., Yalamanchili, R., VanArsdale, T., Ware, C., and Kieff, E. (1995). The Epstein-Barr virus transforming protein LMP1 engages signaling proteins for the tumor necrosis factor receptor family. Cell *80*, 389-399.

Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996). FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. Cell *85*, 817-827.

Nakanishi, M., Robetorye, R. S., Adami, G. R., Pereira-Smith, O. M., and Smith, J. R. (1995). Identification of the active region of the DNA synthesis inhibitory gene p21Sdi1/CIP1/WAF1. EMBO J. *14*, 555-563.

Nguyen, L. T., Duncan, G. S., Mirtsos, C., Ng, M., Speiser, D. E., Shahinian, A., Marino, M. W., Mak, T. W,, Ohashi, P. S., and Yeh, W. C. (1999). TRAF2 deficiency results in hyperactivity of certain TNFR1 signals and impairment of CD40-mediated responses. Immunity *11*, 379-389.

Nishioka, Y., and Lipsky, P. E. (1994). The role of CD40-CD40 ligand interaction in human T cell-B cell collaboration.J. Immunol. *153*, 1027-1036.

O'Connell, J., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. (1996). The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J. Exp. Med. *184*, 1075-1082.

Oehm, A., Behrmann, I., Falk, W., Pawlita, M., Maier, G., Klas, C., Li-Weber, M., Richards, S., Dhein, J., Trauth, B. C., et al. (1992). Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily. Sequence identity with the Fas antigen. J. Biol. Chem. *267*, 10709-10715.

Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. (1992). Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature *358*, 80-83.

Oltvai, Z. N., Korsmeyer, S. J. (1994). Checkpoints of dueling dimers foil death wishes. Cell *79*, 189-92.

Paulie, S., Rosen, A., Ehlin-Henriksson, B., Braesch-Andersen, S., Jakobson, E., Koho, H., and Perlmann, P. 1989. The human B lymphocyte and carcinoma antigen, CDw40, is a phosphoprotein involved in growth signal transduction. J. Immunol. *142*, 590-595.

Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massague, J. (1994). Cloning of p27Kip1, a cyclin-

dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell *78*, 59-66.

Poon, R. Y., Toyoshima, H., and Hunter, T. (1995). Redistribution of the CDK inhibitor p27 between different cyclin.CDK complexes in the mouse fibroblast cell cycle and in cells arrested with lovastatin or ultraviolet irradiation. Mol. Biol. Cell *6*, 1197-213.

Poudrier, J., and Owens, T. 1994. CD54/intercellular adhesion molecule 1 and major histocompatibility complex II signaling induces B cells to express interleukin 2 receptors and complements help provided through CD40 ligation. J. Exp. Med. *179*, 1417-1427.

Prives C. (1998). Signaling to p53: breaking the MDM2-p53 circuit. Cell *95*, 5-8.

Ragimov, N., Krauskopf, A., Navot, N., Rotter, V., Oren, M., and Aloni, Y. 1993. Wild-type but not mutant p53 can repress transcription initiation in vitro by interfering with the binding of basal transcription factors to the TATA motif. Oncogene *8*, 1183-1193.

Ranheim, E. A., and Kipps, T.J. (1993). Activated T cells induce expression of B7/BB1 on normal or leukemic B cells through a CD40-dependent signal. J. Exp. Med. *177*, 925-935.

Rothstein, T. L., Wang, J. K. M., Panka, D. L., Foote, L C. Wang, Z., Stanger, B., Cui, H., Ju, S., and Marshak-Rothstein, A. (1995). Protection

against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells. Nature *374*, 163-165.

Saas, P., Walker, P. R., Hahne, M., Quiquerez, A. L., Schnuriger, V., Perrin, G., French, L., Van Meir, E. G., de Tribolet, N., Tschopp, J., and Dietrich, P. Y. (1997). Fas ligand expression by astrocytoma in vivo: maintaining immune privilege in the brain? J. Clin. Invest. *99*, 1173-1178.

Sakata, N., Patel, H. R., Terada, N., Aruffo, A., Johnson, G. L., Gelfand, E. W. (1995). Selective activation of c-Jun kinase mitogen-activated protein kinase by CD40 on human B cells. J. Biol. Chem. *270*, 30823-30828.

Sato, T., Irie, S., and Reed, J. C. (1995). A novel member of the TRAF family of putative signal transducing proteins binds to the cytosolic domain of CD40. FEBS Lett *358*, 113-118.

Schattner, E. J., Keith, B. E.,Tumang, D. J., Krammer, P. H., Crow, M. K., and Friedman, S. M. (1995). CD40 ligation induces Apo-1/Fas on human B lymphocytes and facilitates apoptosis through the Apo-1/Fas pathway. J. Exp. Med. *182*, 1557-1565.

Sherr, C. J., and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. *13*, 1501-1512.

Shim, J., Lee, H., Park, J., Kim, H., and Choi, E. (1996). A non-enzymatic p21 protein inhibitor of stress-activated protein kinases. Nature *381*, 804-806.

Silberstein, L. E, Rao, S. P. (1998). VH-mediated mechanisms in normal and neoplastic B cell development. Immunol. Res. *17*, 253-259.

Snyder, L. C., Trusko, S. P., Freeman, N., Eshleman, J. R., Fakharzadeh, S. S., and George, D. L., (1988). A gene amplified in a transformed mouse cell line undergoes complex transcriptional processing and encodes a nuclear protein. J. Biol. Chem. *263*, 17150-17158.

Spriggs, M. K., Armitage, R. J., Strockbine, L., Clifford, K. N., Macduff, B. M., Sato, T. A., Maliszewski, C. R., and Fanslow, W. C. (1992). Recombinant human CD40 ligand stimulates B cell proliferation and immunoglobulin E secretion. J. Exp. Med. *176*, 1543-1550.

Springer, T. A. 1990. Leucocyte adhesion to cells. Scand. J. Immunol. *32*, 211-216.

Stamenkovic, I., Clark, E. A., and Seed, B. (1989). A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. EMBO J. *8*, 1403-10

Staunton, D. E., Marlin, S. D., Stratowa, C., Dustin, M. L., and Springer, T. A. (1988). Primary structure of ICAM-1 demonstrates interaction between

members of the immunoglobulin and integrin supergene families. Cell *52*, 925-933.

Strand, S., Hofmann, W. J., Hug, H., Muller, M., Otto, G., Strand, D., Mariani, S. M., Stremmel, W., Krammer, P. H., and Galle, P. R. (1996). Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells--a mechanism of immune evasion? Nat. Med. *2*, 1361-1366.

Suda, T., Okazaki, T., Naito, Y., Yokota, T., Arai, N., Ozaki, S., Nakao, K., and Nagata, S. (1995). Expression of the Fas ligand in cells of T cell lineage. J. Immunol. *154*, 3806-3813.

Suda, T., Takahashi, T., Golstein, P., and Nagata, S. (1993). Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell *75*, 1169-1178.

Takahashi, T., Tanaka, M., Brannan, C. I., Jenkins, N. A., Copeland, N. G., Suda, T., and Nagata, S. (1994). Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell *76*, 969-976.

Trauth, B. C., Klas, C., Peters, A. M, Matzku, S., Moller, P., Falk, W., Debatin, K. M., and Krammer, P. H. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science *245*, 301-305.

Tsukada, T., Tomooka, Y., Takai, S., Ueda, Y., Nishikawa, S., Yagi, T., Tokunaga, T., Takeda, N., Suda, Y., Abe, S., et al. (1993). Enhanced proliferative potential in culture of cells from p53-deficient mice. Oncogene *8*, 3313-3322.

Uckun, F. M., Schieven, G. L., Dibirdik, I., Chandan-Langlie, M., Tuel-Ahlgren, L., and Ledbetter, J.A. (1991). Stimulation of protein tyrosine phosphorylation, phosphoinositide turnover, and multiple previously unidentified serine/threonine-specific protein kinases by the Pan-B-cell receptor CD40/Bp50 at discrete developmental stages of human B-cell ontogeny. J. Biol. Chem. *266*, 17478-17485.

Van Kooten, C., and Banchereau, J. 1996. CD40-CD40 ligand: a multifunctional receptor-ligand pair. Adv. Immunol. *61*, 1-77.

Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature *369*, 574-578.

Watanabe-Fukunaga, R., Brannan, C. I., Itoh, N., Yonehara, S., Copeland, N. G., Jenkins, N. A., and Nagata, S. (1992). The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J. Immunol. *148*, 1274-1279.

Weintraub, H., Hauschka, S., and Tapscott, S. J. (1991). The MCK enhancer contains a p53 responsive element. Proc. Natl. Acad. Sci. U. S. A. *88*, 4570-4571.

Wu, X., Bayle, J. H., Olson, D., and Levine, A. (1993). The p53-mdm-2 autoregulatory feedback loop. Genes Dev. *7*, 1126-1132.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science *275*, 1129-1132.

Yang. E., Zha, J., Jockel, J., Boise, L. H., Thompson, C. B., Korsmeyer, S. J. (1995). Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell *80*, 285-291.

Zhang, H., Hannon, G. J., and Beach, D. (1994). p21-containing cyclin kinases exist in both active and inactive states.Genes Dev. *8*, 1750-1758.

Chapter 2

Crosslinking CD40 induces apoptosis of mature phenotype B lymphoma cells through upregulation of p53 and $p21^{Cip1/Waf1}$

Qiaoke Gong and Trevor Owens

Neuroimmunology Unit, Montreal Neurological Institute, McGill University, Montreal, QC, H3A 2B4, Canada.

Address correspondence to Dr. Trevor Owens, Montreal Neurological Institute, 3801 University Street, Montreal, QC, Canada H3A 2B4. E-mail: Trevor@med.mcgill.ca. FAX#: 514-398-7371.

Abbreviations used in this paper: CKI, cyclin dependent kinase inhibitor; PCNA, Proliferating cellular nuclear antigen; TRAF, tumor necrosis factor receptor-associated factors; LMP 1, latent membrane protein 1.

Summary

CD40 is implicated in the survival, growth and differentiation of B lymphocytes. There is also evidence for a role for CD40 in growth inhibition of malignancies. We investigated the effect of CD40 on biochemical regulators of cell cycle and its progression, in a panel of murine B lymphoma cell lines. Our results show that crosslinking ligation of CD40 induced apoptosis in 3 B lymphoma cell lines of a mature phenotype (A20, M12 and TA3), but not in 2 lymphomas of an immature phenotype (WEHI-279 and WEHI-231). Crosslinking CD40 induced expression of the tumor suppressor p53 and the p53-regulated CKI $p21^{Cip1/Waf1}$ in all 3 mature B cell lymphomas, and induced the CDKI $p27^{Kip1}$ in A20. Neither p53 nor CKI's were affected by CD40 treatment of immature lymphomas. Co-immunoprecipitation showed that mdm2 association with p53 was reduced in anti-CD40-treated A20. The expression of the apoptosis-regulatory proteins Bcl-2 and Bax correlated with B cell phenotype and susceptibility to CD40-induced apoptosis. Bax was expressed at high levels in mature lymphomas, whereas Bcl-2 was expressed at high levels in immature cell lines. Neither Bax not Bcl-2 levels were affected by CD40 ligation, in any of the lines. These results suggest that differential effects of anti-CD40 treatment correlate with the developmental stage at which B lymphocytes undergo oncogenic transformation, and that CD40 signaling for growth inhibition acts through p53 to target CKI's and induce apoptosis.

Introduction

CD40 is a member of the tumor necrosis factor receptor family that is expressed on B cells, follicular dendritic cells, macrophages, epithelial cells and some carcinomas (Kehry 1996; Noelle et al., 1992; Grewal et al., 1998). CD40 is one of the key molecules involved in the survival, growth and differentiation of B lymphocytes. While crosslinking CD40 induces B cell proliferation, and prevents apoptosis of germinal center B cells, an increasing literature describes a role for CD40 in inhibition of growth of malignancies. CD40 ligation induces apoptotic cell death in transformed cells of mesenchymal and epithelial origin as well as in many B lymphoma cell lines (Heath et al., 1993; Hess and Engelmann 1996; Tutt et al; 1998; French et al., 1999). However, the intracellular signal transduction pathways utilized by CD40 to generate its potent effect on B cells are not yet clearly established.

p53, a tumor suppressor, has been shown to play important roles in control of cell cycle, DNA repair, differentiation, tumor formation and apoptosis. Accumulation of p53 is often associated with apoptosis of cells (Vogelstein and Kinzler 1992; Harris and Hollstein 1993). Expression of wild-type p53 in some p53-deficient tumor cell lines resulted in spontaneous cell death (Yonish-Rouach et al., 1991; Sheikh et al., 1995). Thymocytes and hematopoeitic cells from mice deficient in the p53 gene showed resistance to radiation and drug induced apoptosis (Clarke et al., 1993; Clarke et al., 1994). Also, p53 has been shown to be involved in receptor-mediated apoptosis of WEHI-231 B lymphoma cells (Wu et al., 1997). Activity of p53 is controlled by either altering the level of p53

mRNA or via post-translational modifications and alteration in p53-binding proteins. Mdm2, an oncogenic protein, represses p53 transcriptional activity by interaction with p53 and targets p53 for rapid proteosomal degradation (Prives 1998; Haupt et al., 1997., Kubbutat et al., 1997).

A growing number of cyclin-dependent kinase inhibitors (CKI) have been shown to play a major role in controlling cell cycle progression. The first mammalian CKI to be identified was $p21^{Cip1/Waf1}$, which exists in a quaternary complex with cyclins, cdk, and proliferating cellular nuclear antigen (PCNA). Through this association $p21^{Cip1/Waf1}$ converts active cyclin-dependent kinases to inactive forms, thus controlling and coordinating the cell cycle, especially G_1 to S progression, as well as inhibiting DNA replication (Xiong et al., 1992; Waga et al., 1994; Flores-Rozas et al., 1994). The $p21^{Cip1/Waf1}$ protein has been recognized as a down stream mediator of the tumor suppressor p53 in regulating cell cycle progression through the G_1-S checkpoint. The increase in intracellular $p21^{Cip1/Waf1}$ levels elicited by p53, itself induced by cellular damage caused by irradiation or other toxic agents, leads to cyclin-dependent kinase inhibition and cell cycle arrest. Furthermore, $p21^{Cip1/Waf1}$ has been implicated in apoptosis. Ectopic $p21^{Cip1/Waf1}$ expression induces cell death in MCF-7 breast carcinoma cells (Sheikh et al., 1995) and WEHI-231 B lymphoma cells (Wu et al., 1997). Stimulation of splenic B cells with an anti-IgM that induces apoptosis also induced $p21^{Cip1/Waf1}$expression (Solvason et al., 1996). $p21^{Cip1/Waf1}$ was also associated with anti-IgM-induced G1 growth arrest of murine B cell lymphomas (Marches et al., 1998). $P27^{kip1}$ is another CKI that shares

partial identity with $p21^{Cip1/Waf1}$, and so belongs to the $p21^{Cip1/Waf1}$ family. However, it differs from $p21^{Cip1/Waf1}$ in that it does not bind to PCNA (Kuo et al., 1995). $p27^{Kip1}$ has been shown to accumulate following serum starvation as well as during cell-cell contact inhibition (Polyak et al et al., 1994). The activity of $p27^{Kip1}$ is induced by various antimitotic agents (Polyak et al., 1994; Nourse et al., 1994) and decreased following mitogen induced re-entry of quiescent cells into S phase (Coats et al., 1996). Induction appears to be under translational regulation (Hengst et al., 1996). Accumulation of $p27^{Kip1}$ inactivates G1 cyclin-cdk complexes and causes cell cycle arrest, but $p27^{Kip1}$-induced growth arrest need not depend on p53 (Polyak et al., 1994).

We were interested in the role of the p53 and $p21^{Cip1/Waf1}$ pathway in CD40 signaling in B cells. We investigated the effect of CD40 on B lymphoma cell lines A20, M12, TA3, WEHI-279 and WEHI-231 through analysis of cell cycle and related biochemical signal transduction. Our data show a role for p53, $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ in CD40-induced apoptosis of mature phenotype B lymphomas.

Materials and Methods

Cell lines

The B cell line A20 was derived from a BALB/cAnN lymphoma (IgG^+) (ATCC; Kim et al., 1997; Laskov et al., 1981) , M12 were derived from a BALB/c lymphoma (IgM^+, IgD^+) and TA3 is a hybridoma produced by fusion of LPS-stimulated B cells with M12 (Glimcher et al., 1983), WEHI-279 from NZC and WEHI-231 from BALBxNZC lymphomas (ATCC).

Lymphoma lines were generously provided by Dr T. Watts, University of Toronto. Cells were maintained in RPMI 1640 medium (Life Technologies, Montreal, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) 2 mM L-glutamine (Life Technologies, Montreal, Canada) $5x10^{-5}$M 2-mercaptoethanol (Sigma, Montreal, Canada), penicillin (100 units/ml) (Life Technologies), and streptomycin (100 mg/ml) (Life Technologies).

Antibodies and Reagents

The mAb FGK-45 (rat IgG2a anti-murine CD40) (Rolink et al., 1996) was generously provided by Dr P. Hugo, IRCM, Montreal. This and the monoclonal antibodies MAR 18.5 (mouse IgG2a anti-rat k) and 53-7.3 (rat IgG2a anti-murine CD5) (ATCC) were affinity-purified from culture supernatants using Protein G-Sepharose (Pharmacia Biotech Inc., Montreal, Quebec, Canada). Monoclonal anti-$p27^{kip1}$ (F-8), monoclonal anti-p53 (Pab 240) and monoclonal anti-mdm2 (SMP14) were purchased from Santa-Cruz Biotechnologies CA. Monoclonal anti-$p21^{cip1/waf1}$ (SX118), monoclonal anti-Bcl-2 (3F11) and monoclonal anti-Bax (6A7) were purchased from PharMingen, Canada.

Assessment of cell viability

Lymphoma cells were cultured at densities of $1X10^5$ cell/well (A20, M12 and TA3) and of $2X10^5$ cells/well (WEHI-279 and WEHI-231) in the presence or absence of anti-CD40 (FGK-45) and mouse anti-rat IgG. The cells were collected after 24, 48 and 72h and viable cells (excluding trypan blue dye) were counted by examination under light microscopy.

Cell cycle analysis

Approximately $1x10^6$ cells were washed in cold D-PBS and permeabilized with 70% Ethanol for 5 minutes. Cells were then washed with cold D-PBS and incubated at 37^0C in apoptosis buffer (30 mg/ml propidium iodide (Sigma) 50 mg/ml RNAse A (Boehringer Mannheim/Roche Molecular Biochemicals, Laval, Canada) 25% FCS in D-PBS). Propidium iodide (PI) staining was measured using a FACScan (Becton-Dickinson, Oakville, Ontario). Data were analyzed by Cell Quest Software (Becton-Dickinson).

Annexin V-FITC staining

Cells were washed twice with cold PBS and resuspended in 1x binding buffer (PharMingen Canada, Mississauga, Ontario) at a concentration of $1x10^6$ cells/ml. Cells were then incubated for 15 minutes at room temperature in the dark with 5 ml of Annexin V-FITC (PharMingen Canada, Mississauga, Ontario) and 10 µl of PI. Staining was assessed using a FACScan and analyzed using Cell Quest Software.

Preparation of B cell lysates

B cells were incubated in culture medium with mAbs for various amount of time at 37^0C. Cells were collected and washed three times with Dulbecco's PBS and lysed by incubation in lysis buffer (10mM Tris-HCL, pH7.5, 37 mM NaCl, 1% Nonidet P-40 (ICN Biomedicals, Missisauga, Canada) 1 mM EDTA (Anachemia, Canada) 1 mM NaVO4 (ICN Biomedicals, Mississauga, Canada). 2 mM phenylmethylsulfonyl fluoride (Sigma) and 50 mM NaF (K & K Laboratories, Cleveland, Ohio) for 15 min on ice. Cell debris and nulclei were removed by centrifugation (10

min at 900xg). The protein concentration of each lysate was quantified using a bicinchonimic acid assay (Pierce, Aurora, Ontario).

PAGE/Western blot

Lysates were boiled for 5 min with an equal volume of 2X sample buffer containing 5% 2-mercaptoethanol (Sigma) before loading. Equal amounts of protein were loaded in each lane and separated on 12% SDS polyacrymide gels. Following separation by electrophoresis, proteins were then transferred to PVDF membranes (Millipore Corporation, Bedford, MA). at 100 V for 1 hour. Membranes were blocked for 1 h at room temperature or overnight at 4^0C in 5% non-fat milk in TTBS (20mM Tris-HCL, 137mM NaCL, 1M HCL, 1% Tween-20 (Bio-Rad, CA)). Membranes were incubated with anti-$p21^{Cip1/Waf1}$ (1 μg/ml), anti-$p27^{Kip1}$ (1 μg/ml), anti-p53 (1 μg/ml) or anti-mdm2 (1 μg/ml) in TTBS for 1 hour or overnight and detected using the ECL system with horseradish peroxidase-conjugated secondary antibodies (Amersham Corp., Oakville, Ontario).

Immunoprecipitation

Lysates were incubated with 1-2 μg of anti-p53 for 1 h at 4^0C. 35 ul of washed protein G-sepharose beads (Pharmacia, Biotech, Sweden) were added and incubated at 4^0C for 1 hour to overnight. The immune complexes were collected by centrifugation and washed three times with lysis buffer. Proteins in immune complexes were resolved by SDS-PAGE on a 10% gel and transferred onto PVDF membranes. The membranes were probed with anti-mdm2 and binding was detected using the ECL system.

Results

Crosslinking CD40 induces apoptosis in mature but not in immature B lymphoma cells.

We initiated investigation of the effect of CD40 ligation on B lymphoma cells by cell cycle analysis of A20, TA3, M12, WEHI-279 and WEHI-231. Among these 5 lymphoma cells, A20, TA3 and M12 are derived from mature B cells of BALB/c mice, whereas WEHI-279 and WEHI-231 derive from immature B cells of BALBxNZc mice. The latter two lines represent early B cells characterized by high-density membrane IgM, lack of surface IgG and no inducible immunoglobulin secretion (ATCC). All 5 cell lines expressed equivalent levels of CD40 (not shown).

Cells cultured in the presence or absence of anti-CD40 for 24, 48 and 72h were examined by light microscopy. A dye exclusion cell viability assay showed prominent effect of anti-CD40 on growth of A20, M12 and TA3, but not of WEHI-279 (Fig. 2.1A) or WEHI-231 (not shown). Microscopic examination of anti-CD40-treated A20 for 24 hours revealed morphological changes consistent with cell death after CD40 ligation (Fig. 2.1B). To assess cell cycle status of B lymphoma cells after treatment with anti-CD40, cells were stained with propidium iodide and DNA content was analyzed by FACS. The cell cycle profiles show that by 24h, the proportion of apoptotic cells (<2N DNA) increased in anti-CD40-treated A20, TA3 and M12, compared with untreated cells (Fig 2.2A). This effect was most prominent in A20 and M12 (Fig. 2.2B). In contrast, there was no effect of anti-CD40 on WEHI-279 and WEHI-231. That cell death

induced by CD40 was mediated by apoptosis was further confirmed by annexin V staining (Fig. 2.3).

In order to exclude the possibility of an effect of Fc receptor ligation on B lymphoma cells, we used a rat IgG2a anti-mouse CD5 as an isotype-matched control. Cross-linking this mAb on A20 cells did not induce apoptosis (Fig. 2.2A, Fig. 2.3).

CD40-induced apoptosis is accompanied by p53 protein upregulation.

As an initial assessment of the contribution of p53 to CD40-mediated apoptosis of B lymphoma cells, the expression of p53 protein after anti-CD40 treatment was analyzed. Whole cell lysates were obtained from cells treated with anti-CD40 for 24h and were subjected to immunoblotting using mouse monoclonal anti-p53 antibody. As seen in Fig. 2.4A, A20, M12 and TA3 expressed lower levels of endogenous p53 relative to WEHI-279. p53 was upregulated in A20, M12 and TA3 by stimulation with anti-CD40. In WEHI-279, expression of p53 was unaffected by anti-CD40. p53 was not detectable in WEHI-231, before or after CD40 ligation. Thus, elevation of p53 levels correlated with the induction by CD40 of apoptotic cell death.

Reduced association between p53 and mdm2 in anti-CD40 treated A20 cells.

To address whether p53 was functional after anti-CD40 treatment, we investigated its association with mdm2. We immunoprecipitated the same lysates as above with anti-p53 and after Western transfer, we immunoblotted the membrane with anti-mdm2 (Fig. 2.4B). The level of

mdm2 associated with p53 was reduced within 6h of anti-CD40 treatment. These data suggest that CD40-induced apoptosis involved a functional p53 effect.

Ligation of CD40 in mature B lymphoma cells upregulates CDK inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$.

To assess whether and how p53 expression exerted effect, we measured CKI induction. The expression of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ proteins was analyzed after anti-CD40 treatment. Total cellular proteins were obtained from cells treated with anti-CD40 for the same times as above and were subjected to immunoblotting using mouse monoclonal anti-$p21^{Cip1/Waf1}$ and anti-$p27^{Kip1}$ antibodies. As seen in Fig. 2.5A, $p21^{Cip1/Waf1}$ was upregulated upon stimulation of A20, M12 and TA3 with anti-CD40 mAb. Densitometry showed increases of 3 to 4 folds $p21^{Cip1/Waf1}$ in A20 and M12 cells upon treatment with anti-CD40 antibody. In WEHI-279 and WEHI-231 expression of $p21^{Cip1/Waf1}$ was unaffected. The elevation of $p21^{Cip1/Waf1}$ after CD40 treatment therefore correlated with both apoptosis and activation of p53.

$p27^{Kip1}$ was up-regulated in A20 by stimulation with anti-CD40, but not in M12, WEHI-279 or WEHI-231 (Fig. 2.5B).

Ligation of CD40 does not affect levels of Bcl-2 and Bax.

It has been shown that Bcl-2, an anti-apoptotic protein was trans-repressed and Bax, a pro-apoptotic protein was transactivated by p53 (Selvakumaran et al., 1994; Miyashita and Reed 1995; Miyashita et al., 1994). In order to determine whether Bcl-2 and Bax act as down stream effectors of p53 in CD40 signaling, we assessed the expression of Bcl-2

and Bax proteins by immunoblotting analysis. The mature B cell lines A20, M12 and TA3 expressed high levels of endogenous Bax, while WEHI-279 and WEHI-231 expressed high levels of Bcl-2 (Fig.2.6). However, ligation of CD40 did not affect protein expression of either Bcl-2 and Bax. These data suggest that CD40-induced inhibition of B lymphoma cells acts through increase in p53 expression and activation followed by transactivation of $p21^{Cip1/Waf1}$, without affecting Bcl-2 or Bax levels.

Discussion

The major conclusion from these studies is that anti-CD40-mediated apoptosis of B lymphomas is correlated with the expression of the tumor suppressor p53 and the cyclin-dependent kinase inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$. This apoptosis-triggering pathway is specifically induced in lymphomas with a mature B cell phenotype, thus establishing a mechanistic basis for CD40-mediated tumor growth arrest and inter-lymphoma variability.

Signaling through CD40 can rescue germinal center B cells from undergoing apoptosis and evokes pleiotropic effects on B cell activation and survival (Kehry 1996; Noelle et al., 1992; Grewal et al., 1998). However, increasing evidence shows growth inhibitory effects of CD40 signaling. Anti-CD40 inhibited growth of murine B lymphoma cells (Heath et al., 1993; Funakoshi et al., 1994; Henriquez et al., 1999). CD40 ligation induced apoptosis in cell lines of mesenchymal and epithelial origin (Hess and Engelmann 1996). Recombinant human CD40 ligand

inhibited proliferation and induced apoptosis in human breast cancer cells (Hirano et al., 1999). Anti-CD40 antibody eradicated lymphoma in vivo by a mechanism that involved signaling rather than effector mobilisation (Tutt et al., 1998; French et al., 1999). CD40 ligation induces Fas expression on human B lymphocytes and facilitates Fas-mediated apoptosis (Schattner et al., 1995; Wang et al., 1997; Garrone et al., 1995; Rothstein et al 1995). In our study, anti-CD40-mediated apoptosis was effective in 3 mature B lymphoma cell lines, but failed to inhibit growth of immature B lymphoma cells, although both mature and immature B lymphoma cells expressed CD40. Our finding is consistent with a recent study (French et al., 1999) in which A20 lymphoma was sensitive to CD40-mediated killing, while WEHI-231 was resistant. This identifies a new level of complexity of CD40 signaling, demonstrating that the final consequence of CD40 signaling depends on the stage of B cell differentiation or activation state.

While the inhibitory effect of CD40 on B lymphoma growth is clear, the mechanism of CD40-mediated apoptosis remains elusive. CD40, Fas and TNFRI all belong to the TNF receptor family of proteins, but CD40 is distinct from Fas and TNFRI in that it does not contain a cytoplasmic "death domain", and is not considered to be able to induce apoptosis directly. CD40/MHC II association is induced by activation of human B lymphomas (Leveille et al., 1999), and it is possible that this interaction contributes to signaling for apoptosis induction of mature lymphomas (Newell et al., 1993). Alternatively, the cytoplasmic domain of CD40 can recruit TRAF proteins that act as signaling intermediates. TRAF2, TRAF5 and TRAF6 appear to act as inducers of positive signals for cell growth

and proliferation, mediated by kinase cascades and induction of NF-κB and AP-1 (Kehry 1996; Rothe et al., 1995; Arch et al., 1998). In contrast, TRAF3 can have an inhibitory effect on NF-κB activation and has been shown to be involved in the induction of cell death by the lymphotoxin-B-receptor (VanArsdale et al., 1997). Furthermore, TRAF3 has also been implicated as a common mediator in the transduction of growth inhibitory signals generated via the CD40 and LMP1 pathway in human epithelial cells (Eliopoulos et al., 1996).

We have established a correlation between CD40-mediated growth arrest and p53 in B lymphomas. Other studies support a role of p53 in control of B cell fate. Mutations of the p53 gene occur in 41% of Burkitt's lymphoma, and 70% of non-Hodgkin's lymphoma. Mutation is associated with advanced stage of lymphoid disease and poor prognosis (Newcomb 1995; Ichikawa et al., 1993). In addition, p53 abnormalities were reported in 75% of B cell prolymphocytic leukemia (Lens et al., 1997). p53 is actively involved as an apoptosis inducer at an early control checkpoint in B lymphopoiesis (Lu et al., 1999). Analysis of B cell tumors from p53-null mice showed significantly decreased levels of immunoglobulin heavy and light chain deposition compared with tumors from wild-type mice (Shick et al., 1997). Differential involvement of p53 in mature versus immature B cells has been suggested by a number of studies. Treatment of the immature B cell population from p53-null mice with anti-IgM resulted in hyperproliferation rather than the apoptotic response seen with immature B cells from normal mice (Shick et al., 1997; Norvell et al., 1995). Furthermore, the p53-null mice contained more immature B cells in the bone marrow than wild-type mice and these immature B cells were

resistant to receptor-mediated killing (Norvell et al., 1995). Moreover, p53 is required for induction of apoptosis in primary Abelson virus-transformed pre-B cells (Unnikrishnan et al., 1999). Ectopic expression of p53 mediated apoptosis in WEHI-231 B lymphoma (Wu et al., 1997), whereas ectopic expression of a dominant-negative p53 mutant blocked LPS-mediated differentiation (Aloni-Grinstein et al 1993). Our results showing that anti-CD40 preferentially induced apoptosis in mature B lymphomas, through up-regulation of p53, are consistent with and extend these observations. It is likely that the role which p53 plays in the control of B cell differentiation is recapitulated in lymphomagenesis.

The CKI's $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ are indicated by our study to be crucial CD40 signaling intermediates. The induction of $p21^{Cip1/Waf1}$ closely paralleled the effect of inhibition of A20, M12 and TA3 by CD40 signaling, whereas the CKI $p27^{Kip1}$ was up-regulated by anti-CD40 treatment only in A20, not in M12 and TA3. This suggests that involvement of $p21^{Cip1/Waf1}$ mediates the CD40 effect, and that the involvement of $p27^{Kip1}$ varies between transformed B cell lines. Ectopic expression of $p21^{Cip1/Waf1}$ or increased expression of $p27^{Kip1}$ induced apoptosis in WEHI-231 lymphoma cells (Wu et al., 1997; Wu et al., 1999), a cell line which in our study failed to induce $p21^{Cip1/Waf1}$ and $p27^{Kip1}$, or apoptose in response to anti-CD40. The discrepancy in $p27^{Kip1}$ involvement between these cell lines may reflect that $p27^{Kip1}$ is not a downstream effector of p53. Transfection of expression vectors encoding $p27^{Kip1}$ into human Saos-2 osteosarcoma cells induced G1 arrest, although Saos-2 cells do not express functional p53 (Polyak et al., 1994). If $p27^{Kip1}$ were not an essential downstream component of p53 signaling,

then a p53-dependent mechanism would not always involve $p27^{Kip1}$. Alternatively, loss of growth-regulatory mechanisms may vary from tumor to tumor, and these discrepancies may also reflect the different origin of these cell lines. Our observations complement other findings. $p27^{Kip1}$ was implicated in anti-IgM-mediated G1 arrest and induction of apoptosis in WEHI-231 (Wu et al., 1999; , Ezhevsky et al., 1996), whereas $p21^{Cip1/Waf1}$ was responsible for anti-IgM-mediated G1 arrest in Daudi cells, which are derived from an EBV^+ Burkitt's lymphoma (Marches et al., 1998). Our data are also consistent with the finding that $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ are cell cycle regulators that contribute to mature B cell growth following antigen receptor and anti-CD40 cross-linking (Solvason et al., 1996).

The simultaneous induction of multiple inhibitors may constitute a stronger response to a growth-inhibitory signal. A single antiproliferative signal can elicit a growth arrest response by inducing the activities of multiple CDK inhibitors (Reynisdottir et al., 1995; Matsuoka et al., 1998). $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ both bind to and inhibit a variety of cyclin-CDK complexes, including cyclin A-CDK2, cyclin E-CDK2 and cyclin D-CDK2 (Hunter and Pines 1994). TGF-ß induces G1 cell cycle arrest through the coordinate action of $p21^{Cip1/Waf1}$, $p27^{Kip1}$ and $p15^{Ink2}$ (Reynisdottir et al., 1995). IFN-a mediates G1 phase arrest through up-regulation of $p19^{Ink4D}$ and $p21^{Cip1/Waf1}$ in mouse macrophages (Matsuoka et al., 1998). The prominent effect of CD40-induced apoptosis in A20 in our study might be attributable to such cooperative action of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$.

It is noteworthy that our data showed high expression of Bax in mature cell lines which are sensitive to CD40-induced apoptosis and high expression of Bcl-2 in immature cell lines which are resistant to CD40-mediated killing. Recent findings showed that Bcl-2 inhibits apoptosis triggered by p53 (Chiou et al; 1994; Ryan et al 1994; Zhan et al., 1999). In addition, overexpression of Bcl-2 can block p53-regulated induction of $p21^{Cip1/Waf1}$. The coincidence of high expression of Bcl-2 and lack of response of p53 or $p21^{Cip1/Waf1}$ to CD40 ligation in immature B lymphoma cells supports a potential repressive role of Bcl-2. Our results parallel observations in testicular and bladder cancer cells. Testicular tumor cells that expressed relatively high level of Bax, and had both functional p53 and p53-responsive $p21^{Cip1/Waf1}$ were more sensitive to etoposide-induced apoptosis than bladder tumor cells which expressed Bcl-2 (Chresta et al., 1996). The ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2 represents a major cell fate check point (Gross et al., 1999). Bcl-2 family proteins have been proposed to regulate apoptosis through both homo- and heterodimerization (Oltvai et al., 1993). Coimmunoprecipitation experiments showed that Bcl-2 bound to Bax in WEHI-279 and WEHI-231, and this was not obvious in mature lines (Fig. 2.7)

Taken together, our findings suggest that CD40-induced inhibition of B lymphoma cells represents the integration of several signaling pathways. Elucidation of the mechanisms whereby B lymphoma cells are sensitive to CD40-mediated killing may suggest novel strategies for treatment of B-cell malignancies.

References

Aloni-Grinstein, R., Zan-Bar, I., Alboum, I. Goldfinger, N., and Rotter, V. (1993). Wild type p53 functions as a control protein in the differentiation pathway of the B-cell lineage. Oncogene *8*, 3297-3305.

Arch, R. H., and Thompson, C. B. (1998). 4-1BB and Ox40 are members of a tumor necrosis factor (TNF)-nerve growth factor receptor subfamily that bind TNF receptor-associated factors and activate nuclear factor kappaB. Mol. Cell. Biol. *18*, 558-565.

Chiou, S. K., Rao, L., and White, E. (1994). Bcl-2 blocks p53-dependent apoptosis. Mol. Cell. Biol. *14*, 2556-2563.

Chresta, C. M., Masters, J. R., and Hickman, J. A. (1996). Hypersensitivity of human testicular tumors to etoposide-induced apoptosis is associated with functional p53 and a high Bax:Bcl-2 ratio. Cancer Res. *56*, 1834-1841.

Clarke, A. R., Gledhill, S., Hooper, M. L,, Bird, C. C., and Wyllie, A. H. (1994). p53 dependence of early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma-irradiation. Oncogene *9*, 1767-1773.

Clarke, A. R., Purdie, C. A., Harrison, D. J., Morris, R. G., Bird, C. C., Hooper, M. L., and Wyllie, A. H. (1993). Thymocyte apoptosis induced by p53-dependent and independentpathways. Nature *362*, 849-852.

Coats S, Flanagan WM, Nourse J, and Roberts JM. 1996. Requirement of $p27^{Kip1}$ for restriction point control of the fibroblast cell cycle. Science *272*, 877-380.

Eliopoulos, A. G., Dawson, C. W., Mosialos, G., Floettmann, J. E., Rowe, M., Armitage, R. J., Dawson, J., Zapata, J. M., Kerr D. J., Wakelam, M. J., Reed, J. C,, Kieff, E., and Young, L. S. (1996). CD40-induced growth inhibition in epithelial cells is mimicked by Epstein-Barr Virus-encoded LMP1: involvement of TRAF3 as a common mediator. Oncogene *13*, 2243-2254.

Ezhevsky, S. A., Toyoshima, H., Hunter, T., and Scott, D. W. (1996). Role of cyclin A and p27 in anti-IgM induced G1 growth arrest of murine B-cell lymphomas. Mol Biol Cell *7*, 553-564.

Flores-Rozas, H., Kelman, Z., Dean, F. B., Pan, Z. Q., Harper, J. W., Elledge, S. J., O'Donnell, M., and Hurwitz, J. (1994). Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. Proc. Natl. Acad. Sci. U. S. A. *91*, 8655-8659.

French, R. R., Chan, H. T., Tutt, A. L., and Glennie, M. J. (1999). CD40 antibody evokes a cytotoxic T-cell response that eradicates lymphoma and bypasses T-cell help. Nat. Med. *5*, 548-553.

Funakoshi, S., Longo, D. L., Beckwith, M., Conley, D. K., Tsarfaty, G., Tsarfaty, I., Armitage, R. J., Fanslow, W. C., Spriggs, M. K., and Murphy, W. (1994). Inhibition of human B-cell lymphoma growth by CD40 stimulation. Blood *83*, 2787-2794.

Garrone, P., Neidhardt, E. M., Garcia, E., Galibert, L., van Kooten, C., and Banchereau, J. (1995). Fas ligation induces apoptosis of CD40-activated human B lymphocytes. J. Exp. Med. *182*, 1265-1273.

Glimcher, L. H., Hamano, T., Asofsky, R., Sachs, D. H., Pierres, M., Samelson, L. E., Sharrow, S. O., Paul, W. E. (1983). IA mutant functional antigen-presenting cell lines. J. Immunol .*130*, 2287-2294.

Grewal, I. S., and Flavell, R. A. (1998). CD40 and CD154 in cell-mediated immunity. Annu. Rev. Immunol. *16*, 111-135.

Gross, A., McDonnell, J. M,, and Korsmeyer, S. J. (1999). BCL-2 family members and the mitochondria in apoptosis. Genes Dev. *13*, 1899-1911.

Harris, C. C., and Hollstein, M. (1993). Clinical implications of the p53 tumor-suppressor gene. N. Engl. J. Med. *329*, 1318-1327.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature *387*, 296-299.

Heath, A. W., Chang, R., Harada, N., Santos-Argumedo, L., Gordon, J., Hannum, C., Campbell, D., Shanafelt, A. B., Clark, E. A., Torres, R., and

Howard, M. (1993). Antibodies to murine CD40 stimulate normal B lymphocytes but inhibit proliferation of B lymphoma cells. Cell. Immunol. *152*, 468-480.

Hengst, L., and Reed, S. I. (1996). Translational control of $p27^{Kip1}$ accumulation during the cell cycle. Science *271*, 1861-1864.

Henriquez, N. V., Floettmann, E., Salmon, M., Rowe, M., and Rickinson, A. B. (1999). Differential responses to CD40 ligation among Burkitt lymphoma lines that are uniformly responsive to Epstein-Barr virus latent membrane protein 1. J. Immunol. *162*, 3298-3307.

Hess, S., and Engelmann, H. (1996). A novel function of CD40: induction of cell death in transformed cells. J. Exp. Med. *183*, 159-167.

Hirano, A., Longo, D. L,, Taub, D. D., Ferris, D. K., Young, L. S., Eliopoulos, A. G., Agathanggelou, A., Cullen, N., Macartney, J., Fanslow, W. C., and Murphy, W. J. (1999). Inhibition of human breast carcinoma growth by a soluble recombinant human CD40 ligand. Blood *93*, 2999-3007.

Hunter, T., and Pines, J. (1994). Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. Cell *79*, 573-582.

Ichikawa, A., Hotta ,T., and Saito, H. (1993). Mutations of the p53 gene in B-cell lymphoma. Leuk . Lymphoma *11*, 21-25 .

Kehry, M. R. (1996). CD40-mediated signaling in B cells. Balancing cell survival, growth, and death. J. Immunol. *156*, 2345-2348.

Kim, K. J., Kanellopoulos-Langevin, C., Merwin, R. M., Sachs, D. H., and Asofsky, R. (1979). Establishment and characterization of BALB/c lymphoma lines with B cell properties. J. Immunol. *122*, 549-554.

Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Laskov, R., Kim, J. K., Woods, V. L., McKeever, P. E., and Asofsky, R. (1981). Membrane immunoglobulins of spontaneous B lymphomas of aged BALB/c mice. Eur. J. Immunol. *11*, 462-428.

Lens, D., De Schouwer, P. J,, Hamoudi, R. A., Abdul-Rauf, M., Farahat, N., Matutes, E., Crook, T., Dyer, M. J., and Catovsky, D. (1997). p53 abnormalities in B-cell prolymphocytic leukemia. Blood *89*, 2015-2023.

Leveille, C., Zekki, H., Al-Daccak, R., and Mourad, W. (1999). CD40- and HLA-DR-mediated cell death pathways share a lot of similarities but differ in their use of ADP-ribosyltransferase activities. Int. Immunol. *11*, 719-730.

Lu, L., Lejtenyi, D., and Osmond, D. G. (1999). Regulation of cell survival during B lymphopoiesis: suppressed apoptosis of pro-B cells in P53-deficient mouse bone marrow. Eur. J. Immunol. *29*, 2484-2490.

Luo, Y., Hurwitz, J., and Massague, J. (1995). Cell-cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. Nature *375*, 159-161.

Marches, R., Scheuermann, R. H., and Uhr, J. W. (1998). Cancer dormancy: role of cyclin-dependent kinase inhibitors in induction of cell cycle arrest mediated via membrane IgM. Cancer Res. 58, 691-697.

Matsuoka, M., Tani, K., and Asano, S. (1998). Interferon-alpha-induced G1 phase arrest through up-regulated expression of CDK inhibitors, $p19^{Ink4D}$ and $p21^{Cip1}$ in mouse macrophages. Oncogene *16*, 2075-2086.

Miyashita, T., and Reed, J. C. (1995). Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell *80*, 293-299.

Miyashita, T., Krajewski, S., Krajewska, M., Wang, H. G., Lin, H. K., Liebermann, D. A., Hoffman, B., and Reed, J. C. (1994). Tumor suppressor p53 is a regulator of bcl-2 and bax gene expression in vitro and in vivo. Oncogene *9*, 1799-1805.

Newcomb, E. (1995). P53 gene mutations in lymphoid diseases and their possible relevance to drug resistance. Leuk. Lymphoma. *17*, 211-221.

Newell, M. K., VanderWall, J., Beard, K. S., and Freed, J. H. (1993). Ligation of major histocompatibility complex class II molecules mediates apoptotic cell death in resting B lymphocytes. Proc. Natl. Acad. Sci. U. S. A. *90*, 10459-10463.

Noelle, R. J., Ledbetter, J. A., and Aruffo, A. (1992). CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B-cell activation. Immunol Today *13*, 431-413.

Norvell, A., Mandik, L., and Monroe, J. G. (1995). Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. J. Immunol.154, 4404-4413.

Nourse, J., Firpo, E., Flanagan, W. M., Coats, S., Polyak, K., Lee, M. H., Massague, J., Crabtree, G. R., and Roberts, J. M. (1994). Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. Nature *372*, 570-573.

Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 74, 609-619.

Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M , Tempst, P., and Massague, J. (1994). Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell *78*, 59-66.

Prives C. 1998. Signaling to p53: breaking the MDM2-p53 circuit. Cell *95*, 5-8.

Reynisdottir, I., Polyak, K., Iavarone, A., and Massague, J. (1995). Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes Dev. *9*, 1831-1845.

Rolink, A., Melchers, F., and Andersson, J. (1996). The SCID but not the RAG-2 gene product is required for S mu-S epsilon heavy chain class switching. Immunity *5*, 319-330.

Rothe, M., Sarma, V., Dixit, V. M., and Goeddel, D. V. (1995). TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. Science *269*, 1424-1427.

Rothstein, T. L., Wang, J. K., Panka, D. J., Foote, L. C., Wang, Z., Stanger, B., Cui, H., Ju, S. T., and Marshak-Rothstein, A. (1995). Protection against Fas-dependent Th1-mediated apoptosis by antigen receptor engagement in B cells. Nature *374*, 163-165.

Ryan, J. J., Prochownik, E., Gottlieb, C. A., Apel, I. J., Merino, R., Nunez, G., and Clarke, M. F. (1994). c-myc and bcl-2 modulate p53 function by altering p53 subcellular trafficking during the cell cycle. Proc. Natl. Acad. Sci. U. S. A. *91*, 5878-5882.

Schattner, E. J., Elkon, K. B., Yoo, D. H,, Tumang, J., Krammer, P. H., Crow, M. K., and Friedman, S. M. (1995). CD40 ligation induces Apo-1/Fas expression on human B lymphocytes and facilitates apoptosis through the Apo-1/Fas pathway. J. Exp. Med. *182*, 1557-1565.

Selvakumaran, M., Lin, H. K., Miyashita, T., Wang, H. G., Krajewski, S., Reed, J. C., Hoffman, B., and Liebermann, D. (1994). Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways. Immediate early up-regulation of bax expression by p53 but not TGF beta 1: a paradigm for distinct apoptotic pathways. Oncogene *9*, 1791-1798.

Sheikh, M. S., Rochefort, H., and Garcia, M. (1995). Overexpression of $p21^{WAF1/CIP1}$ induces growth arrest, giant cell formation and apoptosis in human breast carcinoma cell lines. Oncogene *11*, 1899-1905.

Shick, L., Carman, J. H., Choi, J. K., Somasundaram, K., Burrell, M., Hill, D. E., Zeng, Y. X., Wang, Y., Wiman, K. G., Salhany, K., Kadesch, T. R., Monroe, J. G., Donehower, L. A., and el-Deiry, W. S. (1997). Decreased immunoglobulin deposition in tumors and increased immature B cells in p53-null mice. Cell Growth Differ. *8*, 121-131.

Solvason, N., Wu, W. W., Kabra, N., Wu, X., Lees, E., and Howard, M. C. (1996). Induction of cell cycle regulatory proteins in anti-immunoglobulin-stimulated mature B lymphocytes. J. Exp. Med. *184*, 407-417.

Tutt, A. L., French, R. R., Illidge, T. M., Honeychurch, J., McBride, H. M., Penfold, C. A., Fearon, D. T., Parkhouse, R. M., Klaus, G. G., and Glennie, M. (1998). Monoclonal antibody therapy of B cell lymphoma: signaling activity on tumor cells appears more important than recruitment of effectors. J. Immunol. *161*, 3176-3185.

Unnikrishnan, I., Radfar, A., Jenab-Wolcott, J., and Rosenberg, N. (1999). p53 mediates apoptotic crisis in primary Abelson virus-transformed pre-B cells. Mol. Cell. Biol. *19*, 4825-4831.

VanArsdale, T. L., VanArsdale, S. L., Force, W. R., Walter, B. N., Mosialos, G., Kieff, E., Reed, J. C., and Ware, C. F. (1997). Lymphotoxin-beta receptor signaling complex: role of tumor necrosis factorreceptor-associated factor 3 recruitment in cell death and activation of nuclear factor kappaB. Proc. Natl. Acad. Sci. U. S. A. *94*, 2460-2465.

Vogelstein, B, and Kinzler, K. (1992). p53 function and dysfunction. Cell *70*, 523-526.

Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replicationby interaction with PCNA. Nature *369*, 574-578.

Wang, D., Freeman, G. J., Levine, H., Ritz, J., and Robertson, M. J. (1997). Role of the CD40 and CD95 (APO-1/Fas) antigens in the apoptosis of human B-cell malignancies. Br. J. Haematol. *97*, 409-417.

Wu, M., Bellas, R. E., Shen, J., and Sonenshein, G. E. (1998). Roles of the tumor suppressor p53 and the cyclin-dependent kinase inhibitor p21WAF1/CIP1 in receptor-mediated apoptosis of WEHI 231 B lymphoma cells. J. Exp. Med. 187, 1671-1679.

Wu, M., Bellas, R. E., Shen, J., Yang, W., and Sonenshein, G. E. (1999). Increased p27Kip1 cyclin-dependent kinase inhibitor gene expression following anti-IgM treatment promotes apoptosis of WEHI 231 B cells. J. Immunol. *163*, 6530-6535.

Xiong, Y., Zhang, H., and Beach, D. (1992). D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. Cell *71*, 505-514.

Yonish-Rouach, E., Resnitzky, D., Lotem, J, Sachs, L., Kimchi, A., and Oren, M. (1991). Wild-type p53 induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. Nature *352*, 345-347.

Zhan, Q., Kontny, U., Iglesias, M., Alamo, I. Jr., Yu, K., Hollander, M. C., Woodworth, C. D., and Fornace, A. J. Jr. (1999). Inhibitory effect of Bcl-2 on p53-mediated transactivation following genotoxic stress. Oncogene *18*, 297-304.

Figure 2.1: Crosslinking CD40 inhibits mature B lymphoma growth and survival. (A) B lymphoma cells A20, M12, TA3 and WEHI-279 were cultured in the presence or absence of anti-CD40 for 24, 48 and 72h. Viable cells (excluding Trypan blue) were counted under light microscopy. Each point shows the mean of triplicate cultures, bars indicate SD. (B) Microscopic examination of anti-CD40-treated A20 for 24 hours revealed morphological changes consistent with cell death after CD40 ligation.

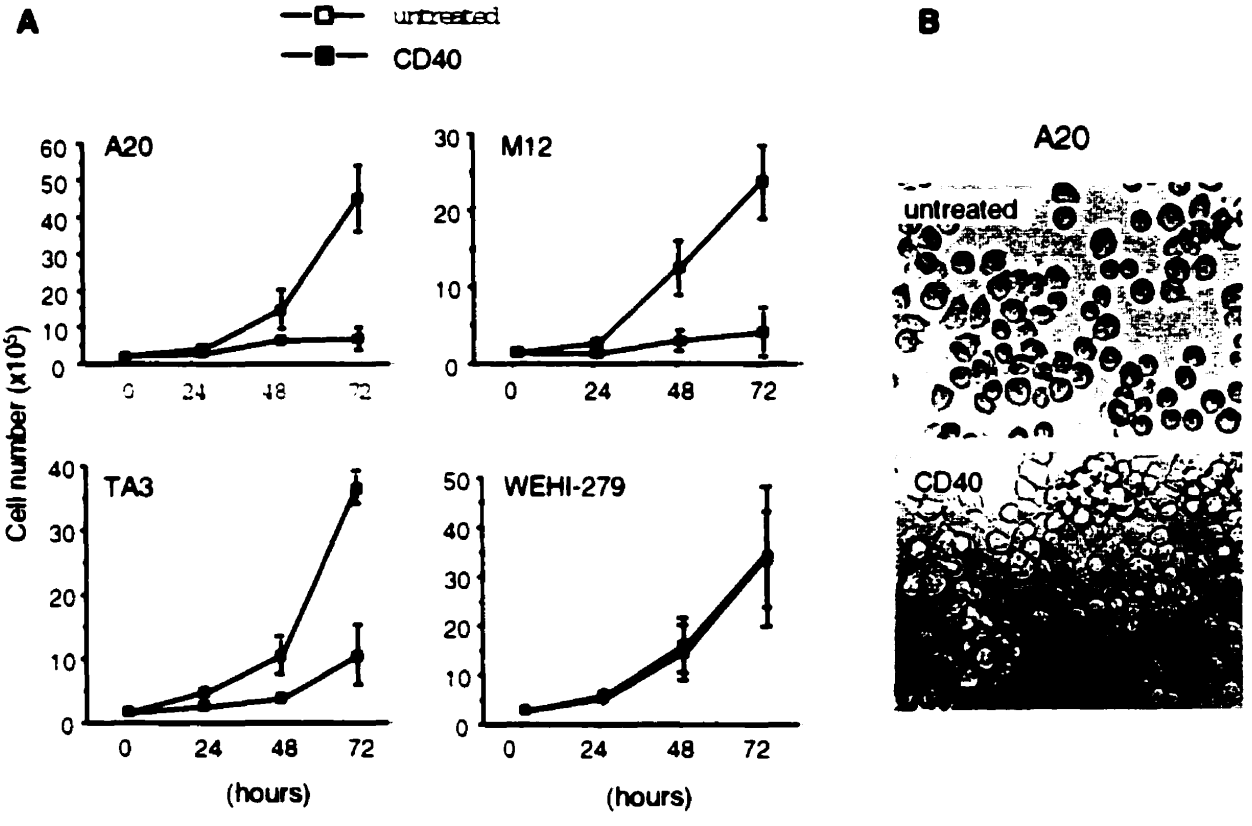

A
untreated
CD40
A20
M12
TA3
WEHI-279
Cell number (x10⁵)
(hours)
(hours)
B
A20
untreated
CD40

Figure 2.2: Ligation of CD40 induces apoptosis in mature, but not in immature B lymphomas. (A). Cell cycle analysis of mature (A20, M12, TA3) and immature (WEHI-279 and WEHI-231) B lymphomas. Cell were treated with rat anti-mouse CD40 (FGK-45), crosslinked with a mouse anti-rat Ig, for 24 hours. DNA content was measured by intracellular PI staining and flow cytometry. Only the mature lines show reduction in proportion of cells in cycle (>2N DNA) and increased apoptosis (% with <2N DNA). Crosslinking rat IgG2a anti-mouse CD5 on A20 did not show this effect. (B) Means of 3 cell cycle analyses in which proportions of cells with less than 2N DNA content were determined, by flow cytometric analysis of PI-stained populations, as in (A). Bars show mean ±SD. Visual inspection of anti-CD40-treated cultures showed increased proportions of apoptotic bodies and a reduction in general cell viability and number.

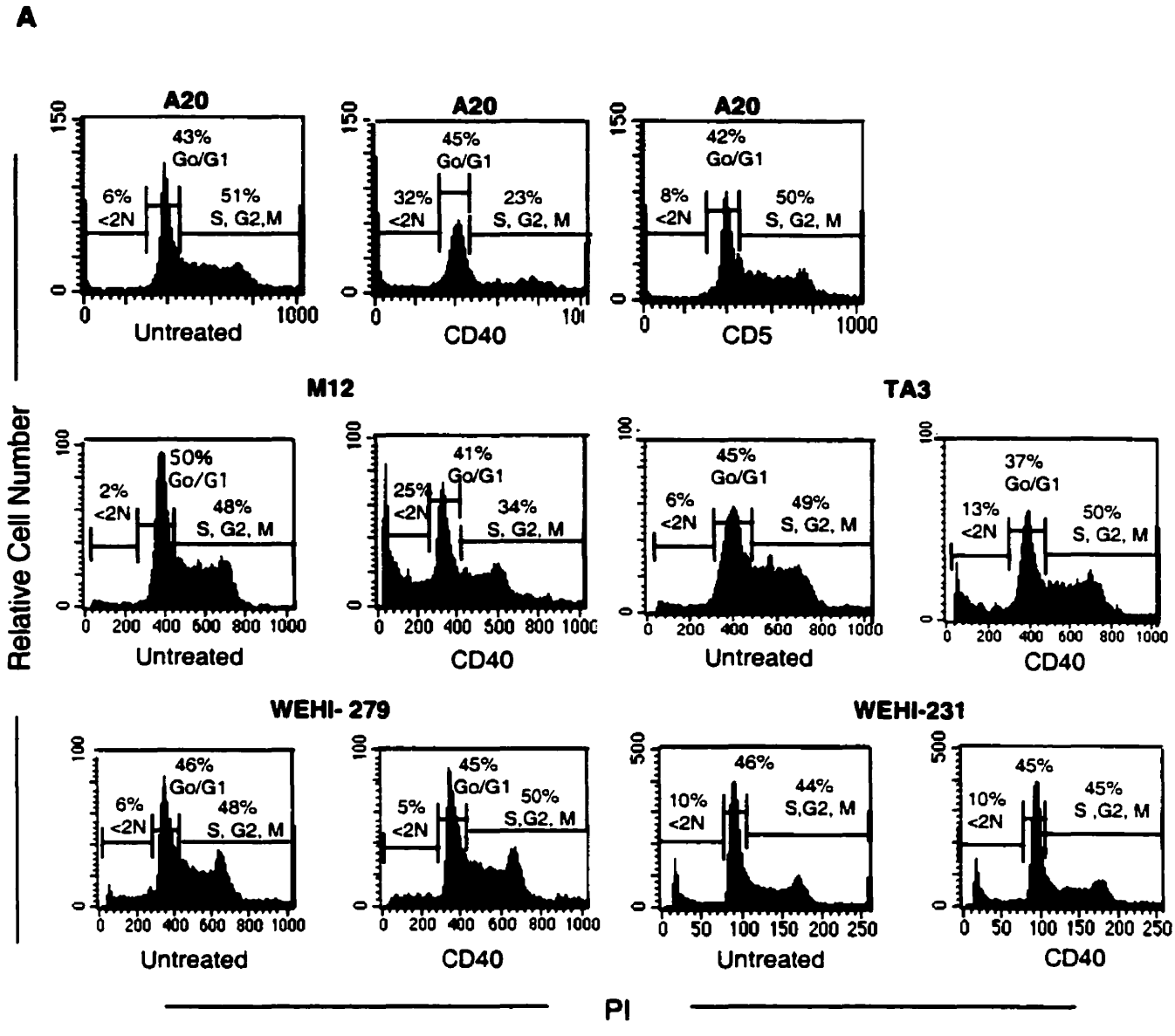

A
A20
43%
Go/G1
6%
<2N
51%
S, G2,M
Untreated
A20
45%
Go/G1
32%
<2N
23%
S, G2, M
CD40
A20
42%
Go/G1
8%
<2N
50%
S, G2, M
CD5
Relative Cell Number
M12
50%
Go/G1
2%
<2N
48%
S, G2, M
Untreated
41%
Go/G1
25%
<2N
34%
S, G2, M
CD40
TA3
45%
Go/G1
6%
<2N
49%
S, G2, M
Untreated
37%
Go/G1
13%
<2N
50%
S, G2, M
CD40
WEHI- 279
46%
Go/G1
6%
<2N
48%
S, G2, M
Untreated
45%
Go/G1
5%
<2N
50%
S,G2, M
CD40
WEHI-231
46%
10%
<2N
44%
S,G2, M
Untreated
45%
10%
<2N
45%
S ,G2, M
CD40
PI
B
Day 1
Day 2
Day 3
Untreated
CD40
% < 2N
A20
TA3
M12
WEHI-279
WEHI-231
B lymphoma cell lines

Figure 2.3: Annexin V staining of CD40-treated B lymphomas. A20, M12, TA3 and WEHI-279. Lymphoma cells were treated with anti-CD40, or with anti-CD5 (A20 only), both crosslinked with mouse anti-rat Ig for 24h. Cells were stained with Annexin V-FITC and analyzed by flow cytometry. Fluorescence levels corresponding to positive staining were defined for untreated samples and are represented by the horizontal marker. Percentage of stained cells are shown. above the marker.

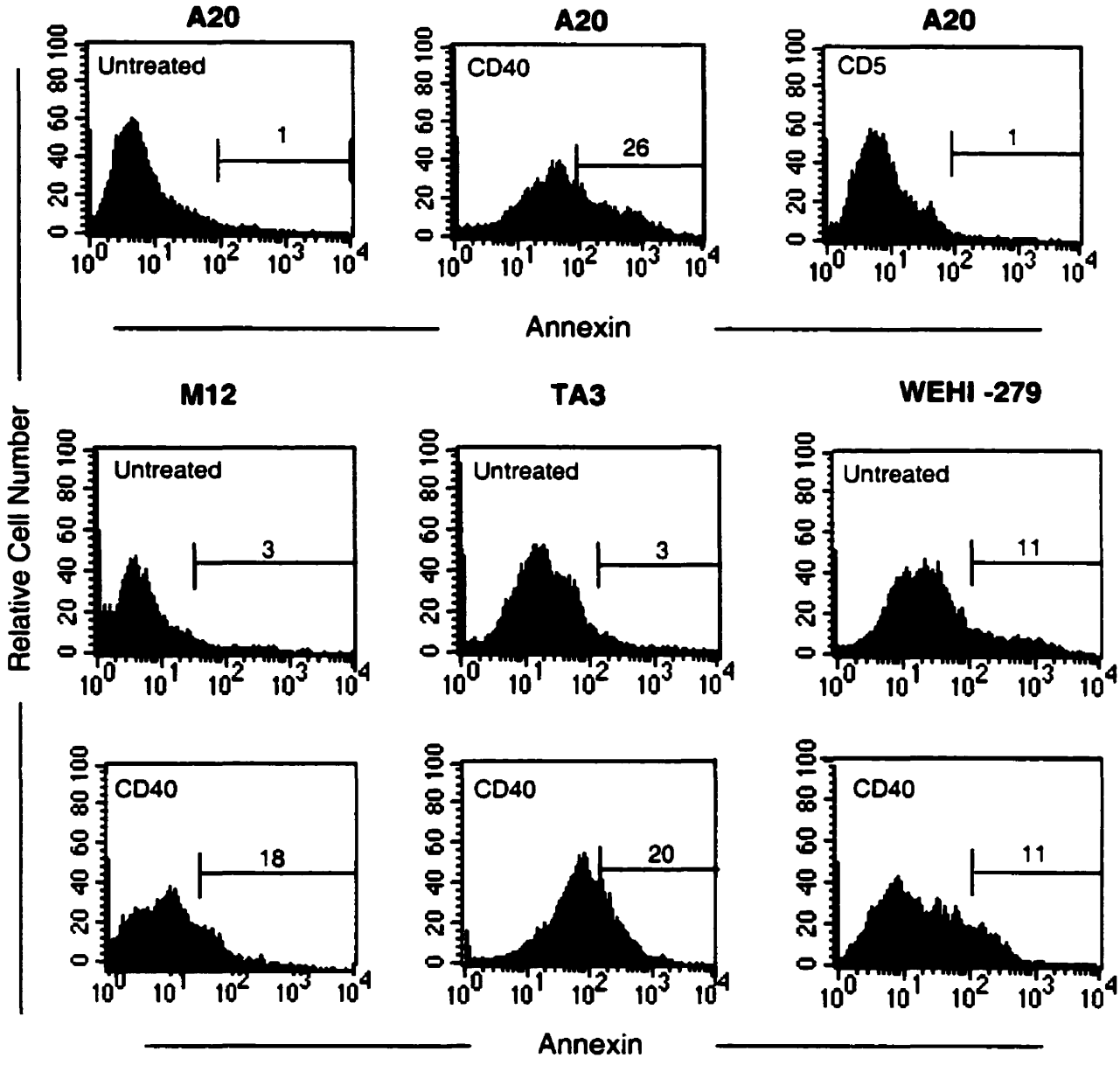

A20
Untreated
1
A20
CD40
26
A20
CD5
1
Annexin
M12
Untreated
3
TA3
Untreated
3
WEHI -279
Untreated
11
Relative Cell Number
CD40
18
CD40
20
CD40
11
Annexin

Figure 2.4: Cross-linking CD40 upregulates p53 in A20, M12 and TA3, but not in WEHI-279 or WEHI-231. (A) B lymphoma cells were incubated for various amounts of time with anti-CD40 and MaR 18.5. Cells were lysed and lysates were separated by 12% SDS-PAGE. Following Western transfer, the membrane was immunoblotted with anti-p53 and binding detected using ECL. CD40 crosslinking induced expression of p53 in mature B lymphomas (A20, TA3 and M12), but not in immature lines (WEHI-279). p53 was not detectable in WEHI-231. (B) Association between p53 and mdm2 is reduced by treatment with anti-CD40. A20 cells were treated with anti-CD40 for the indicated times, then lysates were immunoprecipitated with anti-p53 (IP p53) followed by immunoblotting with anti-mdm2 (IB mdm2). Results show clear reduction in p53-associated mdm2 in CD40-treated cultures.

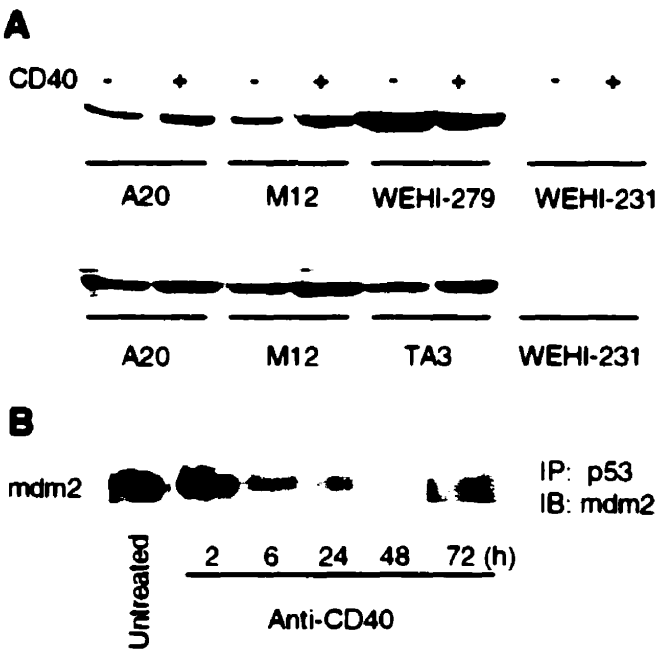

A
CD40 - + - + - + - +
A20
M12
WEHI-279
WEHI-231
A20
M12
TA3
WEHI-231
B
mdm2
IP: p53
IB: mdm2
Untreated
2 6 24 48 72 (h)
Anti-CD40

Figure 2.5: CKI levels in anti-CD40-treated B lymphomas. (A) B lymphoma cells were incubated for various times with anti-CD40 and MaR 18.5. Cells were lysed and lysates were separated by 12% SDS-PAGE. Following Western transfer, the membrane was immunoblotted with anti-$p21^{cip-1}$ and binding detected using ECL. CD40 crosslinking increased expression of $p21^{cip1}$ in mature B lymphomas (A20, TA3 and M12), but not in immature lines (WEHI-279 and WEHI-231). (B) Following Western transfer, the membrane was immunoblotted with anti-$p27^{Kip1}$ and binding detected using ECL. CD40 crosslinking increased expression of $p27^{Kip1}$ in A20, but not in M12, WEHI-279 or WEHI-231.

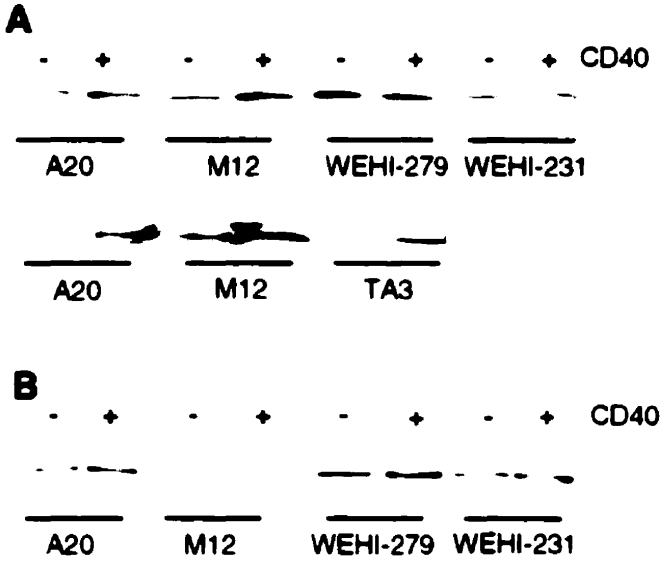

A
- + - + - + - + CD40
A20
M12
WEHI-279
WEHI-231
A20
M12
TA3
B
- + - + - + - + CD40
A20
M12
WEHI-279
WEHI-231

Figure 2.6: Expression of Bax and Bcl-2 in anti-CD40-treated B lymphomas. B lymphoma cells were incubated for various amounts of time with anti-CD40 and MaR 18.5. Cells were lysed and lysates were separated by 12% SDS-PAGE. Following Western transfer, the membrane was immunoblotted with anti-Bax and anti-Bcl-2 and binding detected using ECL. High expression of Bax was detected in mature B lymphoma cell lines (A20 and M12) and high expression of Bcl-2 in immature cell lines (WEHI-279 and WEHI-231). CD40 cross-linking did not affect the level of either Bax or Bcl-2 protein.

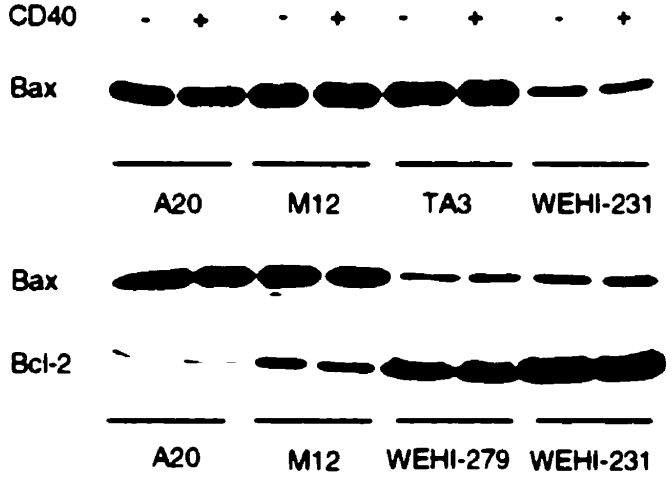
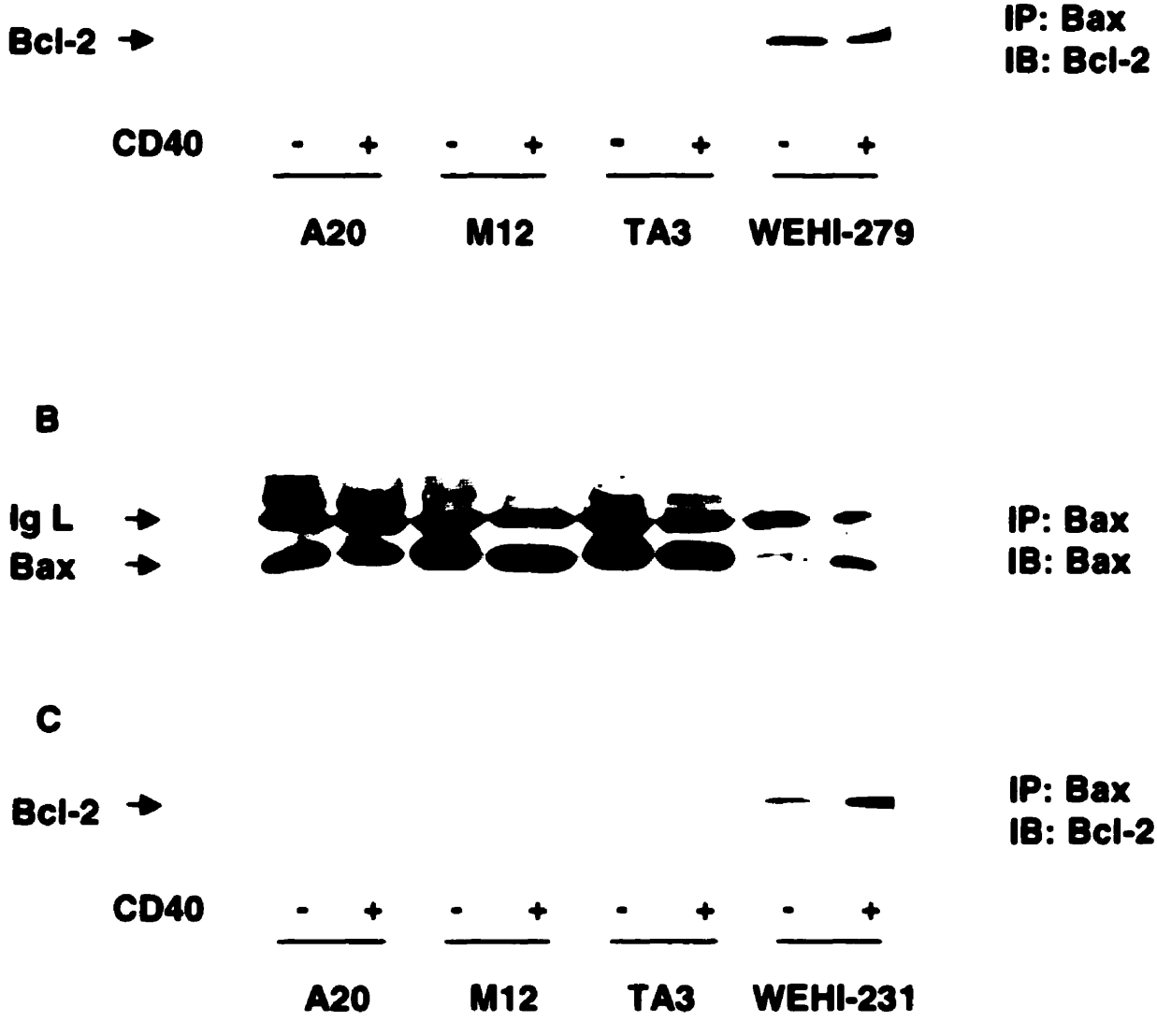

CD40
- + - + - + - +
Bax
A20
M12
TA3
WEHI-231
Bax
Bcl-2
A20
M12
WEHI-279
WEHI-231

Figure 2.7. Bcl-2 binds to Bax in immature cell lines (WEHI-279 and WEHI-231), but not obvious in mature lines (A20, M12 and TA3). B lymphoma cells were treated with anti-CD40 for 24h or left untreated. cells were lysed and lysates were immunoprecipitated with anti-Bax (IP) followed by immunoblotting with Bcl-2 (IB) (A). Lysates were immunoprecipitated with anti-Bax (IP) followed by immunoblotting with anti-Bcl-2 (IB) (B). IgL denotes IgG light chain. The blot shown in (B) was stripped and developed with anti-Bcl-2 (C).


A
Bcl-2
IP: Bax
IB: Bcl-2
CD40
- + - + - + - +
A20 M12 TA3 WEHI-279
B
Ig L
Bax
IP: Bax
IB: Bax
C
Bcl-2
IP: Bax
IB: Bcl-2
CD40
- + - + - + - +
A20 M12 TA3 WEHI-231

Chapter 3

Regulation of growth of B lymphoma by Fas, FasL and ICAM-1

Introduction

Fas/CD95/APO-1 is a cell surface protein belonging to the TNF receptor family. Binding of either Fas ligand or anti-Fas antibodies to Fas triggers apoptosis of Fas-expressing cells (Yonehara et al., 1989; Trauth et al., 1989; Debatin et al., 1993). This Fas-mediated apoptotic death signal has a number of important immunological roles. Fas-mediated apoptosis is involved in thymocyte clonal deletion and tolerance acquisition (Yonehara et al., 1994), T cell activation-induced cell death (Alderson et al. 1995) and T cell-mediated cytotoxicity. Fas receptor expression and response are variable in human lymphoid (Debatin and Krammer, 1995; Shima et al. 1995) and nonlymphoid malignancies (Owen-Schaub et al. 1994; Natoli et al. 1995). Resistance to Fas-mediated cytotoxicity may contribute to tumor immune escape and to tumorigenicity (Wright et al. 1994).

Fas ligand is a key molecule in normal immune development, homeostasis, modulation, and function (Nagata and Golstein, 1995). Fas ligand (FasL) has been found to be predominantly expressed by activated T cells (Suda et al., 1995; Vignaux et al. 1995), NK lymphoma and T cell-type large granular lymphocyte leukemias (Tanaka et al 1996). FasL has been found to be functionally expressed by some cancer cells, and shown to mediate apoptosis of T cells (O'Connell et al., 1996; Strand et al., 1996). It has been reported that FasL can transduce signals, inducing apoptosis in $CD4^+$ T cells (Desbarats et al., 1998).

The intercellular adhesion molecule 1 (ICAM-1, CD54) is an adhesion molecule belonging to the immunoglobulin superfamily (Staunton et al., 1988) that is expressed constitutively on lymphocytes, vascular endothelium and other cell types (Dustin et al. 1986; Wawryk et al. 1989). Its ligands are the membrane-bound integrin receptors LFA-1 and Mac-1 on leukocytes, CD43, the soluble molecule fibrinogen, the matrix factor hyaluronan, and rhinoviruses (Clark and Brugge 1995). ICAM-1 was originally considered to act purely as an adhesion molecule, strengthening weak interaction between cells. However, there is increasing evidence to suggest that ICAM-1 plays a signaling role (Rothlein et al 1988; Chirathworn et al. 1995). Co-cross-linking of ICAM-1 and MHC II induced expression of a functional IL-2 receptor on murine B cells (Poudrier and Owens, 1994). In addition, an anti-ICAM-1 mAb modulated the release of interferon-γ, tumor necrosis factor α and IL-1 by T lymphocytes and monocytes (Geissler et al., 1990). All these results strongly support the idea that ICAM-1 is involved in a signal transduction process.

A recent publication from our lab showed that cross-linking of ICAM-1 on the B lymphoma cell line A20 induced an increase in tyrosine phosphorylation of several cellular proteins. This resulted in activation of Raf-1 and mitogen-activated protein kinases. These represent important components in the cascade of signals that link to various ICAM-1 elicited cellular responses (Holland and Owens, 1997). However, questions still remain as to how ICAM-1 activates these kinases and whether it transduces signals through other pathways. The mechanism of signal transduction remains unknown.

Recent finding showed that ICAM-1 play a significant role in regulating Fas and FasL activity. Upregulation of ICAM-1 was observed in liver endothelial cells treated with Fas agonist (Cardier et al., 1999). Inhibition of ICAM-1 modulated the activity of Fas ligand in FasL transfected cells (Sieg et al., 1997). Blocking the interaction between LFA-1 and ICAM-1 completely suppressed Fas-dependent B cell lysis (Wang and Lenardo, 1997). These data suggest that Fas/FasL may transduce signals, at least partly, dependent on ICAM-1.

To determine whether or not Fas and Fas ligand on B lymphoma cells are able to transduce a signal for apoptosis, I performed cell cycle analysis and Annexin V apoptotic assay on B lymphoma cells treated with anti-Fas or anti-FasL mAb.

I have also investigated whether ICAM-1 is phosphorylated on cytoplasmic tyrosine residues, and whether it is associated with phosphorylated proteins that transduce signals.

Materials and Methods

Cell lines

The B cell line A20 was derived from a BALB/cAnN lymphoma (IgG^+) (ATCC) , TA3 and M12 were derived from a BALB/c lymphoma (IgM^+, IgD^+), WEHI-279 from NZC and WEHI-231 from BALBxNZC lymphomas (ATCC). Cells were maintained in RPMI 1640 medium (Life Technologies, Montreal, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) 2 mM L-glutamine (Life Technologies, Montreal, Canada) $5x10^{-5}M$ 2-mercaptoethanol (Sigma,

Montreal, Canada)), penicillin (100 units/ml) (Life Technologies), and streptomycin (100 μg/ml) (Life Technologies).

Antibodies and Reagents

mAb used were YN1/1.7.4.1(rat IgG2b anti-murine CD54/ICAM-1), MAR 18.5 (mouse IgG2a anti-rat κ), PY72.10.5 (mouse IgG1 anti-phosphotyrosine). Monoclonal antibodies were affinity-purified from culture supernatants using Protein G-Sepharose (Pharmacia Biotech Inc., Montreal, Quebec, Canada). The hamster monoclonal anti-mouse Fas (Jo2) and hamster monoclonal anti-mouse FasL (MFL3) were purchased from PharMingen, Canada. Armenian hamster IgG was from Jackson ImmunoResearch Laboratories, NJ, USA.

Flow cytometry

Staining was performed with 20-minutes incubations of $5x10^5$ cells/tube at 4^0c with biotinylated mAb at concentrations ranging between 2 and 20 μg/ml, with FITC-coupled streptavidin. Fluorescence was analyzed using a FACScan.

Cell cycle analysis

Lymphoma cells were cultured at densities of $1X10^5$ cell/well (A20, M12 and TA3) and of $2X10^5$ cells/well (WEHI-279) in the presence or absence of anti-Fas or anti-FasL at concentration ranging between 1-2μg/ml. The cells were collected after 24 hour, washed in cold D-PBS and permeabilized with 70% Ethanol for 5 minutes. Cells were then washed with cold D-PBS and incubated at 37^0C in apoptosis buffer (1 μg/ml propidium iodide (Sigma) 10 mg/ml RNAse A (Boehringer

Mannheim/Roche Molecular Biochemicals, Laval, Canada) 25% FCS in D-PBS). Propidium iodide (PI) staining was measured using a FACScan (Becton-Dickinson, Oakville, Ontario). Data were analyzed by Cell Quest software (Becton-Dickinson).

Annexin V-FITC staining

Cells were washed twice with cold PBS and resuspended in 1x binding buffer (PharMingen Canada, Mississauga, Ontario) at a concentration of $1x10^6$ cells/ml. Cells were then incubated for 15 minutes at room temperature in the dark with 5µl of Annexin V-FITC (PharMingen Canada, Mississauga, Ontario) and 10 µl of PI. Staining was assessed using a FACScan and analyzed using Cell Quest Software.

Preparation of B cell lysates

B cells were incubated in culture medium with mAbs for various amount of time at 37^0C. Cells were collected and washed three times with Dulbecco's PBS and lysed by incubation in lysis buffer (10mM Tris-HCL, pH7.5, 37 mM NaCl, 1% Nonidet P-40 (ICN Biomedicals, Missisauga, Canada) 1 mM EDTA (Anachemia, Canada) 1 mM NaVO4 (ICN Biomedicals, Mississauga, Canada) 2 mM phenylmethylsulfonyl fluoride (Sigma) and 50 mM NaF (K & K Laboratories, Cleveland, Ohio) for 15 min on ice. Cell debris and nuclei were removed by centrifugation (10 min at 900xg). The protein concentration of each lysate was quantified using a bicinchonimic acid assay (Pierce, Aurora, Ontario).

PAGE/Western blot

Lysates were boiled for 5 min with an equal volume of 2X sample buffer containing 5% 2-mercaptoethanol (Sigma) before loading. Equal amounts of protein were loaded in each lane and separated on 10% SDS polyacrymide gels. Following separation by electrophoresis, proteins were then transferred to PVDF membranes (Millipore Corporation, Bedford, MA). at 100 V for 1 hour. Membranes were blocked for 1 h at room temperature or overnight at 4^0C in 5% non-fat milk in TTBS (20mM Tris-HCL, 137mM NaCL, 1M HCL, 1% Tween-20 [Bio-Rad, CA]). Membranes were incubated with PY72 in TTBS for 1 hour or overnight and detected using the ECL system with horseradish peroxidase-conjugated secondary antibodies (Amersham Corp., Oakville, Ontario).

Immunoprecipitation

Lysates were incubated with 1-2 μg of YN1/1.7.4.1 (rat IgG2b anti-murine CD54/ICAM-1) for 1 h at 4^0C. Thirty-five μl of washed Protein G-sepharose beads (Pharmacia Biotech, Sweden) were added and incubated at 4^0C for 1 hour to overnight. The immune complexes were collected by centrifugation and washed three times with lysis buffer. Proteins in immune complexes were resolved by SDS-PAGE on a 10% acrylamide gel and transferred onto PVDF membranes. The membranes were probed with PY72 and detected using the ECL system.

Results

Anti-Fas mAb induced apoptosis in A20 B lymphoma, not in M12, TA3 or WEHI-279

Anti-Fas mAb has been reported to induce apoptosis of cells expressing Fas antigen (Yonehara et al., 1989; Trauth et al., 1989). Immunofluorescence analysis showed that Fas antigen was expressed on all of the 4 lymphoma cell lines, A20, M12, TA3 and WEHI-279. A20 expressed the highest level of Fas. Expression of Fas and FasL was not obvious in WEHI-231 (Fig 3.1). To determine whether or not Fas induces apoptosis of B lymphoma, A20, M12 TA3 and WEHI-279 were cultured with anti-Fas mAb (Jo1) for 24 hours, cells were stained with propidium iodide and DNA content was analyzed by FACS. The cell cycle profiles show that by 24h, the proportion of apoptotic cells (<2N DNA) increased in anti-Fas-treated A20, compared with untreated cells (Fig 3.2). In contrast, there was no effect of anti-Fas on M12, TA3 or WEHI-279. That cell death induced by anti-Fas was mediated by apoptosis was confirmed by annexin V staining (Fig 3.3). Although there is a decrease (53% Vs 43%) in the number of WEHI-279 cells in the G2/M phase upon treatment with anti-Fas, there was no increase in the intensity of the Annexin V staining (mean fluorescence intensity=89 vs. 75 for control). This does not support the possibility that anti-Fas induced apoptosis in WEHI-279. Normal hamster Ig was used as a control and had no effect on B lymphoma cell viability (Fig 3.4; Fig 3.4).

Anti-FasL did not induce apoptosis in B lymphoma cells

Ligation of FasL induces apoptosis in $CD4^+$ T cells (Desbarats et al., 1998). To assess whether anti-FasL induced growth inhibition of B lymphoma cells, A20, M12, TA3 and WEHI-279 were treated with anti-FasL for 24h, cells were collected and stained with propidium iodide, and DNA content was analyzed by flow cytometry. Cell cycle analysis showed that anti-FasL had no effect on B lymphoma cells (Fig 3.4) even although all these 4 lymphoma cells expressed FasL (Fig 3.1)

Induction of tyrosine phosphorylation by cross-linking ICAM-1 A20

To investigate the intracellular signaling events mediated by ICAM-1, we examined changes in protein tyrosine phosphorylation. A20 cells were incubated with anti-ICAM-1, crosslinked with mouse anti-rat Ig, for different times as indicated. Anti-phosphotyrosine immunoblotting of whole cell lysates showed an increase in protein phosphorylation detectable at 2 min, decreasing 30 min after ligation of anti-ICAM-1 (Figure 3.5)

Immunoprecipitation of ICAM-1 from biotinylated cell lysates

To confirm our immunoprecipitation protocol for ICAM-1, A20 cells were biotinylated by incubation with sulfo-NHS-L-C-biotin and lysed with NP-40 lysis buffer. Lysates were precleared by incubation with Pansorbin and precipitated with YN1/1.7.4.1 (rat IgG2b anti-murine CD54/ICAM-1). After Western transfer, membranes were probed with horseradish peroxidase conjugated streptavidin and detected using the ECL system. One band in the molecular mass range of 90-100KDa was observed,

consistent with the molecular weight of ICAM-1. Thus, YN1 can be used to immunoprecipitate ICAM-1 (Figure 3.6)

Phosphorylation of Immunoprecipitated ICAM-1 from A20

To assess whether the ICAM-1 molecule itself is phosphorylated upon ICAM-1 ligation, A20 cells were stimulated by crosslinking ICAM-1 with MAR and lysates were immunoprecipitated with anti-ICAM-1 plus Protein G sepharose. This was done without preclearing, because Pansorbin beads bind IgG2a. Immunoprecipitates were subjected to SDS-PAGE and transferred to PVDF membranes. After Western transfer, membranes were blotted with PY72. Two hyperphosphorylated bands were observed in the molecular weight of 900-100KD, the other in the molecular mass range of 35-40KD (Figure 3.7).

Effect of ICAM-1 crosslinking on A20 cells

To determine whether ICAM-1 crosslinking had an effect on cell growth in A20 cells, we incubated A20 with rat anti-mouse ICAM-1 and mouse anti-rat Ig for 2 days. Cells were then stained with Propidium Iodide after permeabilization or with Annexin V, and analyzed by FACS. Neither cell cycle analysis nor Annexin V staining showed any effect of ICAM-1 crosslinking on growth of A20. (Figure 3.8)

References

Chirathaworn, C., Tibbetts, S. A., Chan, M. A., and Benedict, S. H. (1995). Cross-linking of ICAM-1 on T cells induces transient tyrosine phosphorylation and inactivation of cdc2 kinase. J. Immunol. *155*, 5479-5482.

Clark, E. A., and Brugge, J. S. Integrins and signal transduction pathways: the road taken. 1995. Science *268*, 233-239.

Debatin, K. M., Goldman, C. K., Waldmann, T. A., and Krammer, P. H. (1993). APO-1-induced apoptosis of leukemia cells from patients with adult T-cell leukemia. Blood *81*, 2972-2977.

Debatin, K. M., and Krammer, P. H. (1995). Resistance to APO-1 (CD95) induced apoptosis in T-ALL is determined by a BCL-2 independent anti-apoptotic program. Leukemia. *9*, 815-820

Desbarats, J., Duke, R. C., and Newell, M. K. (1998). Newly discovered role for Fas ligand in the cell-cycle arrest of CD4+ T cells. Nat. Med. *4*, 1377-1382.

Dustin, M. L, Rothlein, R., Bhan, A. K., Dinarello, C. A., and Springer, T. A. (1986). Induction by IL 1 and interferon-gamma: tissue distribution, biochemistry, and function of a natural adherence molecule (ICAM-1). J. Immunol. *137*, 245-254.

Geissler, D., Gaggl, S., Most, J., Greil, R., Herold, M., and Dietrich, M. (1990). A monoclonal antibody directed against the human intercellular adhesion molecule (ICAM-1) modulates the release of tumor necrosis factor-alpha, interferon-gamma and interleukin 1. Eur. J. Immunol. *20*, 2591-2596.

Holland, J., and Owens, T. (1997). Signaling through intercellular adhesion molecule 1 (ICAM-1) in a B cell lymphoma line. The activation of Lyn tyrosine kinase and the mitogen-activated protein kinase pathway. J. Biol. Chem. *272*, 9108-9112.

Nagata, S., and Golstein, P. (1995). The Fas death factor. Science *267*, 1449-56.

Natoli, G., Ianni, A., Costanzo, A., De Petrillo, G., Ilari, I., Chirillo, P., Balsano, C., and Levrero, M. (1995). Resistance to Fas-mediated apoptosis in human hepatoma cells. Oncogene *11*, 1157-1164.

O'Connell, J., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. (1996). The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J. Exp. Med. *184*, 1075-1082.

Owen-Schaub, L. B., Radinsky, R., Kruzel, E., Berry, K., and Yonehara, S. (1994). Anti-Fas on nonhematopoietic tumors: levels of Fas/APO-1 and bcl-2 are not predictive of biological responsiveness. Cancer Res. *54*, 1580-1586.

Poudrier, J., and Owens, T. 1994. CD54/intercellular adhesion molecule 1 and major histocompatibility complex II signaling induces B cells to express interleukin 2 receptors and complements help provided through CD40 ligation. J. Exp. Med. *179*, 1417-1427.

Rothlein, R., Czajkowski, M., O'Neill, M. M., Marlin, S. D., Mainolfi, E., and Merluzzi, V. J. (1988). Induction of intercellular adhesion molecule 1 on primary and continuous cell lines by pro-inflammatory cytokines. Regulation by pharmacologic agents and neutralizing antibodies. J. Immunol. *141*, 1665-1669.

Shima, Y., Nishimoto, N., Ogata, A., Fujii, Y., Yoshizaki, K., and Kishimoto, T. (1995). Myeloma cells express Fas antigen/APO-1 (CD95) but only some are sensitive to anti-Fas antibody resulting in apoptosis. Blood. *85*, 757-764.

Staunton, D. E., Marlin, S. D., Stratowa, C., Dustin, M. L., and Springer, T. A. (1988). Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell *52*, 925-933.

Strand, S., Hofmann, W. J., Hug, H., Muller, M., Otto, G., Strand, D., Mariani, S. M., Stremmel, W., Krammer, P. H., and Galle, P. (1996). Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells--a mechanism of immune evasion? Nat. Med. *2*, 1361-6.

Suda, T., Okazaki, T., Naito, Y., Yokota, T., Arai, N., Ozaki, S., Nakao, K., and Nagata, S. (1995). Expression of the Fas ligand in cells of T cell lineage. J. Immunol. *154*, 3806-3813.

Tanaka, M., Suda, T., Haze, K., Nakamura, N., Sato, K., Kimura, F., Motoyoshi, K., Mizuki, M., Tagawa, S., Ohga, S., Hatake, K., Drummond, A. H., and Nagata, S. (1996). Fas ligand in human serum. Nat. Med. *2*, 317-322.

Trauth, B. C., Klas, C., Peters, A. M., Matzku, S., Moller, P., Falk, W., Debatin, K. M., and Krammer, P. H. (1989). Monoclonal antibody-mediated tumor regression by induction of apoptosis. Science *245*, 301-305.

Vignaux, F., Vivier, E., Malissen, B., Depraetere, V., Nagata, S., and Golstein, P. (1995). TCR/CD3 coupling to Fas-based cytotoxicity. J. Exp. Med. *181*, 781-786.

Wawryk, S. O., Novotny, J. R., Wicks, I. P., Wilkinson, D., Maher, D., Salvaris, E., Welch, K., Fecondo, J, and Boyd, A. W. (1989). The role of the LFA-1/ICAM-1 interaction in human leukocyte homing and adhesion. Immunol. Rev. *108*, 135-161.

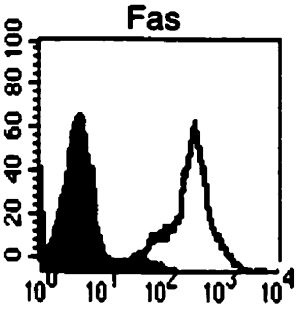
Wright, S. C., Zhong, J., and Larrick, J. W. (1994). Inhibition of apoptosis as a mechanism of tumor promotion. FASEB J. *8*, 654-60.

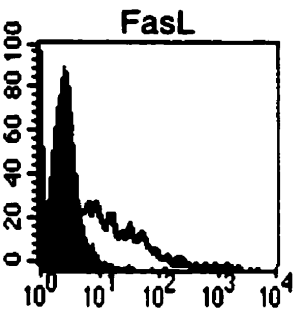
Yonehara, S., Ishii, A., and Yonehara, M. (1989). A cell-killing monoclonal antibody (anti-Fas) to a cell surface antigen co-downregulated with the receptor of tumor necrosis factor. J Exp. Med. *169*, 1747-1756.

Yonehara, S., Nishimura, Y., Kishil, S., Yonehara, M., Takazawa, K., Tamatani, T., and Ishii, A. (1994). Involvement of apoptosis antigen Fas in clonal deletion of human thymocytes. Int Immunol. *6*, 1849-1856.

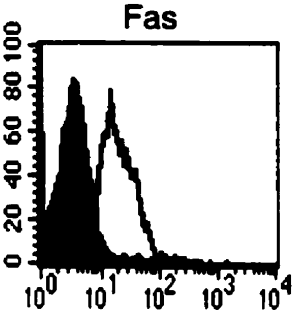
Figure 3.1: Expression of Fas and Fas ligand by B lymphoma cells A20, M12, TA3 and WEHI-279. Cells were collected, washed, and stained with biotin-conjugated hamster anti-mouse Fas (Jo2) or FasL (MFL3) followed by phycoerythrin-coupled streptavidin. Solid profiles show staining controls, and open profile show Fas or FasL staining.

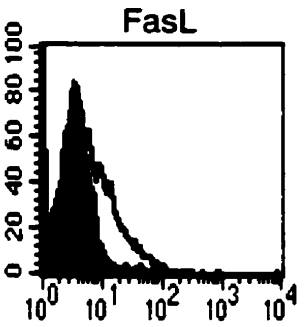
A20



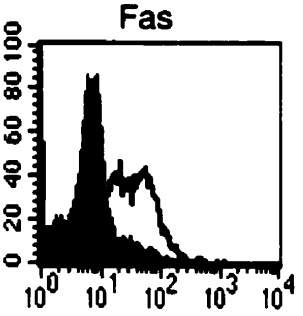


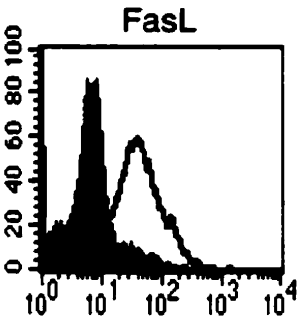
M12



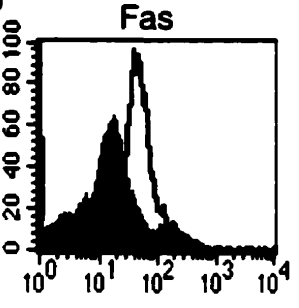


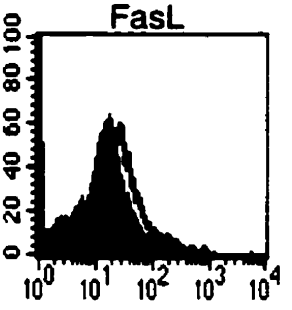
TA3



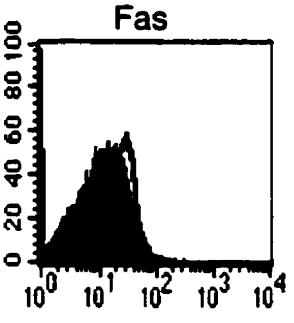


WEHI-279





WEHI-231



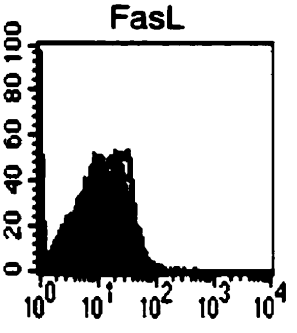
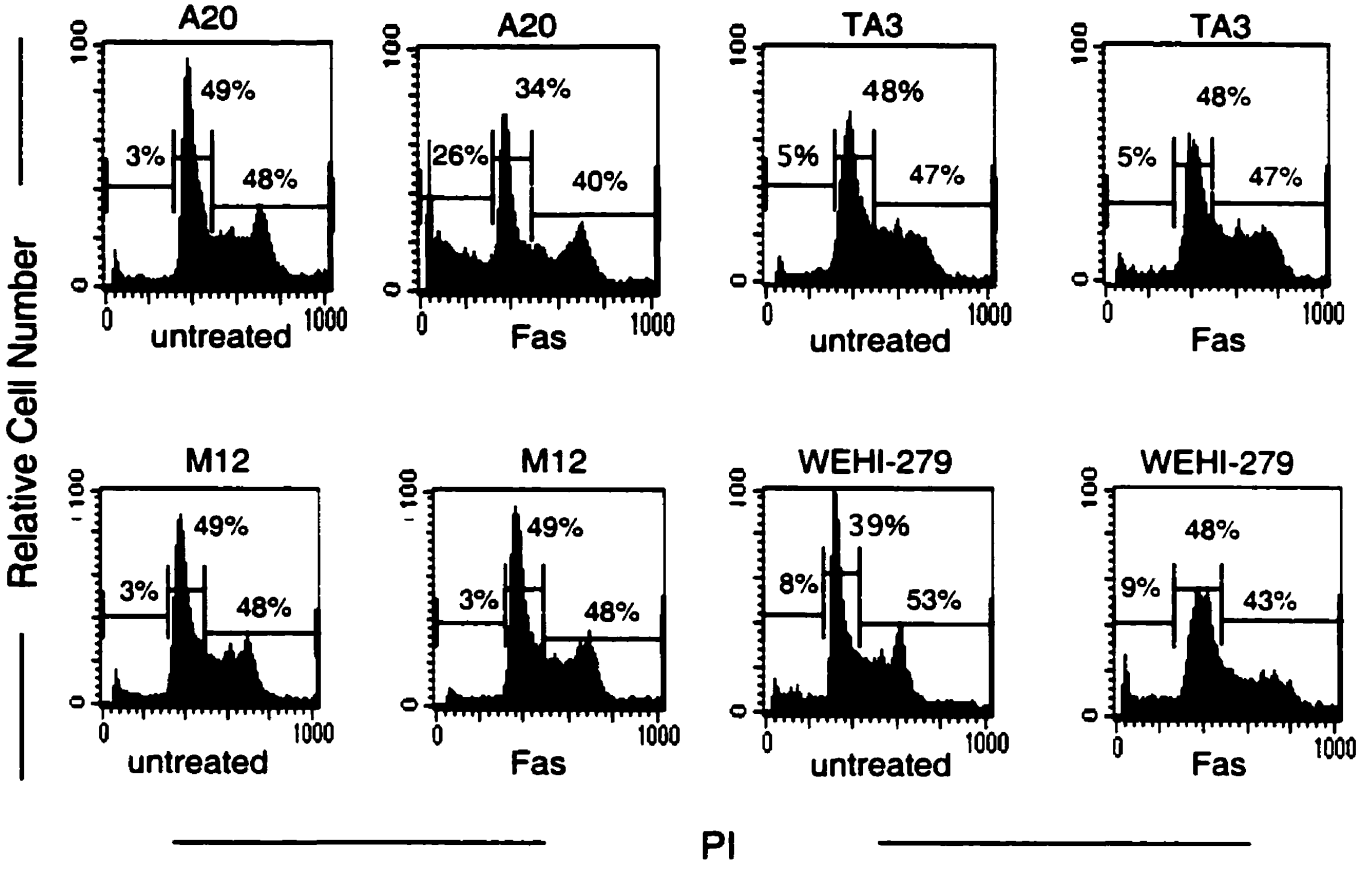


Figure 3.2: Induction of apoptosis in A20 by anti-Fas, not in M12, TA3 or WEHI-279. (A) Cell cycle analysis of A20, M12, TA3 and WEHI-279. Cells were treated with hamster anti-mouse CD95 (Jo1) for 1 day. DNA content was measured by intracellular PI staining. Only A20 showed growth arrest (reduction in % with >2N DNA) and apoptosis (increase in % with <2N). Hamster Ig did not produce this effect (Fig. 3.4). (B) Proportions of cells with less than 2N DNA content were determined by flow cytometric analysis of PI-stained populations, as in (A). Bars show means of 3 experiments ±SD. Visual inspection of anti-Fas-treated cultures showed increased proportions of apoptotic bodies.

A


A20
49%
3%
48%
untreated
A20
34%
26%
40%
Fas
TA3
48%
5%
47%
untreated
TA3
48%
5%
47%
Fas
M12
49%
3%
48%
untreated
M12
49%
3%
48%
Fas
WEHI-279
39%
8%
53%
untreated
WEHI-279
48%
9%
43%
Fas
Relative Cell Number
PI

B

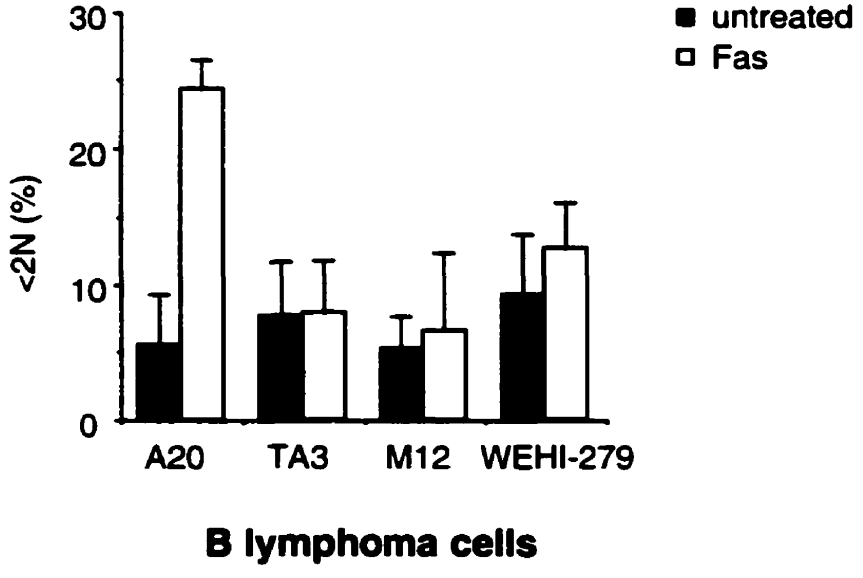

untreated
Fas
<2N (%)
30
20
10
0
A20
TA3
M12
WEHI-279
B lymphoma cells

Figure 3.4: Lack of effect of anti-FasL on B lymphoma cells. A20, M12, TA3 and WEHI-279 cells were cultured with hamster anti-mouse FasL (MFL3) for 1 day. DNA content was measured by intracellular PI staining and flow cytometry. Hamster IgG used as a control. Anti-FasL did not show any effect on B lymphoma cells.

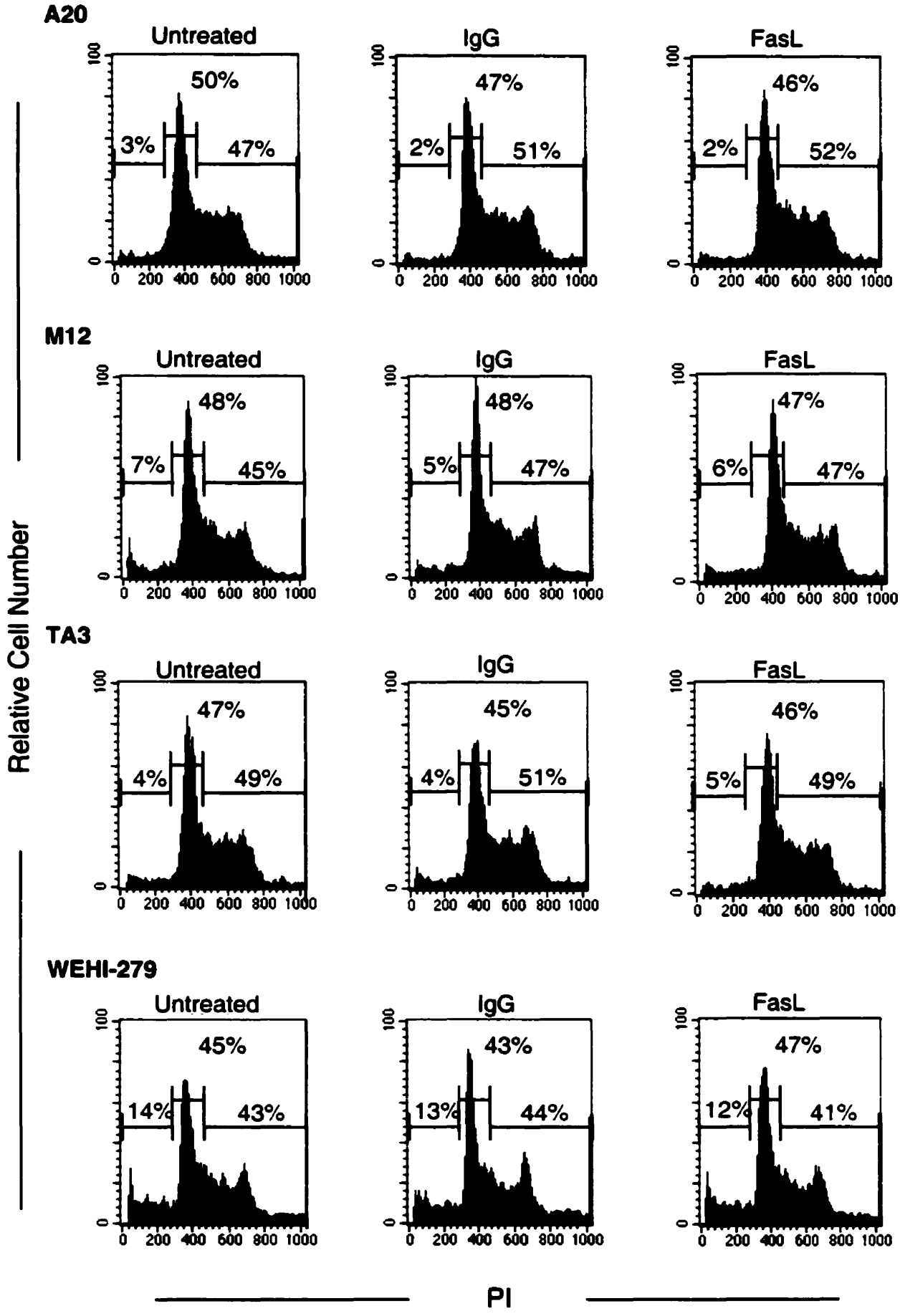

A20
Untreated
IgG
FasL
50%
3%
47%
47%
2%
51%
46%
2%
52%
M12
Untreated
IgG
FasL
48%
7%
45%
48%
5%
47%
47%
6%
47%
TA3
Untreated
IgG
FasL
47%
4%
49%
45%
4%
51%
46%
5%
49%
WEHI-279
Untreated
IgG
FasL
45%
14%
43%
43%
13%
44%
47%
12%
41%
0
200
400
600
800
1000
100
Relative Cell Number
PI

Figure 3.3: Annexin V Staining of CD95-treated B lymphomas A20, M12, TA3 and WEHI-279. Lymphoma cells were left untreated, treated with anti-CD95, or with hamster IgG. Cells were stained with Annexin V-FITC and analyzed by flow cytometry. Fluorescence levels corresponding to positive staining were defined by reference to untreated samples as represented by the horizontal line. Percentages of stained cells are shown.

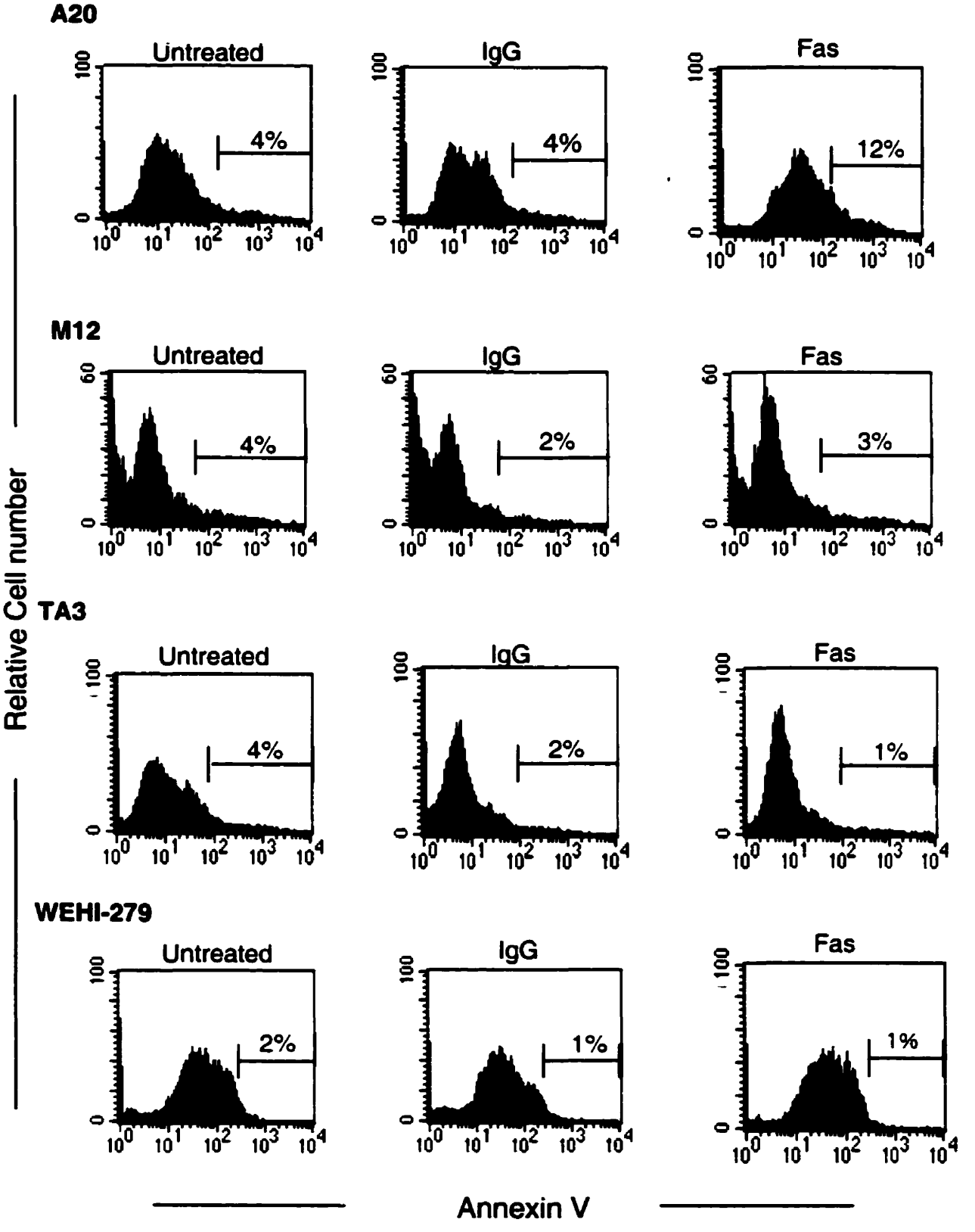

A20
Untreated
IgG
Fas
4%
4%
12%
M12
Untreated
IgG
Fas
4%
2%
3%
TA3
Untreated
IgG
Fas
4%
2%
1%
WEHI-279
Untreated
IgG
Fas
2%
1%
1%
Relative Cell number
Annexin V

Figure 3.5 Cross-linking ICAM-1 induces tyrosine phosphorylation in A20. A20 cells were incubated at 37^{0}C in culture medium with YN1 and MAR antibodies for various times as indicated. Cells were lysed in NP40 buffer and lysates were subjected to 10% SDS-PAGE. Following Western transfer, membranes were immunoblotted with PY72.10.5 followed by horseradish peroxidase conjugated goat anti-mouse Ig. Blots were developed with the ECL substrate. Crosslinking ICAM-1 induced hyperphosphorylation from 2 min, which decreased after 30 min afterwards.

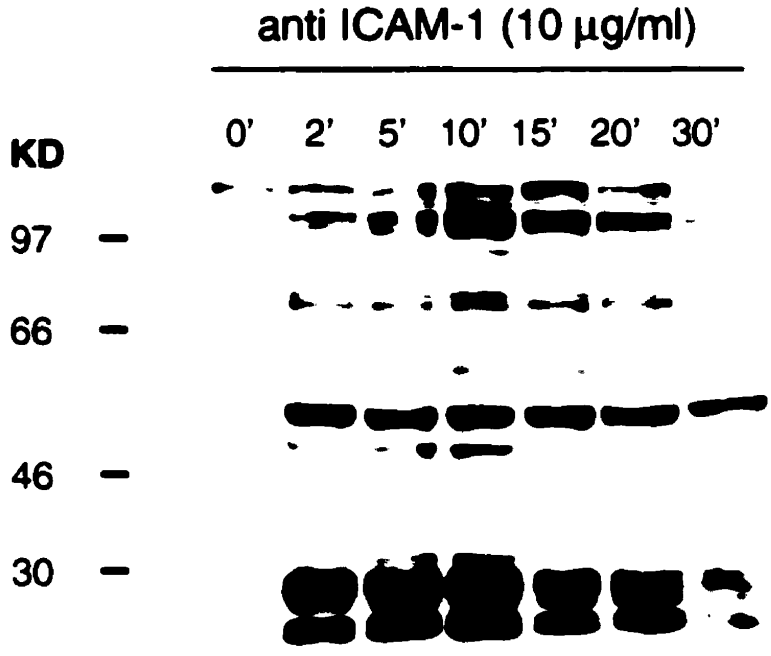

anti ICAM-1 (10 µg/ml)
0' 2' 5' 10' 15' 20' 30'
KD
97
66
46
30

Figure 3.6 Immunoprecipitation of ICAM-1 from biotinylated cell lysates. Biotinylated A20 B cells were lysed. Lysates were incubated with YN1/1.7.4.1 followed by incubation with Protein G Sephorose. Immunoprecipitates were loaded onto SDS-PAGE. After Western transfer, membranes were probed with horseradish peroxidase conjugated streptavidin and detected using the ECL system.

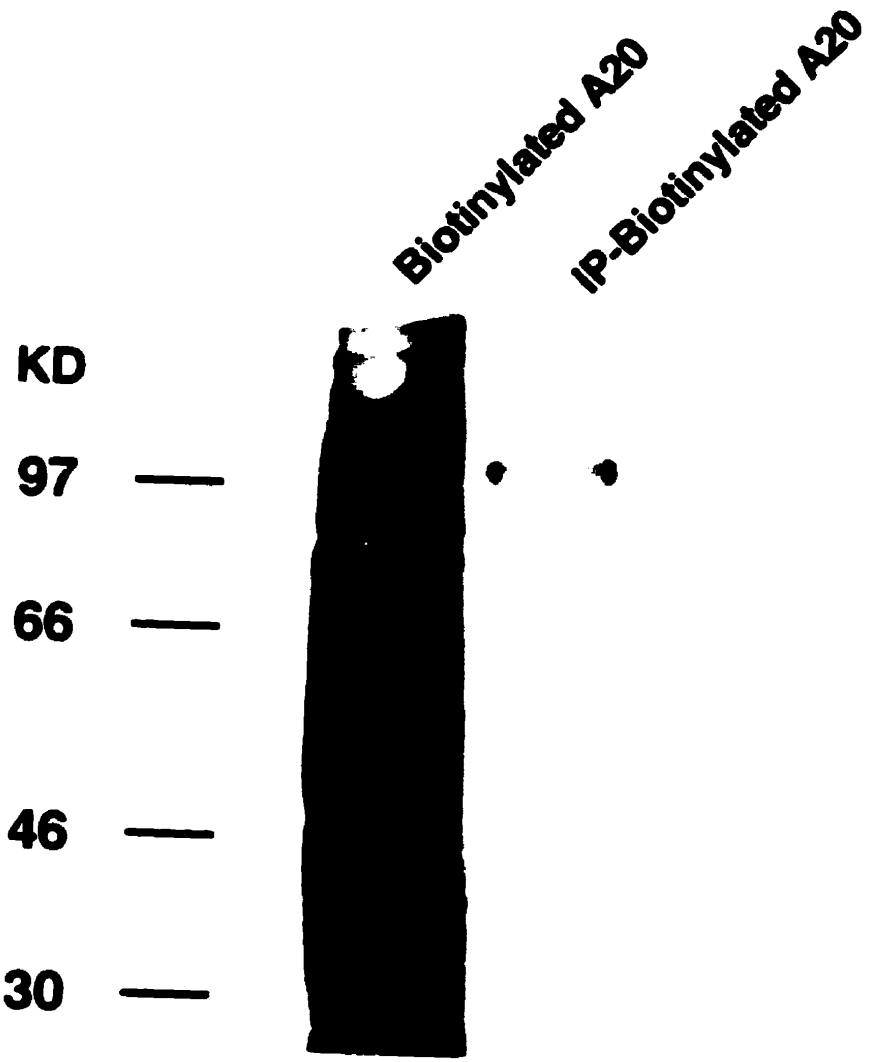
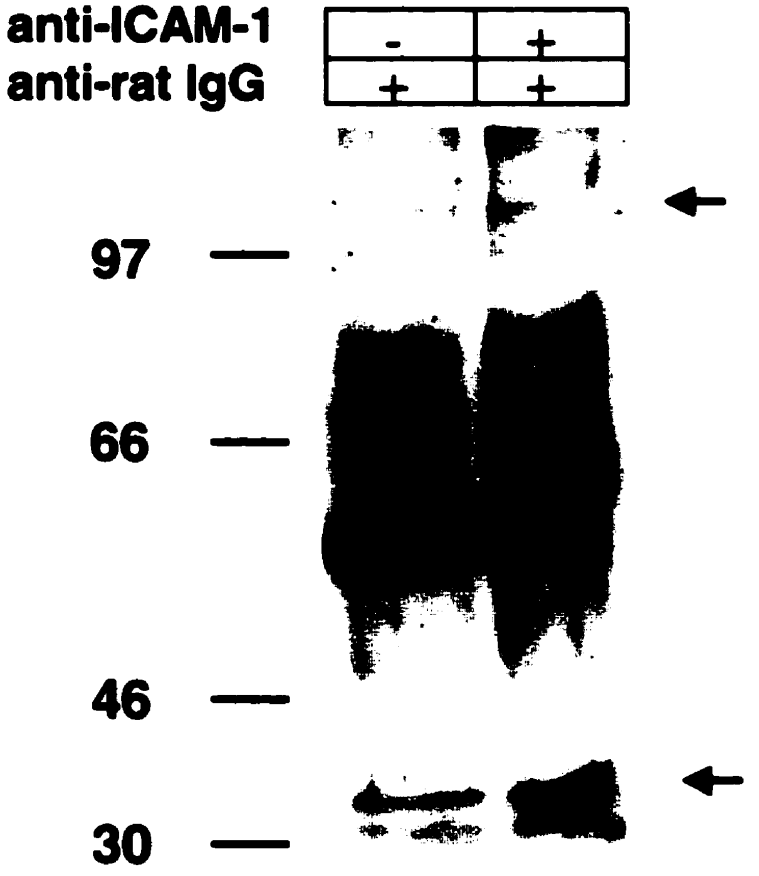
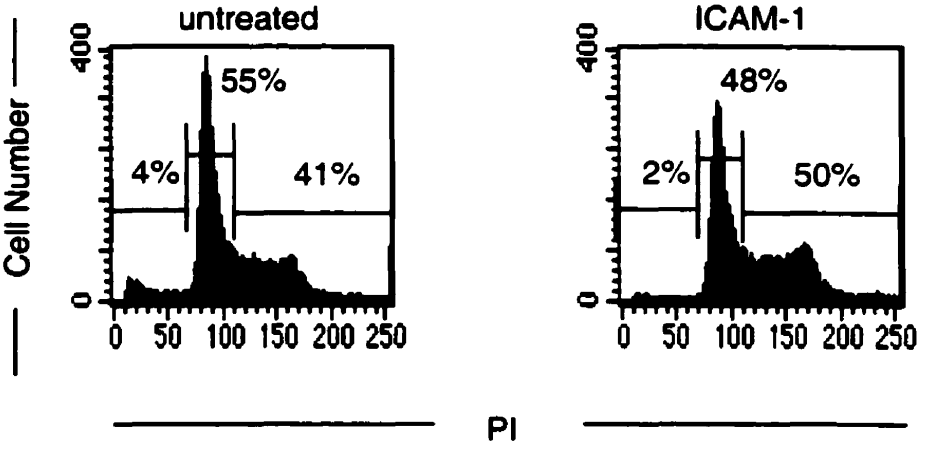

Biotinylated A20
IP-Biotinylated A20
KD
97
66
46
30

Figure 3.7 Phosphorylation of ICAM-1 immunoprecipitated from stimulated A20 cell lysates. A20 cells were treated by crosslinking ICAM-1 with MAR and lysates were immunoprecipitated with anti-ICAM-1-coupled Protein G sepharose and immunoprecipitates were separated by 10% SDS-PAGE. After Western transfer, membranes were blotted with PY72.


anti-ICAM-1
anti-rat IgG
-
+
+
+
97
66
46
30

A


untreated
ICAM-1
Cell Number
400
0
55%
4%
41%
48%
2%
50%
0 50 100 150 200 250
PI

B

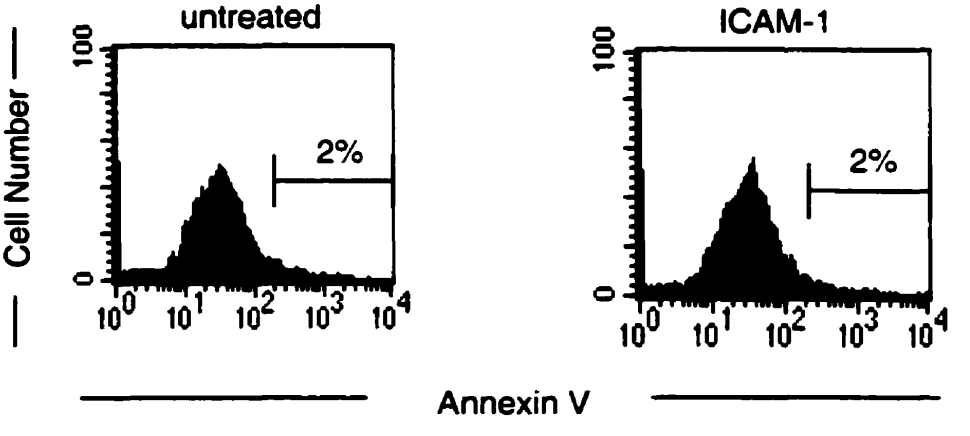

untreated
ICAM-1
Cell Number
100
0
2%
2%
10^0 10^1 10^2 10^3 10^4
Annexin V

Figure 3.8 ICAM-1 crosslinking had no effect on growth of A20 cells. A20 cells were incubated with YN1/1.7.4.1 and MaR 18.5 or left untreated for 2 days. Intracellular DNA content was determined by PI staining (A). Annexin V staining was used to assess apoptosis of A20 after iCAM-1 ligation (B).

Chapter 4

Conclusion

Fas engagement contributes to cell fate determination in a variety of cell types, and plays an important role in modulating immune response and regulating autoreactive B lymphocytes (Rathmell et al., 1996; Foote et al., 1998). The results reported here showed that B lymphoma cells, M12, TA3 and WEHI-279 are resistant to Fas killing.

Emergence of resistance to apoptosis in tumor cells is common and may be fundamental to tumor progression (O'Connell et al., 1999). Mechanisms of Fas resistance are highly variable, including downregulation of Fas expression (Moller, P. et al., 1994), deletion of Fas (Strand et al., 1996), the expression of soluble Fas lacking a transmembrane domain (Natoli et al., 1995), downregulation of caspase in breast cancer and leukemia cells (Keane et al., 1996; Tamura et al., 1996), expression of high level of FLIP in melanoma cell lines (Irmler et al., 1997), and high expression of Fas-associated phosphatase-1 (FAP-1) (Sato et al., 1995). Recently, a novel gene was identified, termed "FAIM" which opposes Fas mediated apoptosis when overexpressed in a B cell line, and whose expression in primary B cells is coordinately regulated with sIg signals that block Fas killing (Schneider et al., 1999). Although the cause of Fas resistance in B lymphoma cells remains to be elucidated, our results showing that these 4 lymphoma cells all express Fas antigen eliminate depletion of cell surface Fas receptor as a cause. One possible reason why anti-Fas-mediated apoptosis occured only in A20 cells may be that this cell line expressed the highest levels of Fas as

determined by cell surface staining and FACS analysis. The resistance of M12, TA3 and WEHI-279 to Fas-mediated killing may also suggest a defect at a later point in the apoptosis signal transduction pathway.

There is evidence that members of Bcl-2 family regulate Fas-mediated apoptosis in some cell types. Ectopic expression of Bcl-2 rescued human Burkitt lymphoma Ramos B cells from apoptosis induced by Fas (Alam et al., 1997) and overexpression of Bcl-2 in WR19L B lymphoma cells resulted in inhibition of Fas-induced cell death (Itoh et al., 1993). Breast cancer cell lines that express low levels of Bax showed resistance to apoptosis induced by CD95 (Bargou et al., 1995). Bax was shown to play a critical function in Fas-mediated cell death in Burkitt's lymphoma cell lines (Gutierrez et al., 1999) Overexpression of Bax in these cancer cells restored the sensitivity to Fas-triggered apoptosis (Bargou et al., 1996). Upregulation of Bcl-2 inhibited anti-CD95-induced apoptosis in human glioma cell lines (Weller et al., 1995). Bcl-XL was induced in primary B cells after sIg engagement that produces Fas resistance (Schneider et al., 1997). Our data show that sensitivity of B lymphoma cells correlated poorly with expression of Bcl-2 family proteins. The lymphoma cells M12 and TA3 that express a high level of endogenous Bax and low level of Bcl-2 are resistant to anti-Fas mAb, whereas A20 that expresses the highest level of Bcl-X was sensitive to Fas-mediated killing. So no obvious correlation was observed between susceptibility to anti-Fas mAb and Bcl-2 protein expression in B lymphoma cells A20, M12, TA3 and WEHI-279. Our data parallel other findings that Bcl-2 did not interfere with Fas antigen-mediated apoptosis in myeloma cells (Shima et al., 1995) and that sensitivity to anti-Fas-mediated apoptosis was independent of the density of Fas expression on the cell surface and

independent of the amount of Bcl-2 in T-ALL lymphoblastoid leukemia (Debatin and Krammer, 1995).

Oncogene and tumor suppressor gene mutation may be linked with Fas resistance in some cancer cell lines. Mutation of p53 interferes with apoptosis and impairs Fas signaling (O'Connell et al., 1997). Recent evidence indicated that c-myc, in addition to controlling proliferation, can also induce apoptosis via the Fas pathway (Hueber et al., 1997). Further study is required to elucidate the mechanism for the acquisition of resistance against anti-Fas by B lymphoma cells.

FasL was initially thought to be expressed only in cells of the lymphoid/myeloid lineages including T cells, natural killer cells, B cell and phagocytes. It has also been shown to be expressed by non-lymphoid cells, where it contributes to immune privilege by inducing apoptosis in infiltrating immunocytes (Griffith et al., 1995; Bellgrau et al., 1995). This mechanism is analogous to the established role of FasL in mediating immune privilege in mice. FasL expressed in tissues and at sites of immune privilege, such as the testis (Bellgrau et al., 1995) and the eye (Griffith et al., 1995), induces apoptosis in activated lymphocytes that infiltrate these sites. We demonstrated that B lymphoma cells express Fas ligand through analysis of Fas receptor expression by flow cytometry. Coexpression of Fas and FasL by B lymphoma cell does not lead to increased cell death. Our results were consistent with another study in which colon cancer cells expressed both Fas and functional FasL, and killed T cells, suggesting a Fas counterattack model as a mechanism of immune escape in cancer cell (O'Connell et al. 1996). Further experiments should assess whether Fas ligand expressed in B lymphoma cells is functional.

Anti-CD40 induced apoptosis in mature phenotype B lymphoma cells (A20, M12 and TA3) through upregulation of tumor suppressor p53 and CDKI p21 and p27. High expression of Bax is linked to mature phenotype, high expression of Bcl-2 to immature lines. Anti-Fas induced apoptosis only in A20, not in M12, TA3 or WEHI-279, independently of the cell phenotype and without obvious correlation with expression of Bcl-2 family proteins. The cells that died in response to anti-CD40 stimulation aggregated (Fig. 2.1B); this morphological change did not occur in non-responding lines (WEHI-279 and WEHI-231), nor in cells in response to anti-Fas-mediated apoptosis. These data suggest a difference in response to anti-CD40 mediated early in the signal transduction cascade between mature and immature B lymphoma cell lines and demonstrate differences between the apoptotic pathway induced by anti-CD40 from that induced by anti-Fas.

CD40 and CD95 are both members of TNF receptor family, showing structural homology of intracellular domains. The pre-eminent role of CD40 is its ability to rescue germinal center B cells from undergoing apoptosis (Liu et al., 1989; Gregory et al., 1991). Interaction of Fas and FasL induces apoptosis of a variety of cell types, including lymphocytes. In the current study, we investigated the effect of CD40 and CD95 in transformed B cells and we showed distinct effects of CD40 and CD95 on B lymphoma cells from those on B cells. It will be interesting to know the mechanisms that underlie this different effect on B lymphoma cells.

We also assessed the phosphorylation of ICAM-1. Our preliminary data showed that ICAM-1 crosslinking on the A20 B lymphoma line induced hyper-phosphorylation of a 90-100 KD band, presumed to be ICAM-1itself. Associated lower molecular weight bands in the 35-40 KD range

were also observed. This suggests that ICAM-1 might be phosphorylated upon stimulation with anti-ICAM-1.

ICAM-1/CD54 is an important adhesion and signaling molecule involved in B cell activation. The cytoplasmic domain of ICAM-1 contains two tyrosine residues. There exists ICAM-1 isoforms and all these isoforms can be expressed at cell surface and recognized by ICAM-1 Abs (King et al., 1995). Signaling from surface receptor usually involve cross-talk with others and are integrated to produce the final response. Induction of CD80 and increased expression of ICAM-1 and CD86 are dependent on the interaction of CD40 and CD40 ligand (Evans et al., 2000). NF-Kappa B activation correlated with the ability of CD40 to induce the upregulation of ICAM-1 and LFA-1 (Hsing et al., 1997). CD40-CD40L interaction upregulated ICAM-1 expression on fibroblasts (Yellin et al., 1995). Blocking antibodies against LFA-1/ICAM-1 system and antisense oligodeoxynucleotides against CD11a canceled the rescue effect of anti-CD40 antibody against anti-IgM-induced DND-39 cell death, showing that the LFA-1/ICAM-1 interaction plays an important role in the CD40-mediated inhibition of surface IgM-mediated negative signals (Mayumi et al., 1995). Adhesion through the CD11a/CD54-dependent pathway has been reported to prevent apoptosis of germinal center B cells (Koopman et al. 1994). Coligation of ICAM-1 and MHC II induced expression of a functional IL-2 receptor and cytokine responsiveness on primary B cell (Poudrier and Owens, 1994). Fas antigen and ICAM-1 were coregulated in tonsillar B cells and expression was also coordinated in follicular B cells and B-cell lymphomas (Moller et al., 1993). These findings suggest that adhesion molecules are implicated in both formation of cellular connections and in signal delivery. However, ICAM-1 deficient mice

develop normally and show normal lymphocyte populations, but these mutant mice exhibited abnormalities of inflammatory and immune function and display resistance to septic shock (Sligh et al., 1993; Xu et al., 1994). In addition, ligation of CD40 induces Fas expression on B lymphocytes and enhances Fas susceptibility of these cells (Schattner et al., 1995). Thus it appears that these molecules actively cooperate with each other to modulate cell function.

Understanding the biochemistry of the surface receptor junction on B lymphocytes is an important step towards understanding how B cells integrate a multitude of signals to produce an appropriate immunological response. To defind the mechanism of these receptors to regulate cellular activity, and the signal transduction pathways utilized by B cell surface molecules and their relationship is a prominent challenge of future studies.

References

Alam, M. K., Davison, S., Siddiqui, N., Norton, J. D., and Murphy, J. J. (1997). Ectopic expression of Bcl-2, but not Bcl-xL rescues Ramos B cells from Fas-mediated apoptosis. Eur. J. Immunol. *27*, 3485-3491.

Bargou, R. C., Daniel, P. T., Mapara, M. Y., Bommert, K., Wagener, C., Kallinich, B., Royer, H. D., and Dorken, B. (1995). Expression of the bcl-2 gene family in normal and malignant breast tissue: low bax-alpha expression in tumor cells correlates with resistance towards apoptosis. Int. J. Cancer. *60*, 854-859.

Bargou, R. C., Wagener, C., Bommert, K., Mapara, M. Y., Daniel, P. T., Arnold, W., Dietel, M., Guski, H., Feller, A., Royer, H. D., and Dorken, B. (1996). Overexpression of the death-promoting gene bax-alpha which is downregulated in breast cancer restores sensitivity to different apoptotic stimuli and reduces tumor growth in SCID mice. J. Clin. Invest. *97*, 2651-2659.

Bellgrau, D., Gold, D., Selawry, H., Moore, J., Franzusoff, A., and Duke, R. C. (1995). A role for CD95 ligand in preventing graft rejection. Nature *377*, 630-632.

Debatin, K. M., and Krammer, P. H. (1995). Resistance to APO-1 (CD95) induced apoptosis in T-ALL is determined by a BCL-2 independent anti-apoptotic program. Leukemia. *5*, 815-820.

Evans, D. E., Munks, M. W., Purkerson, J. M., and Parker, D. C. (2000). Resting B lymphocytes as APC for naive T lymphocytes: dependence on CD40 ligand/CD40. J Immunol. *164*, 688-697.

Foote, L. C., Marshak-Rothstein, A., and Rothstein, T. L. (1998). Tolerant B lymphocytes acquire resistance to Fas-mediated apoptosis after treatment with interleukin 4 but not after treatment with specific antigen unless a surface immunoglobulin threshold is exceeded. J. Exp. Med. *187*, 847-53

Gregory, C. D., Dive, C., Henderson, S., Smith, C. A., Williams, G. T., Gordon, J., and Rickinson, A. B. (1991). Activation of Epstein-Barr virus latent genes protects human B cells from death by apoptosis. Nature. *349*, 612-614.

Griffith, T. S., Brunner, T., Fletcher, S. M., Green, D. R., and Ferguson, T. A. (1995). Fas ligand-induced apoptosis as a mechanism of immune privilege. Science. *270*, 1189-92.

Gutierrez, M. I., Cherney, B., Hussain, A., Mostowski, H., Tosato, G., Magrath, I., and Bhatia, K.. (1999). Bax is frequently compromised in Burkitt's lymphomas with irreversible resistance to Fas-induced apoptosis. Cancer Res. *59*, 696-703.

Hsing, Y., Hostager, B. S., and Bishop, G. A. (1997). Characterization of CD40 signaling determinants regulating nuclear factor-kappa B activation in B lymphocytes. J Immunol *159*, 4898-906

Hueber, A. O., Zornig, M., Lyon, D., Suda, T., Nagata, S., and Evan, G. I. (1997). Requirement for the CD95 receptor-ligand pathway in c-Myc-induced apoptosis. Science . *278*, 1305-1309.

Irmler, M., Thome, M., Hahne, M., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schroter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997). Inhibition of death receptor signals by cellular FLIP. Nature. *388*, 190-195.

Itoh, N., Tsujimoto, Y., and Nagata, S. (1993). Effect of bcl-2 on Fas antigen-mediated cell death. J Immunol. *151*, 621-627.

Keane, M. M., Ettenberg, S. A., Lowrey, G. A., Russell, E. K., and Lipkowitz, S. (1996). Fas expression and function in normal and malignant breast cell lines. Cancer Res. *56*, 4791-4798.

King, P. D., Sandberg, E. T., Selvakumar, A., Fang, P., Beaudet, A. L., Dupont, B. (1995). Novel isoforms of murine intercellular adhesion molecule-1 generated by alternative RNA splicing. J. Immunol.*154*, 6080-6093

Koopman, G., Keehnen, R. M., Lindhout, E., Newman, W., Shimizu, Y., van Seventer, G. A., de Groot, C., and Pals, S. T. (1994). Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. J Immunol. *152*, 3760-3767.

Liu, Y. J., Joshua, D. E., Williams, G. T., Smith, C. A., Gordon, J., and MacLennan, I. C. (1989). Mechanism of antigen-driven selection in germinal centres. Nature. *342*, 929-931.

Mayumi, M., Sumimoto, S., Ohshima, Y., Katamura, K., Heike, T., Hata, D., Kanazashi, S., and Furusho, K. (1995). Role of LFA-1/ICAM-1-dependent cell adhesion in CD40-mediated inhibition of anti-IgM antibody-induced B-cell death. J Allergy Clin Immunol. *96*, 1136-1144.

Moller, P., Henne, C., Leithauser, F., Eichelmann, A., Schmidt, A., Bruderlein, S., Dhein, J., and Krammer, P. H. (1993). Coregulation of the APO-1 antigen with intercellular adhesion molecule-1 (CD54) in tonsillar B cells and coordinate expression in follicular center B cells and in follicle center and mediastinal B-cell lymphomas. Blood *81*, 2067-2075.

Moller, P., Koretz, K., Leithauser, F., Bruderlein, S., Henne, C., Quentmeier, A., and Krammer, P. H. (1994). Expression of APO-1 (CD95), a member of the NGF/TNF receptor superfamily, in normal and neoplastic colon epithelium. Int. J. Cancer.*57*, 371-7.

Natoli, G., Ianni, A., Costanzo, A., De Petrillo, G., Ilari, I., Chirillo, P., Balsano, C., and Levrero, M. (1995). Resistance to Fas-mediated apoptosis in human hepatoma cells. Oncogene *11*, 1157-1164.

O'Connell, J., Bennett, M. W., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. (1997). The Fas counterattack: a molecular mechanism of tumor immune privilege. Mol. Med. *3*, 294-300

O'Connell, J., Bennett, M. W., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. (1999). The Fas counterattack: cancer as a site of immune privilege. Immunol Today. *20*, 46-52.

O'Connell, J., O'Sullivan, G. C., Collins, J. K., and Shanahan, F. (1996). The Fas counterattack: Fas-mediated T cell killing by colon cancer cells expressing Fas ligand. J. Exp. Med. *184*, 1075-1082.

Poudrier, J., and Owens, T. (1994). CD54/intercellular adhesion molecule 1 and major histocompatibility complex II signaling induces B cells to express interleukin 2 receptors and complements help provided through CD40 ligation..J. Exp. Med. *179*, 1417-1427.

Rathmell, J. C., Townsend, S. E., Xu, J. C., Flavell, R. A., and Goodnow, C. (1996). Expansion or elimination of B cells in vivo: dual roles for CD40- and Fas (CD95)-ligands modulated by the B cell antigen receptor. Cell 87, 319-329.

Sato, T., Irie, S., Kitada, S., and Reed, J. C. (1995). FAP-1: a protein tyrosine phosphatase that associates with Fas. Science. *268*, 411-415.

Schattner, E. J., Elkon, K. B., Yoo, D. H., Tumang, J., Krammer, P.H., Crow, M. K., and Friedman, S. M. (1995). CD40 ligation induces Apo-

1/Fas expression on human B lymphocytes and facilitates apoptosis through the Apo-1/Fas pathway. J Exp Med. *182*, 1557-1565.

Schneider, T. J., Fischer, G. M., Donohoe, T. J., Colarusso, T. P., and Rothstein, T. L. (1999). A novel gene coding for a Fas apoptosis inhibitory molecule (FAIM) isolated from inducibly Fas-resistant B lymphocytes. J. Exp. Med. *189*, 949-956.

Schneider, T. J., Grillot, D., Foote, L. C., Nunez, G. E., and Rothstein, T. L. (1997). Bcl-x protects primary B cells against Fas-mediated apoptosis. J Immunol. *159*, 4834-4839.

Sligh, J. E. Jr., Ballantyne, C. M., Rich, S. S., Hawkins, H. K., Smith, C. W., Bradley, A., Beaudet, A. L. (1993). Inflammatory and immune responses are impaired in mice deficient in intercellular adhesion molecule 1. Proc. Natl. Acad. Sci. U. S. A. *90*, 8529-8533.

Strand, S., Hofmann, W. J., Hug, H., Muller, M., Otto, G., Strand, D., Mariani, S. M., Stremmel, W., Krammer, P. H., and Galle, P. R. (1996). Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells--a mechanism of immune evasion? Nat. Med.12, 1361-1366.

Tamura, T., Ueda, S., Yoshida, M., Matsuzaki, M., Mohri, H., and Okubo, T. (1996). Interferon-gamma induces Ice gene expression and enhances cellular susceptibility to apoptosis in the U937 leukemia cell line. Biochem Biophys Res Commun. *229*, 21-26.

Weller, M., Malipiero, U., Aguzzi ,A., Reed, J. C., and Fontana, A. (1995). Protooncogene bcl-2 gene transfer abrogates Fas/APO-1 antibody-mediated apoptosis of human malignant glioma cells and confers resistance to chemotherapeutic drugs and therapeutic irradiation. J. Clin. Invest. *95*, 2633-2643.

Xu, H., Gonzalo, J. A., St Pierre, Y., Williams, I. R., Kupper, T. S., Cotran, R. S., Springer, T. A., Gutierrez-Ramos, J. C. (1994). Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. J. Exp. Med. *180*, 95-109.

Yellin, M. J, Winikoff, S., Fortune, S. M., Baum, D., Crow, M. K., Lederman, S., and Chess, L. (1995). Ligation of CD40 on fibroblasts induces CD54 (ICAM-1) and CD106 (VCAM-1) up-regulation and IL-6 production and proliferation. J. Leukoc. Biol. *58*, 209-216.