Regulation of IgA Class Switch Recombination in the I.29μ B Cell Lymphoma by Cytokines and Inhibitors of Poly(ADP-ribose) Polymerase: A Thesis

Penny E. Shockett
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Regulation of IgA Class Switch Recombination in the I.29μ B Cell Lymphoma by Cytokines and Inhibitors of Poly(ADP-ribose) Polymerase

A Thesis Presented By
PENNY E. SHOCKETT

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfilment of the requirements for the degree of:

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Regulation of IgA Class Switch Recombination in the I.29µ B Cell Lymphoma by Cytokines and Inhibitors of Poly(ADP-ribose) Polymerase

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ABSTRACT

Heavy chain isotype switch recombination is preceded by the appearance of RNA initiating 5' of the specific switch region which will undergo recombination. In an effort to understand the potential function of germline transcripts in switch recombination and the degree to which the regulation of germline transcripts correlates with the regulation of switching, we studied this process in the murine B-lymphoma cell line I.29μ, which in the presence of bacterial lipopolysaccharide (LPS) switches primarily to IgA and less frequently to IgE. Levels of α-germline transcripts initiating upstream of α switch (Sa) sequences are elevated in clones of this line which switch well as compared to clones which switch less frequently. TGFβ1 has been shown to increase α-germline transcripts and switching to IgA expression in LPS-stimulated murine splenic B-cells. We now demonstrate in I.29μ cells that TGFβ also increases switching to IgA and increases the level of α-germline transcripts 5 to 9 fold. Nuclear run-on analysis shows that this increase is at the level of transcription. Thus, TGFβ appears to direct switching to IgA by inducing transcription from the unrearranged Sa-Cα DNA segment. Germline α RNA is quite stable in I.29μ cells, having a half life of about 3 to 5 hours, and we find only slight stabilization in the presence of TGFβ. Levels of e-germline transcripts are not increased by TGFβ. IL-4, which modestly increases switching to IgA in I.29μ cells, slightly increases transcription of α-germline RNA. However, we present evidence suggesting that endogenously produced IL-4 may also act at additional levels to increase switching to IgA. IFNγ, which reduces IgA expression in these cells, also reduces the level of α-germline transcripts. IFNγ also reduces the level of e-germline transcripts induced by IL-4. Our results support the hypothesis that the regulation of
transcription of particular switch sequences by cytokines in turn regulates the specificity of recombination.

In studies aimed at identifying other signalling pathways that promote class switching, we discovered that inhibitors of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) increase lipopolysaccharide (LPS)-induced switching to IgA in the B cell lymphoma I.29μ and to IgG1 in LPS + IL-4-treated splenic B cells. PARP, which binds to and is activated by DNA strand breaks, catalyzes the removal of ADP-ribose from NAD+ and poly(ADP-ribosylation) of chromatin-associated acceptor proteins. This enzyme is believed to function in cellular processes involving DNA strand breaks as well as in modulating chromatin structure. In I.29μ cells, PARP inhibitors increase IgA switching by day 2 and cause a 5-fold average increase in switching on day 3 as assayed by immunofluorescence microscopy. The PARP inhibitor, nicotinamide, also causes a reduced intensity of hybridization of Cμ and Cα specific probes to genomic DNA fragments containing the expressed VDJ-Cμ and the unrearranged Sα-Cα segments, respectively, indicating that PARP inhibition increases rearrangement of these fragments. Induction of switching by PARP inhibitors is not mimicked by treatment with cAMP analogs or reduced by inhibitors of protein kinase A (PKA). Induction of switching by PARP inhibitors does not appear to involve increased levels of transcription of the unrearranged Cα gene, although TGFβ is required for optimal induction by PARP inhibitors, consistent with a requirement for transcription of the unrearranged CH gene. PARP inhibitors do not overcome the requirement for endogenously produced IL-4.
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CHAPTER I
INTRODUCTION

The production by B cells of different classes of antibody with distinct effector functions is a regulated process during an immune response. The antibody class is determined by the constant region of the immunoglobulin heavy chain (CH), the expression of which is controlled by a DNA recombination event, known as the H chain isotype switch. Switch recombination causes the rearranged H chain variable (V), diversity (D), and joining (J) segments, which are initially expressed in association with the IgM constant region gene (C\textsubscript{\mu}), to be subsequently expressed with one of six downstream CH genes, ordered in the mouse 5'-'\gamma3-\gamma1-\gamma2b-\gamma2a-\varepsilon-\alpha-3' (Shimizu et al. 1982).

Rearrangement of V, D, and J gene segments occurs in the bone marrow and thymus during pre B-cell development and early T-cell development, respectively, and determines the specificity of an antigen receptor. In contrast, switch recombination occurs in mature B-cells upon antigen exposure in secondary lymphoid organs, thus allowing the antibody class to be dictated by the antigen. Also unlike V(D)J recombination, the recombination that occurs in isotype switching is not site specific. Recombination instead occurs within or near 2 to 10 kb stretches of guanine rich DNA known as switch (S) regions consisting of tandemly repetitive sequences located 5' of C\textsubscript{\mu} and every C region gene except C\textsubscript{\delta} (Nikaido et al. 1981; Nikaido et al. 1982). These repeat sequences are composed of various combinations of motifs such as GAGCT and GGGGT. DNA between S\textsubscript{\mu} and a downstream S region is deleted (Cory and Adams 1980; Honjo and Kataoka 1978; Rabbits et al. 1980) as a circular
DNA excision product (Iwasato et al. 1990; Matsuoka et al. 1990; von-Schwedler et al. 1990). The precise means by which DNA is cut, aligned, and rejoined is unknown as are the roles in recombination of nuclear proteins that have been shown to bind to S regions (Liao et al. 1992; Marcu et al. 1992; Schultz et al. 1991; Waters et al. 1989; Williams and Maizels 1991; Wuerffel et al. 1992; Wuerffel et al. 1990; Xu et al. 1992). Similarly, the sequences CTGG(G) and CCAG occur near switch junctions with nonrandom frequencies but, the functions of these motifs are not known (Chou and Morrison 1993; Iwasato et al. 1992). The correlation between the rearrangement of Sμ DNA and the S-phase of the cell cycle in cells undergoing switching, and the inhibition of switching with inhibitors of DNA synthesis, support a role for DNA synthesis in switch recombination (Gronowicz et al. 1979; Kenter and Watson 1987; A. Kenter personal communication). The finding of base substitutions, duplications and deletions in switch recombination junctions has led to the proposal that an error-prone DNA synthesis event effects recombination (Dunnick et al. 1993; Dunnick and Stavnezer 1990; Dunnick et al. 1989).

One component of the class switching recombination machinery appears to be isotype nonspecific and can be induced in B cells and B cell lines, by coculture with inducers of B cell activation and proliferation, including anti-IgM, anti-δ dextran, activated helper T cells (TH) or their membrane fractions, anti-CD40, CD40 ligand, other cells in the local environment of the B cell, or by bacterial lipopolysaccharide (LPS) (reviewed in Snapper and Finkelman 1993 and Snapper and Mond 1993; Bergstedt-Lindqvist et al. 1984; Coffman et al. 1988; Gascan et al. 1991; Grabstein et al. 1993; Hodgkin et al. 1990; Jabara et al. 1990; Kearney and Lawton 1975; Noelle et al. 1991; Purkerson et al. 1988; Schrader et al. 1990; Severinsson-Gronowicz et al. 1979; Shapira et al. 1992;
Considerable evidence indicates that this general machinery is directed to a particular isotype, or $\text{CH}_i$ gene, by the cytokine-controlled accessibility of a particular switch sequence (Lutzker et al. 1988; Stavnezer et al. 1988). Several studies in mouse B and pre B cell lines show correlations between the switch recombination activity of a particular $\text{CH}_i$ gene, hypomethylation and DNase sensitivity of that $\text{CH}_i$ gene, and the steady state level of RNA transcripts initiating upstream of and proceeding through the corresponding S region prior to recombination (Lutzker et al. 1988; Schmitz and Radbruch 1989; Stavnezer-Nordgren and Sirlin 1986). Studies in LPS-activated spleen cells have shown that the presence of RNA transcribed from particular unrearranged $\text{CH}_i$ genes correlates with switching to those isotypes (Lutzker et al. 1988; Stavnezer et al. 1988). These RNA, termed germline transcripts, contain upstream exons derived from sequences located 5' of switch regions that are deleted if switch recombination has occurred. These upstream sequences are spliced to segments encoded by $\text{CH}_i$ genes (Gaff and Gerondakis 1990; Gauchat et al. 1990; Gerondakis 1990; Lebman et al. 1990b; Lutzker and Alt 1988; Radcliffe et al. 1990; Rothman et al. 1990; Rothman et al. 1990; Sideras et al. 1989; Xu and Stavnezer 1990).

The function of transcription of these RNA or of the RNA themselves is unknown. A small open reading frame (ORF) with an effective translation initiation codon is found in the upstream exon of the murine $\alpha$-germline transcript (Radcliffe et al. 1990). A minor 1.8 kb species of mouse $\alpha$-germline transcripts contains an open reading frame consisting of approximately 30 bp of sequence of $I_\alpha$ exon spliced to $C_\alpha$ exons (Gaff and Gerondakis 1990). Likewise, the mouse $\gamma_1$ and $\gamma_2\alpha$ germline transcripts contain possible ORFs
consisting of 79 and 33 nucleotides of their respective I exons spliced to C region exons (Collins and Dunnick 1993; Goodman et al. 1993). Use of the γ2a ORF, however, would require bypassing 10 upstream translational start sites and appears unlikely. None of the other mouse or human germline transcripts sequenced contain open reading frames that could encode large proteins (Gerondakis 1990; Lutzker and Alt 1988; Radcliffe et al. 1990; Rothman et al. 1990; Rothman et al. 1990; Sideras et al. 1989). The 1.7 kb mouse α germline transcript has been observed to associate with polysomes, and can be translated in vitro, but attempts to detect a putative peptide encoded by it in cell extracts have been unsuccessful (Radcliffe et al. 1990; D. Waite unpublished data).

It is possible that germline transcripts themselves participate in recombination. The overall structure of all the known germline transcripts is identical. However, the only significant sequence homologies between the 5' exons (I exons) of the corresponding human and mouse germline RNA which have been examined include a 90bp stretch surrounding the start sites of the germline γ RNA (Rothman et al. 1990; Sideras et al. 1989), the 5' flanking region for the ε and α-germline RNA (Gauchat et al. 1990; Lin and Stavnezer 1992; Nilsson et al. 1991; Rothman et al. 1990), and enhancer core-like sequences within the human germline I exons examined (Sideras et al. 1989). These homologies are all suggestive of similar transcription initiation machinery. Comparative searches of secondary structure shared by germline RNA have not been reported.

An attractive hypothesis is that the activation of transcription, by making a particular locus accessible to recombination factors, targets that locus for switch recombination. The idea that transcription promotes
recombination is not unique to switch recombination systems. Transcription has been proposed to increase the frequency of VDJ recombination (Blackwell et al. 1986; Schlissel and Baltimore 1989) and homologous recombination in some yeast systems (Thomas and Rothstein 1989).

It has been well established in limiting dilution studies of murine B-cells activated with LPS or of B-cells clonally activated by T-cells, that cytokines can direct isotype switching to particular isotypes (Bergstedt-Lindqvist et al. 1988; Lebman and Coffman 1988a; Savelkoul et al. 1988). Further evidence that the regulation of transcription determines the specificity of switch recombination comes from the excellent correlation between the effect in vivo and in vitro of cytokines on preferential isotype expression and the induction of germline transcripts in LPS-stimulated mouse B lymphoma lines, pre B lines, and splenic B-cells (Bergstedt-Lindqvist et al. 1988; Berton et al. 1989; Esser and Radbruch 1989; Finkelman et al. 1990; Gauchat et al. 1990; Lebman et al. 1990a; Lutzker et al. 1988; Rothman et al. 1990; Rothman et al. 1988; Savelkoul et al. 1988; Severinson et al. 1990; Stavnezer et al. 1988).

It has been shown in vitro in both polyclonal and antigen specific systems that the ability of one or another type of TH-cell line (or polyclonally activated membrane fractions from either TH type) to support preferential isotype expression by mouse splenic B-cells is largely dependent upon the ability to make (or the presence of) the appropriate lymphokine(s) (Coffman et al. 1988; Hodgkin et al. 1990; Hodgkin et al. 1991; Lebman and Coffman 1988a; Noelle et al. 1991; Stevens et al. 1988). For example, TH2-cells induce IgG1 expression. TH1-cells can support IgG1 expression if supplemented with TH2-cell supernatant or IL-4 and/or anti-IFNγ antibody. (Coffman et al. 1988;
Hodgkin et al. 1991; Stevens et al. 1988). Similarly, activated TH₁-cell membranes supplemented with IL-4 and IL-5 or TH₂ cell supernatant can support IgG₁ responses (Hodgkin et al. 1991; Noelle et al. 1991). Additionally, murine splenic B-cells treated with LPS produce mainly IgG₃ and IgG₂b. When treated with IL-4 in addition to LPS they make increased levels of IgG₁ and IgE (Snapper et al. 1988).

IL-4 is also required for IgE expression in vivo as observed in mice treated with anti-IL-4 antibody or anti-IL-4 receptor antibody during responses to anti-β-dextran or Nippostrongylus brasiliensis (Finkelman et al. 1990). Moreover, IgE responses are absent and IgG₁ responses greatly reduced in mice rendered IL-4 deficient by gene knockout (Kühn et al. 1991). The IL-4-induced expression of these isotypes in normal mouse B cells treated with LPS is preceded by increased levels of γ₁ and e-germline transcripts (Berton et al. 1989; Rothman et al. 1990; Severinson et al. 1990; Stavnezer et al. 1988). Collectively, the data cited above support the model that isotype non-specific recombination machinery is directed to a particular switch region by a mechanism involving cytokine-activated transcription.

Although many studies of cytokine-regulated expression of heavy chain germline transcripts involve analyses of steady state levels of cellular RNA, the available data implicate regulation at the transcriptional level. The induction of DNAse hypersensitive sites 5' to and within the γ₁ switch region upon IL-4 treatment is highly suggestive of transcriptional activation (Berton and Vitetta 1990; Schmitz and Radbruch 1989). However, it is possible that some of the increases in germline transcripts observed after cytokine treatment of B cells occur post-transcriptionally. Regulation of germline transcripts at the
post-transcriptional level might imply that the RNA have additional functions.

Peyer's patches of the gut are major sites of IgA production. In germ-free mice, α-germline transcripts are preferentially detected in Peyer's patch germinal center (PNA high) B-cells after oral immunization with reovirus type 1, suggesting that these germinal centers are sites of IgA switching (Weinstein and Cebra 1991). Transforming growth factor β1 (TGFβ) is a pleiotropic cytokine generally antiproliferative for lymphocytes. Although it has not been directly detected in Peyer's patch germinal centers, for example by in situ hybridization, TGFβ is made by many cell types including T-cells, B-cells and intestinal epithelial cells (Barnard et al. 1989; Kehrl et al. 1986a; Kehrl et al. 1986b; Koyama and Podolsky 1989; McIntire et al. 1993). It has been shown that sIgA⁺, LPS-activated, mouse splenic or Peyer's patch B-cells treated with TGFβ switch to IgA (Coffman et al. 1989; Kim and Kagnoff 1990; Sonoda et al. 1989) and that a 1.3 kb germline α transcript is induced by TGFβ at the steady state level in mouse splenic B-cells prior to switching (Lebman et al. 1990a; Lebman et al. 1990b). In mouse splenic B-cells treated with a variety of different B-cell activators including LPS, anti-δ-dextran, and in polyclonal or antigen-specific T-cell systems, the frequency of surface IgA⁺ (sIgA⁺) cells observed after culture with TGFβ is low (2%), but switching to IgA is increased 4.5 to 16 fold by TGFβ (Coffman et al. 1989; Ehrhardt et al. 1992). TGFβ increases the frequency of recovery of circular DNA resulting from the joining of Sμ to Sα from mouse LPS-activated B-cells (Iwasato et al. 1992; Matsuoka et al. 1990) and TGFβ has also been shown to influence expression of IgA and α-germline transcripts in human B-cells (Defrance et al. 1992; Islam et al. 1991; Lars and Paschalis 1993; Vlasselaer et al. 1992). The only other cytokine shown to influence expression of IgA and to induce α-germline transcripts is IL-4,
although IL-4 is much less effective than TGFβ (Kunimoto et al. 1988; Lebman and Coffman 1988b; Murray et al. 1987; Stavnezer et al. 1988).

The studies reported in this thesis were undertaken to examine the effects of cytokines and other inducers of signaling pathways on the induction of germline transcripts and class switching in the B-cell lymphoma I.29μ. These studies extend earlier studies in the B-cell lymphoma I.29μ and help to solidify the model of transcriptionally directed class switch recombination. Clones of I.29μ cells are induced with LPS to switch to IgA and less frequently to IgE (Stavnezer et al. 1985). The α and ε loci in these cells are hypomethylated, and α and ε germline transcripts are present constitutively (Stavnezer-Nordgren and Sirlin 1986). Additionally, clones of this line which have higher levels of germline α RNA switch to IgA more frequently than lines that have less (Stavnezer et al. 1988; Stavnezer et al. 1990). In this thesis I demonstrate that TGFβ stimulates LPS-induced switching to IgA in the I.29μ B-cell lymphoma. I show directly though nuclear run-on experiments that TGFβ treatment increases the rate of transcription of the endogenous α-germline gene. I also examine the effects of other cytokines on the regulation of α and ε germline transcripts and on the regulation of IgA expression in I.29μ cells. These studies suggest that TGFβ directs, and IFNγ inhibits switching to IgA by mechanisms involving the regulation of α germline transcription. Experiments showing that anti-IL-4-mediated inhibition of IgA switching does not involve reduced levels of α-germline transcripts also suggest that in this system endogenously produced IL-4 regulates switch recombination at a different level.

Moreover, in an attempt to identify additional signalling pathways that can regulate switch recombination in conjunction with transcriptional
targeting, I tested various inducers of second messenger pathways for their ability to increase IgA switching and/or α-germline RNA in I.29μ cells. I discovered that although the α-germline promoter contains a cAMP response-like element (Lin and Stavnezer 1992), methylxanthines which induce cAMP increase IgA switching in I.29μ cells by a cAMP independent mechanism. The pharmacological agents used in this thesis were chosen for their ability to mimic or block pathways known to be affected by methylxanthines. These studies indicate that inhibitors of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) increase IgA switching and associated gene rearrangements in I.29μ cells. This enzyme binds to and is activated by DNA strand breaks and has been proposed to play a role in DNA repair, amplification of tumor promoting genes, sister chromatid exchange and DNA replication (reviewed in Althaus and Richter 1987 and De-Murcia et al. 1991). Several studies suggest that this enzyme can modulate chromatin structure (DeMurcia et al. 1988; Ding et al. 1992; Thibeault et al. 1992). Upon binding DNA ends at strand breaks, PARP is activated, removes ADP-ribose residues from NAD⁺, and attaches them in branching polymers to chromatin-associated acceptor proteins, including histones, topoisomerases, high mobility group (HMG) proteins and PARP itself (Althaus and Richter 1987). Polymers of (ADP-ribose) can be transient due to rapid degradation by poly(ADP-ribose) glycohydrolase. Auto-poly (ADP-ribosylation) of PARP reduces its DNA binding, resulting in inactivation (Ferro and Olivera 1982; Yoshihara et al. 1981; Zahradka and Ebisuzaki 1982). The NAD⁺ substrate analogs used in this study presumably inhibit both enzyme activity and subsequent release from DNA (Buki et al. 1991). Inhibitors of PARP do not appear to induce α-germline transcription in I.29μ cells, but optimal induction of switching does require
that TGFβ1 be present, emphasizing the requirement for transcription of the unrearranged Cα gene. Treatment with PARP inhibitors cannot overcome the requirement for endogenously produced IL-4 for optimal levels of switching. Possible roles of PARP in switch recombination are discussed.
CHAPTER II
MATERIALS AND METHODS

Chemicals: Reagents dissolved in sterile distilled H₂O were: caffeine; 8-(4-chlorophenylthio)-adenosine 3’: 5’-cyclic monophosphate sodium (cpt-cAMP); N⁶, 2’-O-dibutryl adenosine 3’: 5’-cyclic monophosphate sodium (db-cAMP); 8-(6-aminohexyl)-amino-adenosine 3’: 5’-cyclic monophosphate (8-NH₂-cAMP); N₂, 2’-O-dibutryryl guanosine 3’: 5’-cyclic monophosphate sodium (db-cGMP); 8-bromoguanosine 3’: 5’-cyclic monophosphate sodium (8-Br-cGMP); proflavine; cytosine arabinoside HCl (Ara-C); nicotinamide; nicotinic acid (Sigma Chemical Co., St. Louis, MO); 3-methoxybenzamide (3MB), m-anisic acid (MAA) (Aldrich Chemical Co., Milwaukee, WI); and H-89 2HCl (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA). In most experiments, NaOH was used to adjust the pH of the nicotinic acid and m-anisic acid, so that the culture medium remained neutral upon their addition. 3-isobutyl-1-methyl-xanthine (IBMX), forskolin, 2-chloroadenosine (Sigma), dipyridamole (BIOMOL Res. Lab.), actinomycin D (Act D), and 5,6-dichlorobenzimidazole riboside (DRB) (Sigma) were dissolved in 95% ETOH. Ionomycin calcium salt, A23187 (Sigma), R0-201724 (BIOMOL Res. Lab.), and 1,5-dihydroxyisoquinoline (Aldrich) were dissolved in DMSO. Controls with DMSO or ETOH were included to determine effects on switching to IgA.

Cell line. The sIgM⁺ I.29μ B cell line has been described previously (Alberini et al. 1987; Stavnezer et al. 1990; Stavnezer et al. 1985). Briefly, this cell line was derived from an IgM⁺ IgA⁺ B cell lymphoma that arose spontaneously in I/St strain mice. Heavy chain switching in these cells is accompanied by DNA
recombination between S\(\mu\) and S\(\alpha\) switch regions (Stavnezer et al. 1985). Clones 22A10 and 22D of I.29\(\mu\) (Alberini et al. 1987) were used in these experiments.

**Cell culture.** For RNA blots and run-on experiments 0.25 to 0.75 x 10\(^6\) cells/ml were cultured in 20 to 40ml in T75 flasks. For switch assays, except when cells were taken from large scale inductions, 0.1 to 0.25 x 10\(^6\) cells/ml were cultured in 1-ml volumes in 24-well plates. Cells were cultured at 37\(^\circ\)C in an atmosphere of 8% CO\(_2\) in RPMI (Sigma Chemical Co., St. Louis, MO. or JRH Biosciences, Lenexa, KS) complete medium containing 20% defined fetal calf serum (Hyclone Laboratories, Logan, Utah), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 200 U/ml penicillin, 200 mg/ml streptomycin, 0.1 mg/ml kanamycin sulfate (GIBCO Laboratories, Grand Island, NY), 50\(\mu\)M 2-ME (Sigma), and 0.1 U/ml regular purified pork insulin (Squibb and Sons, Inc., Princeton, NJ). Bacterial LPS was mainly E. coli 055:B5 dissolved in RPMI without L-glutamine (RIBI ImmunoChem. Research, Inc. or Sigma). The optimal dose was from 25 to 50 \(\mu\)g/ml. Platelet derived porcine TGF\(\beta_1\) (R&D Systems, Inc., Minneapolis, MN) was reconstituted in 4 mM HCl. Mouse rIL-4 was either purified (given by Steve Gillis of Immunex Corp., Seattle, WA) (used at 850 U/ml) or optimal concentrations of supernatants from the rIL-4 secreting HeLa cell line H-28 (Bergstedt-Lindqvist et al. 1988) or the plasmacytoma cell line X63-Ag8-65 (Karasuyama and Melchers 1988). Recombinant mouse IFN\(\gamma\) was from Shering Corp., USA and was donated as part of the American Cancer Society's program on interferon. IL-5 was supernanant from an X63 myeloma cell line secreting rIL-5 (Karasuyama and
Melchers 1988). Purified IL-6 (Jambou et al. 1988) was from Dana Fowlkes, Univ. of North Carolina. Anti-IL-4 antibody (11B11, monoclonal rat IgG1) (Ohara and Paul 1985) was either hybridoma culture supernatant (given by Eva Severinson, Stockholm, Sweden) or purified antibody (given by David Parker or Robert Woodland, Worcester, MA). AD8 was a purified rat IgG1 anti-ars idiotype antibody (given by Robert Woodland, Worcester, MA).

Analysis of switching in 1.29μ cells. For day 10 to 11 switch assays, cells were cultured as described above with various inducers, which were either removed on day 3 to 4 of culture or slowly diluted as cells were fed and split. LPS was added on day 0 and cytokines were added on day 0 or day 0 and day 1 in most experiments. For days 2 to 5 switch assays, unless otherwise indicated, 50μg/ml LPS was added on day 0 with other inducers and 2ng/ml TGFβ was added on days 0 and 1 or days 0, 1, and 2. On day 1, 0.8 ml medium was removed and replaced with 1 ml fresh medium prior to addition of TGFβ in most experiments. Dilution of LPS enhanced cell viability in the cultures. Cells were analyzed by immunofluorescence microscopy on the days indicated as described (Stavnezer et al. 1985). Briefly, cells dried onto slides coated with poly-L-lysine (Sigma) were fixed in 95% ETOH. Surface and cytoplasmic staining of IgM and IgA was performed in a humid chamber at room temperature using affinity-purified goat anti-mouse IgM-fluorescein isothiocyanate (FITC) and goat anti-mouse IgA-tetramethylrhodamine isothiocyanate (RITC) (Southern Biotechnology Associates, Inc., Birmingham, AL) diluted 1:100 in phosphate buffered saline containing 1% or 1.5% fetal calf serum and 0.1% or 0.2% NaN3. It should be noted that staining of cells expressing both IgA and IgM withstood acid
treatment for 1 min at 4°C with pH 4.0 acetate buffer (0.085M NaCl, 0.005M KCl and 1% FCS) followed by neutralization in PBS or PBS/FCS/NaN₃ essentially as described (Kumagai et al. 1975), both prior to fixation and post staining, ruling out staining of cytophilic Ig or by cytophilic attachment of staining reagents. For most experiments, results are the average of duplicate wells and 1000 cells were counted per well.

Probes. For RNA blots and Southern blots, probes were DNA fragments which were labeled by random priming, by using single-stranded hexanucleotide primers (Boehinger Mannheim, Indianapolis, IN). For nuclear run-on analysis, denatured plasmid DNAs containing various DNA inserts was immobilized on nylon filters. A50 is an 800-bp PstI cDNA fragment that encodes an uninducible mRNA of unknown identity (Nguyen et al. 1983), GAPDH is a 1.27-kb cDNA fragment encoding glyceraldehyde-3-phosphate-dehydrogenase mRNA (Fort et al. 1985), BS is the Bluescript vector (Stratagene, La Jolla, CA) containing no insert, Cµ is a genomic HindIII fragment of the IgM constant region (M2-5B) (Marcu et al. 1980), -820-0 is an 820bp genomic BamHI-HhaI fragment located immediately 5' to the initiation sites of α-germline RNA (Lin and Stavnezer 1992), Iα is a 1.4kb genomic BamHI-HindIII fragment containing the start site(s) and upstream exon(s) of α-germline transcripts from I.29μ cells (Radcliffe et al. 1990), and So3 is a 320-bp genomic Sau3A fragment from the germline α gene in I.29μ cells consisting of four 80-bp consensus repeats from the 5' portion of the α-switch sequence (Dunnick et al. 1989; Waters et al. 1989). This probe is not reiterated in the genome, as shown by hybridization to Southern blots of SacI/PvuII-digested genomic DNA from I.29μ cells. Cα is a fragment from the cDNA clone pJ55813 encoding Cα
sequences (Marcu et al. 1980; Stavnezer et al. 1988), Ce is a 2.1 kb BamHI-HindIII genomic DNA fragment encoding the 5' portion of the Ce gene (Nishida et al. 1981), the PARP probe is a 1.1 kb cDNA fragment containing sequences from the RNA start site to the internal EcoRI site corresponding to the DNA-binding domain of mouse PARP (Huppi et al. 1989), and C\(\kappa\) is a 730 bp fragment containing cDNA sequences coding for C\(\kappa\) and a small portion of the V region of the MOPC384 \(\kappa\) chain isolated from the plasmid pM384\(\kappa\) by Hind III digestion (Stavnezer et al. 1981).

**RNA blot analysis.** RNA isolation was performed essentially as described by Chirgwin et al. (Chirgwin et al. 1979), by homogenization of frozen cell pellets using an SDT Tissuemizer homogenizer (Tekmar, Cincinnati, OH) in a solution containing 6 M guanidinium isothiocyanate (Bethesda Research Laboratories, Life Technologies Inc., Gathersburg, MD). RNA was pelleted for at least 12 h though a cesium cushion consisting of 5.7 M CsCl (CABOT, Revere, PA) and 0.1M EDTA. RNA pellets were resuspended in 10 mM Tris-HCl, pH7.5, 1 mM EDTA containing 0.5% sodium dodecyl sulfate (SDS)(Sigma), extracted twice with phenol (American Bioanalytical, Natick, MA)/chloroform (Mallinckrodt, Paris, KY) and precipitated at -20°C or -70°C in 0.3 M sodium acetate (Sigma) and 2 volumes of 95% ethanol. RNA pellets were washed twice with 70% ethanol and resuspended in double-distilled H\(_2\)O. All solutions were treated with 0.1% diethyl pyrocarbonate (Sigma) prior to use. RNA was quantitated spectrophotometrically, and either 1.5 to 3 \(\mu\)g poly(A\(^{\ast}\)) (selected once on oligo(dT)- cellulose (Collaborative Research, Inc., Bedford, MA) or 10 to 20 \(\mu\)g total cell RNA was fractionated by electrophoresis on 1.0 to 1.4% agarose gels (Boehinger Mannheim Biochemicals, Indianapolis, IN) in 2.2 M formaldehyde.
RNA was blotted to nylon filters (Zetabind, American Bioanalytical, Natick, MA) in 10X SSC (1.5 M sodium chloride, 0.15 M sodium citrate) by capillary transfer. Filters were either prehybridized in 3x SSC, 5x Denhardt's solution (containing 0.1% BSA fraction V (Sigma), 0.1% Ficoll 400 (Pharmacia, Inc., Piscataway, NJ), and 0.1% polyvinylpyrrolidone (Sigma), yeast RNA (1mg/ml) (Type VI; Sigma), 1% SDS, 50 mM sodium phosphate, 10 mM EDTA, and 50% formamide (BRL, Gathersburg, MD) and hybridized in a similar solution containing 1x Denhardt's solution and 5% dextran sulfate (Pharmacia, Uppsala, Sweden), both at 42°C, or prehybridized and hybridized at 60 to 65°C in an aqueous solution containing 0.5M NaHPO4 (pH7.8) (composed of 268.08g Na2HPO4·7 H2O + 4 ml H3PO4 per liter), 7% SDS, 1% BSA, and 1 mM EDTA, pH8.0, essentially by the method of Church and Gilbert (Church and Gilbert 1984). Final washing of filters was in 0.1x SSC, 0.1% SDS, at 60°C for formamide hybridization and in 0.1x SSC, 0.5% SDS, at 60 to 65°C for aqueous hybridization. Autoradiography was performed at ~70°C with an intensifying screen. Quantitation of hybridization was performed by densitometry (Quick Scan R&D, Helena Laboratories, Beaumont, TX or SLR-1D/2D Biomed Instruments, Inc., Fullerton, CA) or on a Betascope 603 blot analyzer (Betagen, Waltham, MA). The densitometry results for poly(A)+ RNA were corrected for RNA loading by subsequent hybridization of blots with probes specific for the uninduced genes A50 or glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Total cell RNA was normalized to densitometric scans of negatives of photographs of 18 S ribosomal RNA.

**Nuclear run-on transcription analysis.** Cells were cultured with various inducers as described above for northern blot analysis. Nuclei isolation and
run-on transcription reactions were performed essentially as previously described (Greenberg et al. 1985). Nuclei (1 to 3 X 10^7), isolated in buffer containing 0.5% Nonidet P-40 (BDH Chemicals Ltd., Poole, England), were incubated at 30°C for 15 min in a mixture consisting of 100 to 150μCi [32P]-UTP (800 Ci/mmole) or [32P]-CTP (400 Ci/mmole) and 0.2 or 0.5 mM CTP, GTP, and ATP. In later experiments DTT (5 mM) was included but appeared to have no effect. For isolation of 32P-labelled RNA, reaction mixtures were placed directly into 6M guanidinium isothyocyanate solution containing yeast RNA (100 μg/ml) as a carrier, and RNA was extracted at 65°C with phenol, equilibrated with 10 mM Tris, pH7.5, 1mM EDTA pH 8, 0.1M sodium acetate pH 5.2, and 1:24 isoamyl alcohol/ chloroform and subsequently precipitated overnight at -20°C with two volumes 95% ethanol, pelleted at 12,000xg at 4°C, washed in 80% ETOH, and resuspended in DEPC H2O (Galli et al. 1987). Attempts were made to hybridize equal numbers of perchloricacetic acid-precipitable counts from each reaction to duplicate filters containing 5 or 10μg of immobilized, linearized, or supercoiled plasmids. The number of counts hybridized varied from 0.5 to 4x10^6 between experiments. Probes specific for uninduced genes were included on the filters and used to normalize the hybridization results. RNA was nicked before hybridization in 25 to 50 mM NaOH on ice for 5 to 15 minutes, and then neutralized with an equivalent concentration of HCl. Denaturing polyacrylamide gels showed that excessive nicking of the RNA was unnecessary, so 5 minutes was used in later experiments. Prehybridization for at least 1 h and and hybridization for at least 72 h were performed at 42°C in solutions containing 50% formamide, as described above for RNA blots. In initial experiments, filters were washed sequentially in 2x SSC and 0.1x SSC, 0.1% SDS, at 65°C, and then incubated at
37°C in 2x SSC containing pancreatic RNAse A (20μg/ml) (Sigma) and RNAse T1 (10U/ml) (Sigma or Boehringer Mannheim). RNAse treatment eliminates any signal due to hybridization of labeled RNA via unlabeled sequences that are nascent at the time the labeling reaction is started. Filters were then washed at 37°C in 2x SSC, 0.5% SDS (Galli et al. 1987). In later experiments, washing was as described by Chen-Kiang and Lavery (Chen-Kiang and Lavery 1989) with 2x SSC, 0.2% SDS, at 42°C and 55°C, then RNAse A (10μg/ml) and RNAse T1 (10U/ml) treatment in 2xSSC, 0.2% SDS at 37°C, and with proteinase K (100μg/ml) in 2x SSC/0.2% SDS at 37°C. Filters were finally rinsed in 2x SSC, 0.2% SDS. Filters were autoradiographed for 1 to 2 weeks at -70°C, and exposures were scanned by densitometry.

**Measurement of α-germline RNA half-life.** Cells were cultured with inducers as above at 0.25x10⁶/ml in 40 ml volumes. In the actinomycin D experiment, cells were cultured with inducers for 36 h at which time 10μg/ml Act D was added to inhibit transcription. This concentration of Act D was shown by [³H]-uridine incorporation into TCA precipitable counts to completely inhibit transcription in I.29μ cells at the times analyzed. Total cell RNA was isolated before addition of Act D (T₀) and at 2, 6, and 14 h post addition. In the 5,6-dichlorobenzimidazole riboside (DRB) experiment, cells were cultured with inducers for 23 h, at which time 30μg/ml (94μM) DRB was added. Total cell RNA was isolated before addition of DRB (T₀) and at 5, 10, and 15 h post addition. RNA was analyzed by RNA blotting and hybridization with the Iα probe to detect α-germline transcripts. RNA half-life was determined by densitometric scanning of various exposures of blots and normalization to scans of 18S
ribosomal RNA from negatives of photographs of the gels. Half-life plots were constructed by linear regression of scatter plots of time vs. % T0 RNA remaining (Simple Curve Fit, Cricket Graph Version 1.3.2, Cricket Software, Malvern, PA).

**Assay of IL-4 in supernatants from 1.29μ cells.** In the measurement of IL-4 activity in 1.29μ cell supernatants, 1 U of IL-4 activity is defined as that amount giving half-maximal proliferation of the IL-2/IL-4-dependent cell line CTLL. Standards included recombinant IL-4-containing supernatants from H-28 cells or X-63 cells. The presence of IL-4 was detected by assay of [3H]-thymidine incorporation after incubation of CTLL cells with 1.29μ culture supernatants in the presence and absence of the murine IL-4- and murine IL-2-specific antibodies 11B11 (Ohara and Paul 1985) and S4B6 (Mossman et al. 1986), respectively. CTLL cells were plated at 1 x 10^4/ml in 100μl volumes in 96 well plates and were pulsed with 1μCi 3H-thymidine/well for the last 4-7 h of a 24 h incubation period at 37°C and 5% CO2.

**Southern blot analysis:** 1.29μ cells were induced until day 3 as described for switch assays, at which time they were expanded in fresh media until day 7. Genomic DNA was isolated by digestion of cells in pronase (750μg/ml), SDS (1%), RNase (100μg/ml) (Sigma) in STE (10mM Tris-HCl, pH 8, 100mM NaCl, 1mM EDTA, pH 8) for 2 hours at 37°C, followed by three phenol-chloroform extractions and ETOH precipitation. DNA was spooled onto glass rods, washed in 70% ETOH and 95% ETOH and resuspended in TE (10mM Tris, pH 8, 1mM EDTA). DNA was analyzed essentially as described (Southern 1975). 10.5 ug of DNA digested with Bgl II and ETOH-precipitated, was loaded in each lane of a 0.8%
agarose gel. After electrophoresis, DNA was transferred to Nitro Plus nitrocellulose membranes (MSI, Westboro, MA) by capillary action. Filters were baked for 2 hours at 80°C. Hybridizations were performed at 65°C for 2 hours in Rapid Hybridization Buffer (Amersham Corporation, Arlington Heights, IL) as described by the manufacturer.

**Analysis of switching in splenic B cells.** Six to seven week old, conventionally housed, BALB/c mice (Jackson Laboratories, Bar Harbor, ME) were killed by cervical dislocation. Spleens were mashed between frosted glass slides in BSS containing 1.5% heat inactivated FCS (BSS 1.5% ΔFCS) and cell debris was removed by gravity. Red blood cells were lysed in 2 ml Tris ammonium chloride (17 mM Tris-HCL, 144 mM NH4Cl, pH 7.2) per spleen for 5 min. at 37°C, and cells were pelleted and washed 1x in BSS 1.5% ΔFCS. Spleen cell suspensions (2x10⁷/ml) were depleted of T lymphocytes by incubation for 30 min. on ice in an anti-T cell antibody cocktail (Beaudoin et al. 1988) consisting of HO-13-4, a mouse IgM anti-Thy 1.2 (ascites 1:160), and 50% ammonium sulfate cuts of GK1.5.6, a rat IgG₂b anti-L3T4 (1:320), 3.168.8, a rat IgM anti-Lyt2 (1:160); and Jijo.10, an IgM rat anti mouse Thy1 (1:320) in BSS 1.5% ΔFCS. Cells were pelleted, resuspended at 2x10⁷ cells/ml and incubated for 30 min on ice in a solution of MAR 18.5, a mouse IgG₂a anti-rat κ-chain antibody (50% NaSO₄ cut, 1:160 in BSS + 1.5% ΔFCS). Low-tox (Accurate) or agarose-adsorbed (Pel Freeze) guinea pig complement was added to 1:20 or 1:10, respectively, and cells were incubated an additional 45 min at 37°C. Live cells were isolated on Ficoll-hypaque, washed at least 1:10 vol:vol (cells + ficol: BSS 1.5% ΔFCS), pelleted, washed two additional times, and then layered onto Percol gradients (Sigma). Small B cells were pooled from the 66-70% and 60-66% Percoll fractions
Surface staining revealed that this population was >83% IgM+. Cytoplasmic staining of saponin-permeabilized cells demonstrated that this cell population was >93% IgM+ after 24 h preinduction with LPS (Sander et al. 1991). Cells were cultured at 0.5-2.0x10⁶/ml in 5 ml RPMI 1640 containing 10% fetal calf serum (as above except insulin was omitted and CO₂ was 5%). For induction of IgG₁ switching, cells were preinduced for 20-26 hours with 0.5-25 μg/ml LPS. Recombinant IL-4 (30, 300 or 10,000 U/ml) (a gift of Dr. Steve Gillis, Immunex Corp., Seattle, WA), and/or 10 mM nicotinamide or nicotinic acid was added on day 1. In some experiments, cells were analyzed on day 3. In other experiments, inducers were diluted 12.5-fold on day 3 and cells analyzed on day 4 or 5. Approximately 1x10⁶ cells per 100μl were surface-stained for analysis by fluorescence-activated cell sorting (FACS) at 4°C for 30-45 min. with goat anti-IgM-FITC (as above) and affinity purified goat anti-IgG₁-R-phycoerytherin (PE) (Southern Biotechnology Associates) diluted 1:200 and 1:120, respectively, in PBS/1.5% FCS/0.2% NaN₃. Cells were washed 3X in PBS/FCS/NaN₃ at 4°C and resuspended in 0.5ml PBS/FCS/NaN₃. Paraformaldehyde (4%) was added (0.2ml), and cells were vortexed and fixed 5 min. at room temperature. Cells were washed 2X in PBS/FCS/NaN₃ and resuspended in 0.4 ml PBS/FCS/NaN₃ for FACS analysis. Affinity purified goat anti-IgA-FITC and goat anti-IgA-PE (Southern Biotechnology Associates) served as negative staining controls.

Statistical Analysis: In the experiments herein, many values are reported as the average fold increase in several experiments ± the Standard Error of the Mean (SEM) and significance (p). The fold increase is given because levels of
switching and germline transcripts are somewhat variable in 1.29μ cells from experiment to experiment. For switch assays with TGFβ and with inhibitors of PARP, the average raw values ± SEM and p values are also reported. P values were determined for both fold increase and raw values by a paired, two sample, two-tailed, student's t-test for means (Microsoft Excel Version 4.0, Microsoft Corp.). In tables in which the average fold increase is reported, the log_{10} of raw values were used to convert ratios into differences for t-tests. Where multiple comparisons were made, p values were multiplied by the total number of comparisons. This correction, the Bonferroni adjustment, compensates for the increased chance of rejecting the null hypothesis when making multiple comparisons.
CHAPTER III

EFFECT OF CYTOKINES ON SWITCHING TO IgA AND α-GERMLINE TRANSCRIPTS IN THE B LYMPHOMA I.29μ: TGFβ1 ACTIVATES TRANSCRIPTION OF THE UNREARRANGED Cα GENE

TGFβ augments switching to IgA in I.29μ cells. To ask whether TGFβ increased LPS-induced IgA switching in I.29μ cells, as it does in splenic B cells, we determined the percent of cells that stained on their surface or in their cytoplasm with both anti-IgA and anti-IgM antibodies. This assay is the best measure of switching in this system since these cells have recently undergone switch recombination and they cannot be derived by outgrowth or selection of IgA+ cells present at the initiation of the cultures (Stavnezer et al. 1985). We observed increases in LPS-induced switching with TGFβ on days 3-5 (Table I and Fig 1). Photographs of double-positive cells from a day 5 experiment are shown in Figure 2. No increase with TGFβ treatment was observed in the absence of LPS (Table I). Only a 2-fold average increase in IgA expression was observed on day 10 with addition of TGFβ (Table I). Since the optimal fold increase in the percent of double expressing cells with TGFβ occurred on day 3 with TGFβ added on days 0, 1, and 2, this protocol was used for most of the switch assays in this thesis. The average raw values (± SEM) for switching by uninduced I.29μ cells were 0.01% (± 0.01%, n=17), 0.2% (± 0.1%, n=7), and 0% (± 0%, n=4), for double positive cells on days 3, 4, and 5, respectively. The percent of total IgA+ cells averaged, 0.7% (± 0.08%), 0.93% (± 0.1%), and 0.45% (± 0.2%) in the same experiments. Table I summarizes the effects of TGFβ on switching to IgA in I.29μ cells. A summary of the average percent switching to IgA in the presence of LPS or LPS + TGFβ on days 3, 4 and 5 is shown in Figure 1.
TGFβ increases the steady state level of 1.7kb and 1.3kb α-germline transcripts in I.29μ cells. To determine whether the increase in IgA switching with TGFβ treatment was preceded by increased levels of α-germline transcripts, I.29μ cells were incubated for 47 h with increasing concentrations of porcine TGFβ in the presence and absence of 50 μg/ml LPS. Northern blot analysis was performed on poly(A)+ RNA using a probe specific for the Iα upstream exon of the 1.7kb α-germline transcript previously described (Radcliffe et al. 1990) and shown on the map in Figure 3. As shown in Figure 4, in the absence of LPS, TGFβ treatment results in a dose dependent increase in the level of 1.7kb germline α transcript. The mean induction of this RNA in three experiments, after treatment for 43 to 47 h with 2ng/ml TGFβ in the absence of LPS, was 4.9-fold above the untreated control (SE, ± 1.0) (Table I). All hybridization results reported in this thesis were corrected for RNA loading by normalization to signals from uninduced genes (GAPDH and/or A50) or to 18S rRNA.

Although LPS increases switching to IgA in I.29μ cells (Stavnezer et al. 1985), it has little effect on the level of α-germline transcripts (see Figs. 4, 9A, and 9B). In seven experiments in which cells were treated with LPS alone (50μg/ml) for 46 to 48 h, the mean induction of α-germline RNA was 0.9-fold (SE, ± 0.1). However, after longer treatment with LPS, α-germline RNA levels sometimes were found to increase up to about 2-fold (see below). In the presence of LPS, TGFβ at 2ng/ml for 48 h caused a 5.6-fold mean increase in the level of the 1.7kb α-germline RNA, relative to LPS alone (Table I). In general and as seen in Figures 4, 5 and 6, the fold-induction by TGFβ is comparable in the presence and absence of LPS, but the overall level of germline α RNA is reduced in the presence of LPS (Table I).
Subsequent hybridization of the blot in Figure 4 with a probe specific for the C\(\alpha\) gene segment, which detects smaller \(\alpha\)-germline RNA species, showed that the 1.3kb \(\alpha\)-germline transcript was also induced by TGF\(\beta\). This RNA is the same size as the major \(\alpha\)-germline transcript induced by TGF\(\beta\) in murine spleen cells (Lebman et al. 1990a; Lebman et al. 1990b). It contains an upstream exon that is shorter than that seen in the 1.7kb \(\alpha\)-germline transcript resulting from a more 5' splice donor site (Gaff and Gerondakis 1990). The membrane forms of the \(\alpha\)-germline transcript also appear to be induced by TGF\(\beta\) (Fig. 4, upper). The 1.0-kb germline transcript was not induced. The induction of germline RNA by TGF\(\beta\) is specific for the IgA isotype, because the 1.6kb \(\varepsilon\)-germline RNA was not induced (Fig. 4). In two experiments the \(\varepsilon\) germline RNA appeared to be reduced by TGF\(\beta\) treatment in the absence of LPS.

**Kinetics of induction of \(\alpha\)-germline RNA.** Optimal induction of germline \(\alpha\) RNA in 1.29\(\mu\) cells occurred after 24 h of treatment with TGF\(\beta\). The mean induction of \(\alpha\)-germline RNA observed after 22 to 26 h of treatment with 2ng/ml TGF\(\beta\) in the absence of LPS was 8.8-fold (SE \(\pm\) 2.1) (Table I). Twelve-hour treatment with TGF\(\beta\) yielded smaller increases in \(\alpha\)-germline RNA (mean-fold \(\pm\) range, 3.2 \(\pm\) 1.3, in two experiments). In order to examine more carefully the kinetics of induction of \(\alpha\)-germline transcripts by TGF\(\beta\), 1.29\(\mu\) cells were induced for 24 h with 2ng/ml TGF\(\beta\) in the presence or absence of 50 \(\mu\)g/ml LPS. At 24 h, a portion of the cells were removed and analyzed for the presence of \(\alpha\)-germline transcripts. The cells remaining were removed from inducers, diluted two-fold, and recultured for a total of 48 and 72 h. As shown in Figure 5, in the absence of LPS, the 1.7kb \(\alpha\)-germline transcript was induced by TGF\(\beta\)
20-, 17-, and 4.5-fold relative to the control at 24, 48, and 72 h, respectively. In the presence of LPS + TGFβ, α-germline RNA increased 7-, 3-, and 2-fold relative to LPS alone at these same times. LPS alone caused a 2-fold increase in α-germline RNA at 48 and 72 h.

**TGFβ increases transcription of the α-germline gene in 1.29μ cells.** In order to determine whether TGFβ causes an increase in the level of α-germline transcripts by inducing transcription, nuclear run-on experiments were performed. As shown in Figure 6A, nuclei isolated from 1.29μ clone 22D treated for 4, 8, and 18 h with 2ng/ml TGFβ, showed a 2-, 2.4-, and 2.9-fold increase, respectively, of transcription of RNA hybridizing to the $I_\alpha$ probe. As shown in Figure 3, this probe contains sequences corresponding to the $I_\alpha$ upstream exon of the 1.7kb α-germline RNA. Transcription of RNA hybridizing to the $S_{α3}$ (α-switch region) and $C_{α}$ (α-constant region) probes is also increased. Sequences upstream of the transcription initiation sites had background levels of hybridization (-820-0 probe). Although α-germline transcription is much reduced in 1.29μ cells in the presence of LPS, TGFβ treatment still increased transcription (Fig. 6B and Table I). There appears to be no difference in the level of α-germline transcription induced by 0.5ng/ml or 2ng/ml TGFβ in the presence of LPS. The dose dependence of the increase in α-germline transcripts induced by TGFβ on northern blots might be explained by a more sustained increase in transcription with 2ng/ml TGFβ compared to 0.5ng/ml TGFβ.

The greatest increases in transcription of the α-germline gene occurred after 12 h of treatment with TGFβ (Fig. 7 and Table II). The average induction by TGFβ of RNA hybridizing to the $I_\alpha$ and/or $S_{α3}$ probe was 5.6-fold at 12 h in
two experiments.

Although the increases in the steady state level of α-germline RNA induced by TGFβ are sometimes greater than increases observed in transcription assays, the results of two experiments measuring the effect of TGFβ on the stability of α-germline RNA after addition of either of two inhibitors of transcription, 5,6-dichlorobenzimidazole-riboside (DRB) or actinomycin D (Act D), indicated that TGFβ has little or no effect upon the half-life of this RNA, either in the presence or in the absence of LPS (Fig. 8). The half-life of the 1.7kb α-germline RNA averaged 5.5 h (range, ± 2.1) with LPS and 4.9 h (range, ± 0.5) with LPS + TGFβ. These inhibitors were added after different lengths of treatment with LPS (24 h for DRB and 36 h for Act D). Although a small increase in stability was observed with TGFβ in the DRB experiment (less than 2-fold), and we cannot rule out the possibility that TGFβ affects other post-transcriptional events such as RNA processing, the main action of TGFβ on α-germline transcripts appears to be transcriptional.

**IL-4 increases switching to IgA and α-germline transcripts in I.29μ cells.** As shown in Figure 7 and Table III, 12 h of treatment with IL-4 caused a 1.5-fold increase in transcription of the α-germline gene. This amount of induction correlates with the increase in α-germline RNA on RNA blots both in the presence and absence of LPS and the amount of switching induced by IL-4 in I.29μ cells (Table III, Figs. 9B, 10, and (Stavnezer et al. 1988). We detected less than a 2-fold increase in the half-life of α-germline RNA in cells treated with IL-4 in one experiment. The combination of IL-4 and TGFβ does not greatly increase transcription over that induced by TGFβ alone (4.3-fold vs. 3.7-fold over control) (Fig. 7 and Table III). IgA expression on day 10 after treatment
with LPS plus TGFβ is also not significantly increased by addition of IL-4 (Table III). Although IL-4 induces α germline transcripts in the absence of LPS, IL-4 does not induce switching to IgA in the absence of LPS.

**IFNγ reduces the level of α-germline transcripts and expression of IgA in I.29μ cells.** The only other cytokine found to affect the levels of α-germline transcripts or IgA expression in these cells was IFNγ. As shown in the time course experiment in Fig. 9A, IFNγ reduced the level of α-germline transcripts at day 1 and day 2 by 22% and 80%, respectively, in the presence of LPS. The reduction at day 2 is also observed in the presence of IL-4 (43% reduction relative to LPS plus IL-4) (Fig. 9B). IFNγ also reduced IgA expression induced by LPS, both in the presence and in the absence of IL-4 (Fig. 10). IFNγ did not inhibit TGFβ-induced α-germline transcripts in the presence or absence of LPS in one experiment. Table III summarizes the effects of IL-4, IL-5, IL-6, and IFNγ on germline α transcripts, and day 10 expression of IgA in I.29μ cells.

Treatment with IL-5 or IL-6 for 18 to 24 h did not induce transcription of the α-germline gene, nor did these cytokines increase IgA expression (Table III). These results do not rule out the possibility that IL-5 acts on IgA+ I.29μ cells to increase secretion, as it does in IgA+ Peyer's patch B-cells, splenic B-cells, and the CH12LX B-cell lymphoma line (Beagley et al. 1988; Harriman et al. 1988; Kunimoto et al. 1988).

**IL-4 and IFNγ regulate ε-germline transcripts and switching to IgE in I.29μ cells.** I.29μ cells switch at a low frequency to IgE expression. IL-4, which increases this switching, also induces ε-germline transcripts in these cells (Stavnezer et al. 1988; Stavnezer et al. 1990). Because expression of IgE
(Snapper et al. 1988; Snapper and Paul 1987) and the level of ε-germline transcripts (Severinson et al. 1990) are reciprocally regulated by IL-4 and IFNγ in splenic B cells, we determined whether the same was true in I.29μ cells. Consistent with studies in spleen cells, IFNγ reduced the expression of ε-germline transcripts both in the presence and absence of IL-4. IFNγ also inhibited LPS induced switching to IgE by 80% in two experiments, in which 1.0% (± 0.1%, range) of cells were IgE+ in the presence of LPS, and 0.2% (± 0.2%, range) of cells were IgE+ in the presence of LPS + IFNγ.

I.29μ cells synthesize IL-4, and anti-IL-4 inhibits IgA switching in I.29μ cells. During these studies I noticed that I.29μ cells (clone 22D) secrete IL-4 upon LPS treatment. In supernatants from 22D cells treated for 36 or 48 h with LPS, I detected levels of IL-4 ranging from 0.3 to 4.0 U/ml as measured by proliferation of the IL-2/IL-4 dependent cell line, CTLL (Fig.11). Low levels of IL-4 were also detected after 6 h with LPS and after 48 h with LPS + TGFβ. The lower level of CTLL proliferation in supernatants from LPS + TGFβ-treated cells compared to those from LPS-treated cells is most likely due to the approximate 2-fold inhibition of CTLL proliferation by TGFβ. Interestingly, C19, a clone of I.29μ cells which has relatively low levels of α-germline transcripts and which switches poorly to IgA (Stavnezer et al. 1988) made no detectable IL-4 after 36 h of LPS treatment.

The endogenously synthesized IL-4 induced by LPS treatment appears to influence the ability of 22D cells to switch to IgA. We observed that LPS-induced IgA expression on day 10 was inhibited by 67% (SE, ± 9.7%) in 4 experiments by treatment with 2 to 5% supernatant containing anti-IL-4 (11B11, added on day 0) and by 55% in one experiment with purified 11B11 (4.2
μg/ml, added on days 0, 1, 2). The percentage of IgA+ cells on day 10-11 averages 4.9 ± 1.3 in the presence of LPS and 1.9 ± 0.8 with LPS + anti-IL-4 (p<0.05). Figures 12A and B demonstrate that this effect was dose-dependent with 11B11 concentration. At day 10-11, LPS + TGFβ1-induced IgA expression was inhibited 46.9% (range ± 11.2 %) in two experiments. The experiment shown in Fig. 12C demonstrates that the inhibition of IgA expression by anti-IL-4 is reversed when rIL-4 is added back to the cultures. LPS + TGFβ-induced switching to IgA on days 2, 3 and 5 was also inhibited by 11B11 supernatant. LPS + TGFβ-induced expression of IgA+ IgM+ cells and total IgA+ cells were reduced an average of 68.9% (SEM ± 7.2%) and 62.3% (SEM ± 12.1%), respectively, in single experiments performed at each of these times. An irrelevant isotype matched control antibody, AD8, did not inhibit IgA switching on day 3 or 5 (day 2 not examined). Moreover, as shown in the next chapter, analysis of IgA expression induced by other inducers of switching in this system also showed a requirement for endogenously produced IL-4 in the expression of IgA+ IgM+ and IgA+ cells on days 3 and 5.

Endogenously produced IL-4 does not appear to influence the levels of α-germline transcripts in switching cells. 11B11 did not affect levels of α-germline transcripts in cells treated with LPS + TGFβ in two experiments or with LPS alone in three experiments (Fig. 13). Of note is that the quantity of 11B11 supernatant used in these experiments was sufficient to completely block maximal IL-4 induced CTLL proliferation but not IL-2-induced CTLL proliferation. Culture of I.29μ cells for 48 h with 2.5% 11B11 supernatant, in addition to LPS or LPS + TGFβ, completely abolished the recovery of IL-4 activity from culture supernatants whether LPS at 10, 25 or 50μg/ml was used. Levels of CTLL proliferation induced by culture supernatants (and thus IL-4
activity) were comparable regardless of the LPS concentration used. Since LPS does not induce α-germline transcription and anti-IL-4 does not inhibit α-germline transcripts, it is likely that endogenously synthesized (LPS-induced) IL-4 acts at a different level to increase switching to IgA, perhaps to increase recombinase or other activities required for recombination. Anti-IL-4 does not appear to affect cell recovery or ³H-thymidine incorporation in these experiments, ruling out a requirement for IL-4 for DNA synthesis or cell division. It is likely that the relatively low levels of switch induction obtained when IL-4 is added exogenously to LPS stimulated 1.29μ cells is due to the fact that it is made endogenously.
CHAPTER IV

INHIBITORS OF POLY(ADP-RIBOSE) POLYMERASE INCREASE ANTIBODY CLASS Switching

*Methylxanthines but not cAMP increase IgA switching in 1.29μ cells.* Because the α-germline promoter from 1.29μ cells contains an ATF/CRE element (Lin and Stavnezer 1992), we tested whether inducers of cAMP and cAMP analogs would induce switching to IgA by 1.29μ cells. Addition of isobutyl-methylxanthine (IBMX), an inhibitor of cAMP-specific phosphodiesterase, for the first 12 to 48 h of culture, increased the percent of IgA+ cells at day 10 post induction with LPS + TGFβ, as assayed by immunofluorescence microscopy (Fig 14A and B). Similar increases were observed with IBMX alone or in combination with forskolin (an activator of adenylate cyclase).

To more precisely measure switching, we determined the percent of cells expressing both IgA and IgM in their cytoplasm on days 3 to 5. As previously stated, we chose to assay double-positive cells because they cannot be derived by outgrowth or selection of IgA+ cells in the culture (Stavnezer et al. 1985). By these criteria, IBMX significantly increased LPS-induced IgA switching, while forskolin did not (Table IV). These results suggested that the increased switching caused by IBMX was independent of cAMP. Caffeine, another methylxanthine which inhibits phosphodiesterase, increased switching to IgA in the presence of LPS + TGFβ (Table IV).

To further analyze the effect of cAMP, 1.29μ cells were treated with chlorophenylthio (cpt)-cAMP (a phosphodiesterase resistant cAMP analog), dibutyryl (db)-cAMP, or 8-NH2-cAMP in the presence of LPS + TGFβ. Switching to IgA was not significantly increased whether or not TGFβ was present (Table...
IV and Fig. 15). Doses of 1 to 3 mM cpt-cAMP or dbcAMP did not significantly increase the % IgA+ IgM+ cells induced by TGFβ at day 3. Since different isozymes of protein kinase A may be activated by various combinations of cAMP analogs (Beebe et al. 1984), we also examined switching to IgA in I.29μ cells after treatment with combinations of cAMP analogs, but this was also ineffective (Fig. 16).

Several pathways affected by methylxanthines do not increase IgA switching in I.29μ cells. Since the above results indicated that IBMX and caffeine augment LPS-induced switching in I.29μ cells by a cAMP-independent mechanism, we screened compounds known to mimic or antagonize several other signalling pathways influenced by methylxanthines for their ability to influence IgA switching in I.29μ cells. None of the compounds tested could mimic or block the methylxanthine-induced increases in IgA switching, except inhibitors of the nuclear enzyme poly(ADP-ribose polymerase). The results are summarized in Tables IV and V.

Inhibitors of poly(ADP-ribose) polymerase increase IgA switching in I.29μ cells. Because a reported effect of methylxanthines is to inhibit the nuclear enzyme poly(ADP-ribose) polymerase (PARP) (Levi et al. 1978), we tested NAD+ substrate analogs known to more specifically inhibit PARP (Preiss et al. 1971; Purnell and Whish 1980) for their ability to increase LPS- and LPS + TGFβ-induced IgA switching in I.29μ cells. Figure 17 summarizes the raw values of several experiments with the inhibitors nicotinamide and 3-methoxybenzamide (3-MB) and their inactive analogs, nicotinic acid and m-anisic acid (MAA), respectively. The average percent of total IgA+ cells on day
3 is approximately 2% after induction with LPS + TGFβ. After induction by LPS + TGFβ + 3-MB or nicotinamide, 9-11% of the cells are IgA+ on day 3. Similarly, cells expressing both IgA and IgM average 1.5% on day 3 after treatment with LPS + TGFβ and 5-7% after addition of LPS + TGFβ + 3MB or nicotinamide.

Figure 18 shows photographs taken at low magnification of IgM+ cells and IgA+ cells induced by LPS + TGFβ ± nicotinamide or nicotinic acid from an experiment in which a five-fold increase in total IgA+ and IgA+IgM+ cells was induced by nicotinamide at day 3. Photographs taken at high magnification demonstrating double expressing cells like those observed in this study were shown in the previous chapter (Shockett and Stavnezer 1991). Figure 19 demonstrates that both single and double positive cells are resistant to weak acid treatment and thus not likely due to binding of cytophyllic Ig. The percents of IgA+IgM+ and total IgA+ cells induced by nicotinamide or 3MB in the presence of LPS + TGFβ are the highest we have observed at this time. The average induction by PARP inhibitors is 5-7 fold over LPS alone and 5-fold over that obtained using LPS + TGFβ, as measured by the appearance of IgA+IgM+ cells on day 3 (Table V). A 3-fold and 5-fold average increase over LPS alone and LPS + TGFβ alone, respectively, is seen in the percent of total IgA+ cells.

The inactive analogs m-anisic acid (MAA) and nicotinic acid do not cause significant increases in IgA switching (Fig. 17, 18, 20 and Table V). The differences between the fold increase in double positive cells in the presence of active vs. inactive analogs are statistically significant with p<0.004 and p<0.003 for 3MB vs. MAA in the presence of LPS or LPS + TGFβ, respectively, and p<0.04 and p<0.001 for nicotinamide vs. nicotinic acid in the presence of LPS or LPS + TGFβ, respectively.
Switch induction by 3MB is dose responsive and optimal by 2mM and induction by nicotinamide is comparable at 10mM (Fig. 20). This difference approximately corresponds to the relative inhibitory activities of these inhibitors of PARP, since the $K_i$ for 3MB is approximately 10-fold lower than that of nicotinamide (Banasik et al. 1992; Rankin et al. 1989). These compounds do not increase switching in the absence of LPS. Although all of these compounds are somewhat anti-proliferative in this system there was no correlation between effects on cell recovery and ability to increase switching.

We also observe increased IgA switching in I.29μ cells with the more potent and specific PARP inhibitor, 1,5-dihydroxyisoquinoline (1,5-di-OH-isoQ). This compound preferentially inhibits the activity of PARP ($K_i$, 0.39μM) over mono(ADP-ribosyl) transferase ($K_i$, 890μM) in vitro (Banasik et al. 1992). (Nicotinamide and 3MB also preferentially inhibit PARP ($K_i$ nicotinamide, 31μM; $K_i$ 3MB, 3.4μM) relative to mono(ADP-ribosyl)transferase ($K_i$ nicotinamide, 3400μM; $K_i$ 3MB, 2700μM)(Rankin et al. 1989). Table VI shows the results of switch assays with 1,5-di-OH-isoQ. Initially we observed a 2.6-fold increase in the percent of IgA+ cells using 1,5-di-OH-isoQ (30nM) on day 3 with LPS + TGFβ but no increase in double-expressing cells (Experiment I). We then assayed on day 2 to see if there was an earlier increase in IgA+M+ cells. Doses of this compound above 50nM are toxic, but doses of 20, 30 or 40nM increase the percent of IgA+ cells an average of 2.6-fold, 3.6-fold, and 3.5-fold, respectively, and the percent of IgA+1gM+ cells 2.2-fold, 2.1-fold, and 2.2-fold, respectively, in the presence of LPS + TGFβ on day 2 in two experiments (Experiments II and III). These levels are similar to those induced by nicotinamide or 3MB in the same experiments. In experiment IV in which this compound was added at 30nM on both days 0 and 1, increases in the percent of
IgA+ and the percent of IgA+IgM+ cells on day 3 were again similar to increases with nicotinamide in the same experiment. Because it is toxic to I.29µ cells, we were forced to use 1,5-di-OH-isoQ at levels 10-fold lower than the $K_i$ for inhibition of PARP. The other inhibitors of PARP were used at levels substantially above their $K_i$'s for PARP inhibition possibly explaining the slightly weaker effect with 1,5-di-OH-isoQ. We cannot speculate on the differences in uptake or metabolism of these compounds in I.29µ cells.

**Inhibitors of PARP induce rearrangement of Cµ and Cα genes in I.29µ cells.**
Previously it has been shown that induction of switching in I.29µ cells is accompanied by switch recombination involving the tandemly repeated switch regions (Dunnick et al. 1989; Stavnezer et al. 1985). To determine whether inhibitors of PARP increase switch recombination events at the Cµ and Cα loci in I.29µ cells, we cultured cells with inducers of switching, purified genomic DNA, and analyzed it by Southern blotting. Maps of the expressed µ gene and the unrearranged α genes in I.29µ cells prior to switching are shown in Figure 21A.

Figure 21B shows hybridization of $Bgl$II digested DNA from cells cultured with no inducers, LPS, LPS + TGFβ, or LPS + TGFβ + nicotinamide. λHindIII markers are loaded in the leftmost lane. Three fragments are detected after hybridization to a probe specific for Cµ. The largest $Bgl$II fragment (15.4kb) contains the Sµ region and segment of the expressed chromosome located 5' to Cµ. The intermediate sized band (6.0kb) represents the 3'Cµ $Bgl$II fragment present on both the expressed and nonexpressed chromosomes. The smallest band represents the 5'Cµ fragment of the nonexpressed chromosome. Hybridization of the same blot to a Cα probe
detects the 16.6 kb unrearranged \textit{BglII} fragment of the expressed and nonexpressed chromosomes which contains both \textit{S\alpha} and \textit{C\alpha}. The \textit{C\kappa} probe detects a 2.75 kb kappa light chain fragment that does not rearrange in I.29\mu cells. To control for DNA loading and transfer, hybridization results have been normalized to those with the \textit{C\kappa} probe. The hybridization signal observed with this probe appears to correspond with the loading of the gel as visualized by ethidium bromide staining.

Densitometry of this blot (Fig. 21C) shows that the band corresponding to the 5′\textit{C\mu} fragment of the expressed chromosome containing \textit{S\mu} is reduced in intensity after culture with inducers of switching. A 60% diminution is seen with LPS + TGF\beta, and a 75% diminution is seen with LPS + TGF\beta + nicotinamide. Diminution of the 3′\mu fragment is intermediate as would be expected if the expressed \mu gene undergoes deletion but the nonexpressed \mu gene does not. In this clone of I.29\mu cells the 5′\textit{C\mu} \textit{BglIII} fragment of the nonexpressed chromosome is 5.7 kb shorter than in the parental I.29\mu cell line (Stavnezer et al. 1985). Thus, extensive deletion of the switch region may have occurred on this allele, preventing rearrangement. The intensity of hybridization to the unrearranged \textit{C\alpha} fragment is also most reduced in the presence of nicotinamide. The very low intensity of hybridization signal with the \textit{C\alpha} probe of DNA from the nicotinamide-treated cells precluded scanning of this band by densitometry.

Since the signals from the nonexpressed \textit{C\mu} fragment and the \textit{C\kappa} fragment are approximately the same, and are not reduced in intensity after induction, nicotinamide does not appear to be causing a nonspecific nicking of the DNA. Figure 21D shows that in this experiment, nicotinamide increased switching 3 to 4-fold as measured both by IgA+IgM+ and IgA+ cells at day 3. On
day 7, 5.6% (± 0.2% range) of cells treated with LPS + TGFβ, and 16.8% (± 2.0% range) of cells treated with LPS + TGFβ + nicotinamide were IgA+, suggesting that much of the rearrangement of μ and α genes that is evident at this time either does not result in immediate IgA expression or results in nonproductive rearrangements.

Inhibitors of PARP do not increase α-germline transcripts in 1.29μ cells. We next determined whether inhibiting PARP increases IgA switching in 1.29μ cells by increasing transcription through the α-switch region. 1.29μ cells were treated with inhibitors of PARP and total cell RNA was isolated 24 hours later. At this time, TGFβ optimally induces α-germline transcripts (Shockett and Stavnezer 1991). Hybridization of RNA blots with the Iα probe showed that inhibitors of PARP do not increase the 1.7kb α-germline transcript in the presence or absence of TGFβ (Fig. 22A). Levels of α-germline RNA are comparable using either inhibitory or non-inhibitory analogs. The 3.8kb PARP mRNA and the 1.3 kb GAPDH mRNA, which are not significantly affected by these inhibitors, serve as an internal controls. In this experiment, replicate cultures of cells were examined for switching on day 2. Nicotinamide and 3-MB increased double-expressing cells by 10-fold on day 2 relative to induction by LPS + TGFβ. In the presence of LPS + TGFβ + nicotinamide or 3MB, 6% of the cells were IgA+ and 4% were IgA+M+, compared to 0.6% IgA+ and 0.4% IgA+IgM+ with LPS + TGFβ (Fig. 22B).

Induction of IgA switching by PARP inhibitors is not due to elevation of intracellular cAMP levels. Since a reported effect of nicotinamide and its
analogs is to inhibit cAMP-specific phosphodiesterase (Shimoyama et al. 1975), we tested whether an inhibitor of protein kinase A (PKA) would inhibit the induction of switching by nicotinamide. 1.29µ cells were treated with LPS + TGFβ + nicotinamide in the presence or absence of the PKA inhibitor H-89 (Chijiwa et al. 1990). H-89 preferentially inhibits PKA with a 10- and 600-fold lower Ki than protein kinase G and protein kinase C, respectively. If nicotinamide increased switching by inhibiting cAMP-specific phosphodiesterase and consequently increasing intracellular cAMP levels, PKA would be a likely cellular target and H-89 would inhibit the effect of nicotinamide. H-89 did not inhibit the increase in switching to IgA caused by nicotinamide (Fig. 15). and surprisingly, at 12µM H-89, a small increase in switching over that induced by nicotinamide was observed. These results and those with cAMP analogs strongly argue that PARP inhibitors do not increase switching in this system by elevating intracellular cAMP levels and subsequently activating PKA. Previously we observed inhibition of switching with high doses of cAMP analogs (Fig. 15), and although this may be explained by toxic effects, it is also possible that PKA activation is detrimental to switching in this system.

*Treatment with PARP inhibitors cannot overcome the requirement for endogenously produced IL-4 in IgA switching by 1.29µ cells.* To determine whether endogenously produced IL-4 was required for switching to IgA in 1.29µ cells in the presence of PARP inhibitors, 1.29µ cells were induced with LPS + TGFβ + nicotinamide, in the presence and absence of 11B11 antibody, or the irrelevant isotype matched control antibody, AD8. Purified 11B11 (10µg/ml) inhibited switching by 86% and 78% as measured by the percent of
IgA+IgM+ and total IgA+ cells, respectively on day 3 (Figure 23). Inhibition was dose dependent with 11B11 concentration and was not observed in the presence of AD8. Inhibition of double staining cells induced by LPS + 3MB (56%) and LPS + TGFβ + 3MB (53%) was also observed with 2.5% 11B11 supernatant on day 5, but not with AD8. These experiments further demonstrate the requirement for endogenously produced IL-4 for IgA switching by 1.29μ cells.

*Induction of switching in normal splenic B cells by PARP inhibitors.* To determine whether inhibitors of PARP could increase switching in normal B-cells, T-depleted, small, mouse spleen cells were cultured with LPS + IL-4 in the presence or absence of nicotinamide and examined for surface IgG1 and IgM expression by FACS analysis. Addition of nicotinamide to these cultures increased IgG1 switching to a variable extent, and larger inductions by nicotinamide were observed in experiments in which switching was suboptimal. Therefore, in Table VII we separated the data into two groups, one including those experiments in which less than 10% of cells were induced to express IgG1 by LPS + IL-4, and one including experiments in which greater than 10% of cells were induced to express IgG1 by LPS + IL-4. A 2.1-fold increase (P<0.008) was seen with nicotinamide in experiments in which switching was <10%, and a 1.3 fold increase (P<0.007) in experiments in which switching was >10%.
CHAPTER V
DISCUSSION

Our experiments strengthen and extend the previously reported finding that TGFβ induces the steady state level of α-germline transcripts and directs switching to IgA in LPS activated B cells (Coffman et al. 1989; Lebman et al. 1990a). We demonstrate through nuclear run-on experiments that TGFβ induces transcription of the α-germline gene in the B cell lymphoma I.29μ. Increased transcription is observed as early as 4 h, is optimal at approximately 12 h and is still observed 18 h after addition of TGFβ. TGFβ increases transcription both in the presence and absence of LPS. This early activation of transcription is consistent with the hypothesis that TGFβ directs heavy chain isotype switching to IgA by inducing transcription through the region of DNA that will undergo subsequent recombination.

The increased transcription of the α-germline gene in I.29μ cells is followed by a corresponding increase in switching to IgA, as there is an 11-fold increase in the percent of IgA+IgM+ cells by day 3 relative to that induced by LPS alone. This result directly demonstrates that TGFβ induces switching rather than outgrowth of IgA+ cells. Double positive cells must be derived by an active switch event, because I.29 IgA+ cells have deleted the Cμ gene from the expressed chromosome (Stavnezer et al. 1985). Additionally, TGFβ, when present longer in the cultures reduces the percent of IgA+ cells at day 10. The fact that TGFβ induces transcription as early as 4 h after addition also supports the idea that the effect of TGFβ on IgA switching is an early event.

Although TGFβ is antiproliferative for normal mouse B cells in the presence of LPS (Kim and Kagnoff 1990) and for I.29μ cells in the absence of
LPS, TGFβ does not inhibit the growth of I.29μ cells in the presence of LPS. In fact, in the presence of LPS, which is required for switching by I.29μ cells, 2ng/ml TGFβ slightly increases, the cell recovery at day 3 (by 1.3-fold ± 0.12, SEM, in 17 experiments), and proliferation of I.29μ cells, as measured by [3H]-thymidine incorporation at 48 h. These observations suggest that the 11-fold average increase in switching observed in I.29μ cells on day 3 does not result from the selective inhibition of IgM+ cell growth with a consequential outgrowth of IgA+ cells, or the selective outgrowth of IgA+ cells.

The steady state accumulation of α-germline RNA observed on RNA blots in the presence of TGFβ peaks at about 24 h post induction both in the presence and absence of LPS. When inducers are effectively removed after 24 h the level remains increased at 48 h and then declines significantly by 72 h. The increase in α-germline transcripts in I.29μ cells is smaller and occurs with faster kinetics than in murine splenic B-cells induced with LPS + TGFβ. Maximal levels of α-germline RNA were observed on day 4 in spleen B cells (Lebman et al. 1990a). It is possible that the induction in spleen cells requires an activation period that is not needed in I.29μ cells because they are already proliferating. The greater induction of α-germline transcripts in spleen cells as compared to I.29μ cells may be explained by the lack of constitutive transcription of the α-germline gene in spleen cells. It is possible that the constitutive expression of α-germline transcripts in I.29μ cells is due to the presence of 100pM levels of TGFβ detected in the cultures. We have been unable to determine whether this TGFβ originates from the cells or FCS, but preliminary studies suggest that anti-TGFβ antibody inhibits switching in this system.

The fold increase in transcription of α-germline RNA induced by TGFβ is
somewhat less than the optimal increases in steady state levels assayed by RNA blotting. TGFβ, however, has only a small effect on the half-life of α-germline RNA. It is possible that TGFβ increases the efficiency of splicing of α-germline RNA precursors. TGFβ has been shown to regulate the splicing pattern of the fibronectin messenger RNA precursor (Borsi et al. 1990).

Consistent with a requirement for transcriptional activation of the α-germline gene for IgA switching, IFNγ-treatment of LPS-treated I.29μ cells reduces the level of α-germline RNA on blots at day 1 and 2 of culture and also reduces the level of LPS-induced expression of IgA on day 10. IFNγ did not reduce levels of α-germline transcripts in the absence of LPS. Inhibition by IFNγ is observed in the presence and absence of IL-4. A reduction in the frequency of switching to IgA by the CH12.LX lymphoma after IFNγ treatment has also been reported (Whitmore et al. 1991). Unfortunately, due to the very low signal obtained in nuclear run-on assays using nuclei from cells cultured with LPS, it was impossible to determine directly whether IFNγ inhibits transcription of α-germline RNA. Although the reduction in levels of α-germline transcripts on day 1 and 2 after addition of IFNγ strongly suggests that IFNγ is acting early to inhibit switching, we have not determined whether IFNγ reduces the percentage of double positive cells at early times after adding LPS to the cultures, as we have only assayed switching on day 10.

Studies of germline RNA promoter regions in transfection experiments demonstrate that they respond to cytokines that induce the specific germline transcripts. Analysis of the promoter region for α-germline RNA by transfection of reporter constructs into I.29μ cells has shown that TGFβ induces a minimal promoter containing 118 bp of sequence upstream of the first RNA
start site and that two copies of an oligonucleotide containing a pair of tandem repeats found within the α-germline promoter confer TGFβ inducibility to a heterologous promoter (Lin and Stavnezer 1992).

Concurrent studies in other systems of cytokine induced switching and germline transcripts corresponding to isotypes other than IgA also show that cytokines that induce preferential isotype expression in vivo and in vitro (Finkelman et al. 1990; Snapper and Finkelman 1993) also induce the corresponding germline RNA prior to switch recombination (Berton et al. 1989; Esser and Radbruch 1989; Gauchat et al. 1990; Lebman et al. 1990a; Lutzker et al. 1988; Rothman et al. 1988; Rothman et al. 1990; Severinson et al. 1990; Shapira et al. 1992; Stavnezer et al. 1988). For example, in LPS treated mouse splenic B cells and/or B cell lines, interleukin-4 (IL-4) induces IgE and IgG1 switching, Cε and Cγ1-germline transcripts, and reporter genes driven by Cε and Cγ1-germline promoters (Bergstedt-Lindqvist et al. 1988; Berton et al. 1989; Esser and Radbruch 1989; Rothman et al. 1991; Rothman et al. 1988; Savelkoul et al. 1988; Stavnezer et al. 1988; Xu and Stavnezer 1992). As we have shown for TGFβ and α-germline transcripts in I.29μ cells, endogenous ε-germline transcripts in the pre B cell line 18.81A20 are also induced at the transcriptional level (as assessed by run-on assay) by IL-4 (Rothman et al. 1991; Shockett and Stavnezer 1991).

Several recent studies directly indicate that germline transcripts and/or their promoters are required for switch recombination. Recently it was shown that IgE switching in 18.81A20 pre B cells is rendered IL-4 independent by replacement of the ε promoter and I-exon (germline exon) with the Ig VH promoter and μ intron enhancer by gene targeting (Xu et al. 1993). Additionally, in mice lacking the promoter and I exon of the γ1-
germline gene on one allele due to gene knockout, isotype switching occurs only on the undisrupted allele (Jung et al. 1993). Thus, in vivo, switch recombination to IgG1 absolutely requires 5' elements upstream of the start sites and/or the I exon of germline γ1 RNA. Similar results have been obtained in chimeric mice derived from injection of RAG-2 deficient blastocysts with embryonic stem (ES) cells containing homozygous, targeted, replacement of the Iγ2b exon and promoter with a neomycin resistance (neoR) gene expressed in reverse orientation (Zhang et al. 1993). In B cells from these mice, LPS-induced IgG2b switching is severely reduced. These results further define a requirement for either transcription in the correct orientation, γ2b-specific promoter elements, or the germline transcripts themselves, for switch recombination. With regard to IgA expression, it was recently shown in the CH12 lymphoma that reducing the level of α-germline transcripts by treatment with antisense oligonucleotides, or by transient transfection of inducible constructs expressing antisense RNA, resulted in an approximately 40% reduction in expression of IgA+IgM+ cells in the presence of TGFβ + IL-4 on days 2-3 (Wakatsuki and Strober 1993).

We have not determined the percent of I.29μ cells that express α-germline transcripts by in situ hybridization. Thus, we do not know whether the increased transcription observed in the presence of TGFβ occurs in all cells with a fraction subsequently switching to IgA or whether α-germline transcription is only increased in the fraction that is induced to switch. In situ hybridization to γ1-germline transcripts in mouse spleen B-cells treated with LPS + IL-4 shows that the frequency of cells producing γ1-germline transcripts correlates with the frequency of cells that switch to IgG1 (Turaga et al. 1993).
Our studies in I.29μ cells and those cited above suggest that cytokine induced transcription is intimately associated with the process of switch recombination. The exact function of transcription in this process is not yet known. Changes in the methylation status or the torsional state of switch region DNA may be influenced by proteins binding to germline RNA promoters. It has been shown in Xenopus oocytes that in microinjected β-globin DNA packaged into chromatin, torsional changes in the promoter (negative supercoiling), as measured by cruciform extrusion, occur coincidentally with the onset of transcription (Leonard and Patient 1991). These physical changes in the DNA do not require transcriptional elongation, because they occur even in the presence of the polymerase elongation inhibitor, α-aminitin. It has also been shown that the vaccinia virus intermediate transcription factor, VITF-B, can melt sequences around the start site of the vaccinia virus intermediate gene promoter in vitro in the absence of RNA polymerase (or any other preinitiation proteins) as measured by potassium permanganate sensitivity (Vos et al. 1991). This unwinding is stimulated by ATP. With regard to switch recombination, it remains to be determined in greater detail by gene knockout or transfected switch recombination substrates, whether the physical act of transcription, the binding of proteins to germline RNA promoters that open, bend, or demethylate chromatin, or the RNA itself is essential for switch recombination and these requirements are most likely not mutually exclusive.

We consistently see a small but reproducible inductive effect by IL-4 on both switching to IgA and α-germline transcripts in I.29μ cells. LPS treatment of I.29μ cells is necessary for switching, as assayed by the appearance of IgA⁺IgM⁺ cells, but LPS causes only minor increases in the level of α-
germline transcripts and a reduction in \( \alpha \)-germline transcription. Although TGF\( \beta \) optimally increases \( \alpha \)-germline transcripts in the absence of LPS, TGF\( \beta \) does not increase switching in the absence of LPS. Therefore, the activation of germline transcription alone is not sufficient for the activation of switch recombination. Supernatants from LPS-treated I.29\( \mu \) cells contain detectable IL-4. Furthermore, LPS-induced switching to IgA is inhibited 67\% by anti-IL-4 antibody. Since LPS does not induce \( \alpha \)-germline transcription, and since anti-IL-4 does not inhibit levels of \( \alpha \)-germline transcripts in the presence of LPS or LPS + TGF\( \beta \), endogenously produced IL-4 appears to increase IgA switching in I.29\( \mu \) cells by a pathway distinct from transcriptional activation. Consistent with this idea is the report that the frequency of switching to IgA, IgG2b and IgG3 are all increased by IL-4 in the the CH12 B lymphoma (Whitmore et al. 1991), even though in spleen cells and pre B cell lines germline transcripts from the \( \alpha \), \( \gamma_2b \) and \( \gamma_3 \) loci are differentially regulated by IL-4 (Lutzker et al. 1988; Rothman et al. 1990; Severinson et al. 1990). IL-4 also augments non-IgE/IgG\( _1 \) isotype production from B cells cultured with activated T-cell membranes, likewise suggesting that IL-4 may affect switching by a mechanism distinct from the activation of germline transcription (Hodgkin et al. 1991). The CH12 B-cell lymphoma, which switches to IgA constitutively, also produces IL-4 mRNA and activity constitutively (O'Garra et al. 1989). The levels of IL-4 activity detected in CH12 cell supernatants are comparable to those detected in I.29\( \mu \) cell supernatants. In contrast to I.29\( \mu \) cells, maintenance of in vitro proliferation by the CH12.LX clone has been shown to require endogenously produced IL-4 (Louie et al. 1993). Switching to IgA, IgG3, or IgG\( _2b \) in these cells was not significantly affected by inclusion of anti-IL-4 antibody, although only low amounts of antibody were used (2.3\( \mu \)g/mL)
(Whitmore et al. 1991); these amounts have been shown to be insufficient for complete neutralization of endogenous IL-4 as measured by CH12 cell proliferation (Louie et al. 1993). It has been proposed that endogenous IL-4 might be produced in B-cell responses to T-cell independent antigens (O'Garra et al. 1989). This IL-4 could activate switching in B-cells in conjunction with signals through the antigen receptor. Production of IL-4 by untransformed B-cells has not been reported. It would be interesting to determine whether IL-4 can be detected in supernatants from non-IgM isotype-positive wells derived from IgM+IgA-IgG2b-IgG3- normal B-cells plated in limiting dilution in the presence of LPS or LPS + TGFβ.

Inhibitors of PARP increase antibody class switching. Our studies also show that inhibitors of the chromatin-associated enzyme, PARP, increase antibody class switching in the B cell lymphoma I.29μ and in splenic B cells. In I.29μ cells, increased IgA switching is observed as early as 2 days after treatment with inhibitors as evidenced by an increase in the percent of cells expressing both IgM and IgA in their cytoplasm. These cells comprise a transient population in the cultures and are less frequently observed on day 7 or later. Unlike the augmentation of IgA switching by TGFβ, the increase due to PARP inhibitors involves a mechanism distinct from induction of α-germline transcription. Preliminary studies suggest that optimal induction of LPS-induced switching by inhibitors of PARP does, however, require TGFβ, since antibody to TGFβ added to the cultures inhibits this induction, suggesting a general requirement for transcription of the unrearranged α gene. Treatment of I.29μ cells with inhibitors of PARP cannot substitute for endogenously produced IL-4.
We also show that inhibitors of PARP induce rearrangement of DNA fragments containing $S_{\mu}$ or $S_{\alpha}$. Previously it has been demonstrated that switching to IgA in I.29$\mu$ cells occurs by a typical switch recombination event between $S_{\mu}$ and $S_{\alpha}$ sequences, deleting the $C_{\mu}$ gene and all other intervening $C_{H}$ genes, resulting in the joining of the expressed VDJ segment to the $C_{\alpha}$ segment on the expressed chromosome (Dunnick et al. 1989; Stavnezer et al. 1985). Since the sites of switch recombination vary over approximately 2kb in $S_{\mu}$ and in $S_{\alpha}$, if many independent switch events are occurring in the cultures studied in this thesis, then in the absence of outgrowth of IgA$^{+}$ cells, detection of a clear, rearranged, $C_{\alpha}$ fragment on Southern blots from cells treated with PARP inhibitors would be unlikely. A diminution in $C_{\mu}$ and $C_{\alpha}$ hybridizing fragments as was observed on the Southern blot of DNA from cells treated with nicotinamide relative to that from untreated cells, would be expected (Hurwitz and Cebra 1982). The reduction in signal intensity is observed in DNA from cells treated with LPS + TGF$\beta$, but is most marked in DNA from cells also treated with the PARP inhibitor, nicotinamide, indicating that nicotinamide increases rearrangement of these fragments.

**Inducers of cAMP do not increase switching to IgA.** The experiments reported in this thesis demonstrate that although methylxanthines increase LPS or LPS + TGF$\beta$ induced switching to IgA in I.29$\mu$ cells, cAMP analogs tested over a wide range of concentrations cannot mimic this effect. These results are consistent with those of Lyke and Strober, who found that IgA expression induced in LPS-treated splenic B cells by treatment with cholera toxin is due to a cAMP independent mechanism (Lycke and Strober 1989). Because their studies utilized a purified preparation of the B-subunit of cholera toxin, thought to be
incapable of activating intracellular cAMP, questions could be asked regarding the purity of the preparation and whether cAMP was actually not induced. Our finding that the PKA inhibitor H-89 does not inhibit IgA switching in our system is also inconsistent with a stimulatory role for cAMP. Although cAMP analogs do not appear to increase switching to IgA in this system, they do cause a small increase in the level of α-germline transcripts, but only in the absence of LPS (data not shown).

Possible mechanisms of increased heavy chain switching by PARP inhibitors. Because of the multi-faceted and elusive function of poly(ADP-ribose) polymerase in eukaryotic cells, several models of inhibitor action in switch recombination are imaginable. First, PARP, which binds to and is dependent on DNA strand breaks for activation, is a major acceptor of poly(ADP-ribose) in vivo (Althaus and Richter 1987). Auto-ribosylated PARP has a reduced affinity for DNA (Ferro and Olivera 1982; Yoshihara et al. 1981; Zahradka and Ebisuzaki 1982). It is possible that the inhibitors, by preventing auto-poly(ADP-ribosylation), keep PARP bound to DNA ends generated by an endonuclease activity involved in switch recombination (Buki et al. 1991). There are several possible consequences of PARP remaining bound to DNA ends. For example, PARP bound to DNA ends could directly interact with switch factors. Alternatively, DNA damage increases PARP activity and it is suggested that PARP activity accelerates DNA repair (Althaus and Richter 1987; Ding et al. 1992; Satoh and Lindahl 1992; Satoh et al. 1993). Recently it was shown in cell extracts that in the presence of PARP, DNA repair is dependent upon NAD\(^+\), suggesting that automodification and subsequent release of PARP from DNA must occur to allow accessibility to repair enzymes (Satoh and Lindahl 1992;
Satoh et al. 1993). PARP bound to DNA ends in switch regions may temporarily reduce accessibility to DNA repair enzymes, thus allowing switch regions to align and recombine by protecting unrecombined DNA ends from religation. This might be especially crucial since actively transcribed DNA sequences, like switch regions prior to switch recombination, are believed to be preferentially repaired over untranscribed DNA (Buratowski 1993; Hanawalt and Mellon 1993). Another possibility is that PARP bound to DNA ends in switch regions may protect the DNA from degradation by cellular nucleases (Sastry et al. 1989).

Second, PARP is capable of binding to unusual DNA structures such as cruciform DNA (Sastry and Kun 1990) and DNA/RNA hybrid molecules (Buki et al. 1991). Recent work has suggested that the α-switch region can form triple stranded DNA structures which, when transcribed, may be stabilized by a RNA/DNA interaction (Collier et al. 1988; Reaban and Griffin 1990). It is possible that PARP could bind and stabilize such a structure.

Third, inhibition of PARP could increase isotype switching by inhibiting poly(ADP-ribosylation) of a protein required for recombination. The activities of most enzymes are inhibited by poly(ADP-ribosylation) (Althaus and Richter 1987; Yoshihara et al. 1985). For example, topoisomerase I and II, enzymes that cut and rejoin DNA, are both inhibited by poly(ADP-ribosylation) (Darby et al. 1985; Ferro et al. 1983; Jongstra-Bilen et al. 1983; Kasid et al. 1989). If their activities are required for switch recombination, inhibition of PARP would enhance recombination. Interestingly, PARP and topoisomerase I have been co-purified (Ferro et al. 1983; Jongstra-Bilen et al. 1983).

A fourth model involves the finding that chromatin condensation and
nucleosome structure can be modulated by levels of poly(ADP-ribosylation) and/or presence of polymer (DeMurcia et al. 1988; Thibeault et al. 1992). It has been shown recently that chromatin from HeLa cells, depleted of endogenous PARP by stable transfection of constructs inducibly expressing PARP antisense RNA has increased DNAse I sensitivity and the cells have delayed DNA single strand break repair kinetics (Ding et al. 1992). It is possible that the inhibition of polymerase dictates a chromatin conformation that increases accessibility to recombination enzymes. However, this model might predict that inhibitors of PARP would influence transcription of α-germline RNA.

A final model predicts that PARP inhibition increases the cellular NAD$^+$ pool, which would be beneficial if switch recombination enzymes require NAD$^+$ as a cofactor or the energy of ATP hydrolysis. Other models of how PARP inhibition may increase switching are possible, and the models above are not mutually exclusive.

Interestingly, 3MB has recently been shown to increase the rate of intrachromosomal homologous recombination between a stably transfected pair of mutant thymidine kinase genes in a mouse fibroblast cell line (Waldman and Waldman 1991). The lack of a preference for crossovers vs. gene conversions suggested that PARP inhibition increased the rate limiting step for homologous recombination, which is the induction of DNA strand breaks. Conversely, 3MB inhibited random illegitimate integration of DNA into the genome for which the rate limiting step is thought to be ligation (Farzaneh et al. 1988; Waldman and Waldman 1990). Based on these results and the fact that PARP inhibitors also increase sister chromatid exchange in Chinese hamster ovary (CHO) cells (Oikawa et al. 1980) and possibly mitotic recombination in Drosophila (Ferro et al. 1984), both of which presumably
involve homologies, one might hypothesize that switch recombination is mechanistically more similar to homologous recombination than to illegitimate recombination, and that inhibiting PARP increases the initiation of switch recombination. In CHO cells it was recently shown that intrachromosomal homologous recombination between direct or indirect repeats of an integrated copy of the neomycin resistance (neo<sup>f</sup>) gene was increased 2 to 7-fold by induction of transcription of the neo<sup>r</sup> gene (Nickoloff 1992). Although switch recombination is not believed to be a classical, reciprocal, homologous recombination process involving large regions of homology, one recent model of switching based on the sequence analysis of many switch junctions is consistent with an error prone DNA synthesis event involving priming from one switch region to another by short sequence homologies (Dunnick et al. 1993; Dunnick and Stavnezer 1990).

Is PARP normally inhibited during antibody class switching? An intriguing finding which suggests a possible mechanism whereby PARP could be inhibited during class switching in vivo is the identification on human and mouse B-cells of the cell surface antigen, CD38, which has homology to ADP-ribose cyclase (Howard et al. 1992; States et al. 1992). ADP-ribose cyclase produces cADP-ribose from NAD<sup>+</sup>, releasing nicotinamide as a byproduct. cADP-ribose has been shown to stimulate intracellular Ca<sup>2+</sup> mobilization (Galione 1993). While Ca<sup>2+</sup> ionophores do not appear to significantly increase switching in this system, if this enzyme was activated in B-cells induced to undergo switching, and if cADP-ribose was produced, nicotinamide would be released and the cellular NAD<sup>+</sup> pool might be depleted. A consequence of either of these events could be inhibition of PARP.
The T-cell signals that activate B-cells and drive them to proliferation and class switching are currently being dissected in several labs (Parker 1993). The role of CD38 in this process has not been studied; however, increased proliferation and calcium fluxes in mouse B-cells treated with anti-CD38 antibody in the presence of LPS + IL-4 have been reported (Howard et al. 1992). It is also possible that that the activation of proliferation of B-cells by T-cells ultimately leads to NAD\(^+\) consumption. Such metabolic changes have been proposed to cause changes in PARP activity (Loetscher et al. 1987). Similarly, B cell activation-induced increases in glycohydrolase activity, which degrades poly(ADP-ribose), could have the same effects as inhibiting PARP. A future goal of these studies is to determine whether known inducers of switching such as LPS, activated T-cells, activated T-cell membranes, CD40 ligand, anti-Ig or anti-\(\delta\)-dextran ultimately result in the inhibition of PARP.

We cannot explain the differing abilities of PARP inhibitors to increase switching in I.29\(\mu\) and spleen cells. While it is possible that effects seen with inhibitors of PARP are isotype specific, we favor the idea that there are inherent differences in PARP activity in the two types of cells. It is possible that inhibitors reach greater intracellular concentrations in I.29\(\mu\) cells than in splenic B-cells, and thus PARP inhibitors have a greater effect in I.29\(\mu\) cells than in spleen cells. It is also possible that in normal splenic B-cells treated with LPS + IL-4, PARP activity is already low enough to support switching. Consistent with this idea is our finding that in experiments in which high levels of switching to IgG1 are induced by LPS + IL-4, nicotinamide has only a marginal effect, whereas in experiments with low levels of switching induced by LPS + IL-4, nicotinamide causes a greater increase. We have attempted to
measure levels of poly(ADP-ribose) or PARP activity in nuclear or cell extracts from I.29µ cells and splenic B-cells treated with switch inducers and PARP inhibitors to obtain a better understanding of the observed difference. However, enzyme assays involving induction of artificially high levels of PARP activation in disrupted cells are not reliable and more reliable measurement of endogenous polymer levels in cells that have not been treated with DNA damaging agents is very difficult.

The involvement of normal cellular DNA repair enzymes in switch recombination has precedent in V(D)J recombination, which is aberrant in cells which are deficient in ubiquitously expressed genes involved in DNA double-strand break repair, including SCID cells and certain CHO cell mutants (Pergola et al. 1993; Taccioli et al. 1993). The human and mouse Sµbp-2 protein, which preferentially binds 5'-phosphorylated, single stranded DNA containing GGGGT and GGGCT motifs, respectively, was recently cloned (Fukita et al. 1993; Mizuta et al. 1993). This protein is ubiquitously expressed, and its mRNA levels increased in mouse spleen cells treated with LPS. It contains helicase motifs found in proteins involved in DNA replication, repair, and recombination, thus implicating a normal cellular enzyme in switch recombination. Definitive proof that inhibition of PARP is involved in switch recombination in I.29µ cells awaits direct inhibition of polymerase via antisense RNA or the inducible overexpression of the DNA binding domain of PARP as a dominant negative inhibitor of the enzyme. We have as yet been unable to accomplish these experiments. DNA binding domain-mediated inhibition of PARP activity and inhibition of alkylation-induced DNA single-strand break repair has been reported in CV-1 monkey cells and human
fibroblasts (Küpper et al. 1990; Molinete et al. 1993). However, interpretation of DNA binding domain experiments could be difficult depending on the role of PARP in switching, because the DNA binding domain, rather than inhibiting PARP, could mimic it.

To our knowledge, the experiments reported in this thesis represent the first evidence that inhibitors of the ubiquitous, nuclear enzyme, PARP can increase recombination of immunoglobulin genes. As well as identifying a possible regulatory point for switching in vivo, this study identifies a potentially useful means for inducing higher levels of class switching for examining the mechanism of heavy chain switch recombination. Together with the previously proposed requirement in switch recombination for DNA synthesis (which is constitutive in I.29μ cells), this study re-emphasizes the requirement for germline transcription and identifies two other potential levels of regulation of switching observable in the I.29μ B cell lymphoma. These include an IL-4-activated signal distinct from DNA synthesis and transcription and the inhibition of poly(ADP-ribose) polymerase.

**A Model For Switch Recombination:** Although many possible models may be proposed for how IgA switching occurs in this system, a plausible model is described below. This model includes findings discussed above, and additional recent preliminary findings.

The mode of signaling by TGFβ in this system is unknown. The TGFβ type II receptor has been shown to contain a functional serine/threonine kinase domain (Lin et al. 1992). Additionally, serine/threonine kinase activity has been implicated in TGFβ-induction of Plasminogen Activator-Inhibitor I (PAI-I) expression and in the inhibition of phosphorylation of the retinoblastoma
susceptibility gene product (RB) during late $G_1$ (Ohtsuki and Massagué 1992). The tandem repeats found within the $\alpha$-germline promoter that are required for TGFβ inducibility of reporter constructs are also found in a few, but not all, other TGFβ-inducible genes. These repeats specifically bind proteins in electrophoretic mobility shift assays (EMSAs) of nuclear extracts from I.29μ cells and spleen cells. Although protein/DNA binding is not induced by TGFβ, it is possible that TGFβ activates the $\alpha$-germline promoter by inducing phosphorylation of the TGFβ response element binding protein or associated proteins (Lin and Stavnezer 1992).

I hypothesize that TGFβ-induced activation of transcription of the $\alpha$-switch region, or the binding of TGFβ-inducible proteins to the $\alpha$-germline promoter, would make it accessible to an endonuclease that recognizes and binds switch region sequences. Alternatively, the endonuclease may bind to proteins that bind to the switch regions or regions upstream of switch regions. Another possibility is that such an endonucleolytic activity might associate with the nascent germline transcript itself, or a RNA/DNA structure formed within the switch region.

Transcription of the switch regions might also make them accessible to proteins with binding specificities for single-stranded DNA, such as the helicase, $S_\mu$BP-2, which is capable of binding to single-stranded switch region sequences and which is induced by LPS (Fukita et al. 1993; Mizuta et al. 1993). Such a helicase could unwind DNA ends after cutting has occurred.

There are many possibilities for the action of PARP at this stage and many of these possibilities have been discussed above. I will consider a plausible scenario, incorporating some of these possibilities and some recent, interesting, preliminary data.
Since PARP binds preferentially to DNA ends it is likely that it would bind switch ends. Since PARP would then become locally activated (and since it appears to be preferentially activated during the S-phase of the cell cycle anyway (Bhatia et al. 1990), i.e., at the same time that switching is believed to occur), if PARP normally promotes immediate DNA repair, it is possible that inhibition of PARP would be required during switching so that endonuclease-induced breaks in switch regions are not immediately repaired (a process that would preferentially occur in switch regions because they are transcriptionally active). Inhibition of PARP in vivo might result from signals that ultimately lead to NAD depletion.

Preliminary data suggest that PARP might also bind to switch regions. In HeLa cell nuclear extracts, PARP, complexed with DNA ligase I and a ligase inhibitor protein, can be isolated from affinity columns containing Chi sequences (Andy Eisen, Albert Einstein Medical College, personal communication). These complexes have also been isolated from I.29μ cells, treated or untreated with LPS. Chi (GCTGGTG) promotes recombination in bacteria. Additionally, sequences which occur at high frequency around switch junctions (CTGG and its complement GACC), are found within the chi sequence (Chou and Morrison 1993). The sequence, CTGG, is also found within binding sites for proteins, with unknown functions in switching, that bind to switch regions, including BSAP, NF-Sμ, and SNAP (Liao et al. 1992; Marcu et al. 1992; Waters et al. 1989; Wuerffel et al. 1992; Wuerffel and Kenter 1992; Wuerffel et al. 1990). Thus, it is possible that PARP, DNA ligase I, and its inhibitor bind directly to switch regions. It remains to be determined whether PARP participates in protein complexes seen in EMSAs performed with DNA fragments containing switch region sequences.
One may further speculate that modification of DNA ligase I or its inhibitor by poly(ADP-ribosylation) regulates their activity. Although DNA ligase I is a known acceptor for poly(ADP-ribose), the effect of poly(ADP-ribosylation) on DNA ligase I is controversial.

These data suggest that the inhibition of DNA repair caused by inhibition of PARP may occur either while PARP binds to DNA ends or at PARP bound to switch regions. It is possible that PARP enhances immediate DNA repair by activating DNA ligase I, and that during the initial stages of switching, inhibition of PARP inhibits repair by inhibiting DNA ligase I. It is not yet known whether PARP bound to DNA ends is complexed with DNA ligase I and its inhibitor.

If single stranded DNA ends from the switch regions are available (due to a switch region associated endonuclease, the inhibition of DNA repair by inhibition of PARP, and an LPS-induced helicase activity), short sequence homologies found within opposing switch regions could allow imprecise pairing and priming of DNA synthesis from one switch region to another, for example, from $S_{\mu}$ to $S_{\alpha}$, as has been previously proposed (Dunnick et al. 1993; Dunnick and Stavnezer 1990). Restoration of cellular NAD levels and subsequent reactivation of PARP at this stage could reactivate DNA ligase I, which might be required for the final stages of switching. Alternatively, PARP and associated proteins may not be needed at this stage, and the reactivation and auto-poly(ADP-ribosylation) of PARP would allow release of PARP from DNA allowing accessibility to other DNA repair enzymes. It is also possible that the homologous pairing of single stranded DNA sequences might displace PARP so that a polymerase (which might be another normal DNA repair enzyme) has access to the DNA.
The role of endogenous IL-4 in this process is also not clear. Based on my results in I.29μ cells, endogenous IL-4 is not inducing IgA switching simply by increasing DNA synthesis, or germline transcription. These results suggest that IL-4 may instead be involved in the activation of enzymes involved in recombination. Of note is the finding that the activation of proliferation of hematopoietic cells by IL-4 (as well as by insulin and insulin-like growth factor I, IGF-I) is accompanied by tyrosine phosphorylation of a 170 kDa protein called the IL-4-induced phosphotyrosine substrate (4PS) and of a protein called the insulin receptor substrate I (IRS-I), both of which associate with phosphatidylinositol (PI) 3-kinase after stimulation (Wang et al. 1993a; Wang et al. 1993b). One may speculate that in I.29μ cells, PI 3-kinase activation, induced by endogenous IL-4, ultimately leads to the activation of a required switch enzyme such as an endonuclease or repair polymerase.
CHAPTER VI

TABLES AND FIGURES
Table I

TGFβ increases accumulation and transcription of α-germline RNA and switching to IgA in I.29μ cells\textsuperscript{a}

<table>
<thead>
<tr>
<th></th>
<th>α-germline RNA accumulation\textsuperscript{b}</th>
<th>α-germline RNA transcription\textsuperscript{c}</th>
<th>Fold increase in %IgA\textsuperscript{+}IgM\textsuperscript{+} cells at Day 3</th>
<th>Fold increase in %IgA\textsuperscript{+} cells at Day 10-11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hours of Treatment</td>
<td>Fold Increase</td>
<td>Hours of Treatment</td>
<td>Fold Increase</td>
</tr>
<tr>
<td>LPS\textsuperscript{d}</td>
<td>23-26</td>
<td>0.7±0.2 (3)</td>
<td>18</td>
<td>0.5±0.2 (3)</td>
</tr>
<tr>
<td></td>
<td>46-48</td>
<td>0.9±0.1 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS + TGFβ\textsuperscript{e,f}</td>
<td>23-26</td>
<td>5.4±1.7 (2)</td>
<td>18</td>
<td>5.2±0.9 (2)</td>
</tr>
<tr>
<td></td>
<td>47-48</td>
<td>5.6±1.7 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGFβ\textsuperscript{d,f}</td>
<td>22-26</td>
<td>8.8±2.1 (7)</td>
<td>12</td>
<td>5.6±1.0 (3)</td>
</tr>
<tr>
<td></td>
<td>43-47</td>
<td>4.5±1.0 (3)</td>
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</tr>
</tbody>
</table>

\textsuperscript{a} In all experiments, I.29μ cells were treated with 50μg LPS and 2ng/ml TGFβ. Values are the mean ± SEM. The range is indicated where two experiments were performed. The number of experiments is shown in parentheses. For days 3, 4, and 5 switch assays, LPS was added for 24 hours, diluted 5- or 6-fold and TGFβ was added on day 0 and 1, or days 0, 1, and 2. Value in table is the average fold increase in % double positive cells. For day 10-11 switch assays, LPS was added on day 0 and TGFβ was added on day 0 or day 0 and 1. The value for each switch assay experiment is the mean of duplicate wells. P values determined by two-tailed students t test. Pairwise comparisons to LPS alone log 10 (raw values).

\textsuperscript{b} Determined by RNA blot analysis at the times indicated.

\textsuperscript{c} Determined by nuclear run-on transcription analysis at the times indicated. Each experiment represents hybridization to the $I_\alpha$ or $S_\alpha$3 probe.

\textsuperscript{d} Values relative to uninduced control.

\textsuperscript{e} Values relative to LPS alone.

\textsuperscript{f} Fold increase in total % IgA\textsuperscript{+} cells was 3.8-, 3.6-, and 2.6-fold with LPS + TGFβ and 0.5-, 0.4-, and 0.6-fold with TGFβ, compared to LPS-induced and uninduced cells, respectively, on days 3, 4 and 5.
Table II

Induction of nuclear run-on transcription of α-germline RNA in I.29μ cells (clone 22D) by TGFβ

<table>
<thead>
<tr>
<th>Exp #</th>
<th>Hours of TGFβ</th>
<th>α</th>
<th>βα3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>-</td>
<td>3.7a</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>7.1</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>2</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>2.4</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>2.9</td>
<td>3.7</td>
</tr>
</tbody>
</table>

Values represent the average fold induction of transcription obtained after treatment of cells with 2ng/ml TGFβ and hybridization to duplicate filters.
Table III: Effects of IL4, IFNγ, IL5, and IL6 on expression of IgA and on α-germline transcripts in I.29μ cells

<table>
<thead>
<tr>
<th></th>
<th>Fold increase in α-germline RNA accumulation⁵</th>
<th>Fold increase in α-germline RNA transcription⁶</th>
<th>Fold increase in %IgA+ cells at day 10-11</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hours of treatment</td>
<td>Fold increase</td>
<td>Hours of treatment</td>
</tr>
<tr>
<td></td>
<td></td>
<td>increase</td>
<td></td>
</tr>
<tr>
<td>IL4d</td>
<td>24 or 48</td>
<td>1.4 ± 0.1 (3)</td>
<td>12 hr</td>
</tr>
<tr>
<td>IL4 + LPSe</td>
<td>48</td>
<td>1.6 ± 0.2 (2)</td>
<td>-</td>
</tr>
<tr>
<td>TGFβ + IL4d</td>
<td>25</td>
<td>8.6 (1)⁴</td>
<td>12 hr</td>
</tr>
<tr>
<td>LPS + TGFβ + IL4e</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>IFNγd</td>
<td>48</td>
<td>1.1 ± 0.1 (2)</td>
<td>-</td>
</tr>
<tr>
<td>IFNγ + LPSe</td>
<td>48</td>
<td>0.4 ± 0.1 (4)</td>
<td>-</td>
</tr>
<tr>
<td>IFNγ + IL4 + LPSe</td>
<td>48</td>
<td>0.7 ± 0.1 (2)</td>
<td>-</td>
</tr>
<tr>
<td>IL5 + LPSe</td>
<td>-</td>
<td>-</td>
<td>18 hr</td>
</tr>
<tr>
<td>IL6 + LPSe</td>
<td>-</td>
<td>-</td>
<td>18 hr</td>
</tr>
</tbody>
</table>

⁵ Cells were treated with 25 or 50μg/ml LPS. Cytokines were added at the following concentrations, TGFβ, 2ng/ml, rIFNγ, 100 U/ml, and rIL-4-containing supernatants, H-28, 8% or 23%, X63, 10%, or rIL-4, 850 U/ml. Values are the mean ± SEM. The range is indicated where two experiments were performed. The number of experiments is shown in parentheses.

⁶ Determined by RNA blot analysis at the times indicated.

⁷ Determined by nuclear run-on transcription analysis at the times indicated.

⁸ Values relative to uninduced control.

⁹ Values relative to LPS alone.

¹⁰ 1.1-fold relative to TGFβ alone. The same result was also obtained in another experiment where 4ng/ml TGFβ was used.

¹¹ 1.2-fold relative to TGFβ alone.

¹² Preliminary results.
TABLE IV: ABILITY OF VARIOUS COMPOUNDS TO INCREASE IgA SWITCHING IN 1.29µ Cells

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Modes of Action</th>
<th>Increased Switching in 1.29µ cells</th>
<th>Fold Increase Relative to LPS&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fold Increase Relative to LPS+TGFβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβ 2ng/ml</td>
<td>Increase α-germline transcription</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>IBMX 1mM</td>
<td>Inhibit cAMP/cGMP phosphodiesterase</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Caffeine 1mM</td>
<td>Adenosine R blockade, Inhibit DNA repair, Inhibit Topo I &amp; Topo II, DNA intercalators, Stimulate Ca++ flux Inhibit poly (ADP-ribose) polymerase</td>
<td>YES</td>
<td>YES</td>
<td>YES</td>
</tr>
<tr>
<td>Forskolin 100µM, 100µM</td>
<td>Stimulate adenylyl cyclase (Increase cAMP)</td>
<td>NO</td>
<td>1.3 ± 0.4(3)</td>
<td>1.4 ± 0.2(3)</td>
</tr>
<tr>
<td>8-OPT-cAMP 1mM</td>
<td>cAMP analogs</td>
<td>NO</td>
<td>1.3 ± 0.3(7)</td>
<td>0.9 ± 0.1(7)</td>
</tr>
<tr>
<td>6-dg-cAMP 1mM</td>
<td>Non-xanthine cAMP phosphodiesterase inhibitor</td>
<td>NO</td>
<td>0.3 (1)</td>
<td>0.6 (1)</td>
</tr>
<tr>
<td>8-NeO-cAMP 500µM</td>
<td>Non-xanthine cGMP phosphodiesterase inhibitor</td>
<td>NO</td>
<td>2.0 (1)</td>
<td>1.3 (1)</td>
</tr>
<tr>
<td>RO-20-1724 500µM</td>
<td>Non-xanthine cAMP phosphodiesterase inhibitor</td>
<td>NO</td>
<td>0.7 ± 0.2(2)</td>
<td>0.9 ± 0.1(2)</td>
</tr>
<tr>
<td>Dipyrindiamide 10mM, 100mM, 1µM</td>
<td>Non-xanthine cGMP phosphodiesterase inhibitor</td>
<td>NO</td>
<td>0.5 ± 0.2(3)</td>
<td>1.0 ± 0.2(3)</td>
</tr>
<tr>
<td>8-Brc-GMP 1mM, 3mM</td>
<td>db-cGMP analogs</td>
<td>NO</td>
<td>0.9 ± 0.1(2)d</td>
<td>0.8 ± 0.0(2)d</td>
</tr>
<tr>
<td>2-CI-adenosine 1mM, 10µM</td>
<td>Adenosine deaminase-resistant adenosine analog</td>
<td>NO&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3 ± 0.0(2)</td>
<td>1.1 ± 0.0(2)</td>
</tr>
<tr>
<td>A23187 100; 250ng/ml</td>
<td>Ca++ ionophores</td>
<td>NO</td>
<td>2.5 ± 1.2(4)f</td>
<td>1.3 ± 0.3(4)</td>
</tr>
<tr>
<td>BAPTA-AM 1µM</td>
<td>Intracellular Ca++ chelator</td>
<td>NO</td>
<td>1 ± 0.0(2)</td>
<td>1.8 ± 0.2(2)</td>
</tr>
<tr>
<td>Proflavin 0.1µM, 1µM</td>
<td>Topo II inhibitor, DNA intercalator</td>
<td>NO</td>
<td>0.3 ± 0.3(2)</td>
<td>0.5 ± 0.2(2)</td>
</tr>
<tr>
<td>Ara-C 0.5; 5; 10µM</td>
<td>Inhibit DNA repair</td>
<td>NO</td>
<td>0.5 ± 0.5(2)</td>
<td>0.4 ± 0.3(2)</td>
</tr>
</tbody>
</table>
Cells were assayed for IgA+IgM+ cells and total IgA+ cells (which includes IgA+IgM+ cells) on day 3 or day 4 by immunofluorescence microscopy. In a few cases, cells were also assayed on day 2 or day 5. Values represent the average fold increase in the % positive cells ± SEM. The number of experiments is shown in parentheses.

In all cases LPS or LPS + TGFβ are included in the cultures, as indicated by the column in which the results are found.

**b** TGFβ fold over LPS:
- Day 3: A+M+ (P<0.0001), A+ (P<0.0001).
- Day 4: A+M+ (P<0.001), A+ (P<0.006).
- Day 5: A+M+ (P<0.01), A+ (P<0.011).

IBMX fold over LPS: A+M+ (P<0.0001), A+ (P<0.0001).

IBMX fold over LPS + TGFβ: A+M+ (P<0.05), A+ (P<0.047).

Caffeine fold over LPS: A+M+ (P<0.05), A+ (P=0.04).

Caffeine fold over LPS + TGFβ: A+M+ (P<0.04), A+ (P<0.005).

Paired two-tailed students t test. Log10(raw values). In a few instances where 0% switching occurred in the presence of LPS alone or LPS + nicotinic acid, a value of 0.1% (the limit of detection of the assay) was used in order to obtain an estimate of fold increase over LPS.

c 1 of 5 experiments is from a large scale experiment where media was not diluted on day 1 and cells were assayed on day 2.

d In 1 of 2 experiments, media was not diluted on day 1.

e Values relative to LPS + IBMX ± TGFβ. No blocking of IBMX effect by 2-C1 adenosine.

f A23187 fold over LPS: A+M+ (P>0.2).
Table V: Fold Increase in IgA Switching by Inhibitors of Poly(ADP-ribose) Polymerase at Day 3 in 1.29μ cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IgA+IgM+</th>
<th>IgA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methoxybenzamide (2mM)</td>
<td>&gt;5.3 +/- 0.6 (8) P&lt;0.0001</td>
<td>2.9 +/- 0.4 (8) P&lt;0.002</td>
</tr>
<tr>
<td>M-anisic acid (2mM)</td>
<td>&gt;2.1 +/- 0.6 (7) P&gt;0.05</td>
<td>1.4 +/- 0.3 (7) P&gt;0.05</td>
</tr>
<tr>
<td>Nicotinamide (10mM)</td>
<td>&gt;6.8 +/- 1.7 (5) P&lt;0.02</td>
<td>3.5 +/- 0.9 (5) P&lt;0.12</td>
</tr>
<tr>
<td>Nicotinic Acid (10mM)</td>
<td>0.8 +/- 0.1 (4) P&gt;0.05</td>
<td>0.7 +/- 0.2 (4) P&gt;0.05</td>
</tr>
</tbody>
</table>

Fold over LPS + TGFβ

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IgA+IgM+</th>
<th>IgA+</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Methoxybenzamide (2mM)</td>
<td>4.9 +/- 1.2 (7) P&lt;0.003</td>
<td>4.6 +/- 0.5 (7) P&lt;0.0001</td>
</tr>
<tr>
<td>M-anisic acid (2mM)</td>
<td>1.7 +/- 0.5 (5) P&gt;0.05</td>
<td>1.6 +/- 0.2 (5) P&gt;0.05</td>
</tr>
<tr>
<td>Nicotinamide (10mM)</td>
<td>5.0 +/- 0.7 (8) P&lt;0.0001</td>
<td>5.4 +/- 0.4 (8) P&lt;0.0001</td>
</tr>
<tr>
<td>Nicotinic Acid (10mM)</td>
<td>0.7 +/- 0.2 (6) P&gt;0.05</td>
<td>0.8 +/- 0.1 (6) P&gt;0.05</td>
</tr>
</tbody>
</table>

a Compounds listed were added in addition to LPS (upper) or LPS + TGFβ (lower).

b IgA+IgM+ designates the fold increase in the percent of cells expressing both IgA and IgM in their cytoplasm. IgA+ designates the fold increase in the percent of total IgA+ cells including the IgA+IgM+ dual expressing cells.

c Average fold Increase in percent positive cells +/- SEM (n). Comparisons to LPS alone (upper) or LPS + TGFβ alone (lower).

d Paired two-tailed students t test with Bonferroni adjustment. Comparisons to LPS (upper) or LPS + TGFβ (lower) log10 (raw values).
Table VI. Induction of IgA Switching in 1.29μ cells by 1,5-dihydroxy-isoquinoline

<table>
<thead>
<tr>
<th>Exp. #</th>
<th>Day of Analysis</th>
<th>Day isoQ Added</th>
<th>Treatment</th>
<th>% IgA+M+ Cells</th>
<th>% IgA+ Cells(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>3</td>
<td>0</td>
<td>LPS + TGFβ</td>
<td>1.9 ± 0.1 (^b)</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + 1,5-diOH-isoQ (30nM)</td>
<td>1.7 ± 0.0</td>
<td>6.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + 3MB</td>
<td>5.9 ± 0.3</td>
<td>11.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + Nicotinamide</td>
<td>8.4 ± 0.3</td>
<td>13.1 ± 0.7</td>
</tr>
<tr>
<td>II.</td>
<td>2</td>
<td>0</td>
<td>LPS + TGFβ</td>
<td>1.7 ± 0.0</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + 1,5-diOH-isoQ (20nM)</td>
<td>3.9 ± 0.7</td>
<td>4.8 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + 1,5-diOH-isoQ (30nM)</td>
<td>3.5 ± 0.6</td>
<td>5.2 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + 1,5-diOH-isoQ (40nM)</td>
<td>3.6 ± 0.3</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + 3MB</td>
<td>5.5 ± 0.6</td>
<td>6.8 ± 1.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + Nicotinamide</td>
<td>3.4 ± 0.1</td>
<td>5.7 ± 0.3</td>
</tr>
<tr>
<td>III.</td>
<td>2</td>
<td>0</td>
<td>LPS + TGFβ</td>
<td>1.5 ± 0.1</td>
<td>2.4 ± 0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + 1,5-diOH-isoQ (20nM)</td>
<td>3.1 ± 0.3</td>
<td>7.4 ± 1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + 1,5-diOH-isoQ (30nM)</td>
<td>3.3 ± 0.5</td>
<td>11.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + 1,5-diOH-isoQ (40nM)</td>
<td>3.4 ± 0.2</td>
<td>10.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + 3MB</td>
<td>5.4 ± 0.1</td>
<td>8.7 ± 0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + Nicotinamide</td>
<td>6.7 ± 0.4</td>
<td>10.9 ± 1.5</td>
</tr>
<tr>
<td>IV.</td>
<td>3</td>
<td>0,1</td>
<td>LPS + TGFβ</td>
<td>3.6 ± 0.1</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + 1,5-diOH-isoQ (30nM)</td>
<td>9.3 ± 0.8</td>
<td>18.7 ± 0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LPS + TGFβ + Nicotinamide</td>
<td>11.6 ± 0.1</td>
<td>18.1 ± 0.2</td>
</tr>
</tbody>
</table>

\(^a\) Includes IgA+IgM+ cells.

\(^b\) Averages of duplicate wells ± range. At least 1000 cells were counted per well.
Table VII. Nicotinamide increases switching to IgG1 induced by LPS + IL-4 treatment of splenic B cells as measured by FACS analysis of surface IgG1 expression

<table>
<thead>
<tr>
<th>Exps. Pooled:</th>
<th>% slgG1+cells induced by LPS + IL-4</th>
<th>LPS + IL-4 + Nicotinamide (10mM)</th>
<th>LPS + IL-4 + Nicotinic Acid (10mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10% IgG1</td>
<td>5.1 ± 0.8 (n=7)</td>
<td>10.5 ± 2.0</td>
<td>2.1 ± 0.3 (P&lt;0.008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10% IgG1</td>
<td>15.5 ± 2.1 (n=6)</td>
<td>20.2 ± 2.0</td>
<td>1.3 ± 0.1 (P&lt;0.007)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>18.7 ± 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.2 ± 0.04 (P&lt;0.02)</td>
</tr>
</tbody>
</table>

a Average ± SEM. P values derived from two-tailed students t-tests with Bonferroni adjustment. Pairwise comparisons verses LPS + IL-4 (log 10 raw values).

b Comparison between fold induction by nicotinamide and nicotinic acid in low level IgG1 switch experiments, P<0.004, n=6.

c Comparison between fold induction by nicotinamide and nicotinic acid in high level IgG1 switch experiments, P>0.05, n=6.

d Change in mean fluorescence intensity (analyzed over a 4 log scale) from IgG1- to IgG1+ population in a typical experiment was 18-fold.
Figure 1. Raw values of TGFβ induced IgA switching in 1.29μ cells on day 3, 4, and 5. Cells were cultured in 50μg/ml LPS, added on day 0, with 2ng/ml TGFβ, added on days 0 and 1, or days 0,1, and 2. Inducers were diluted 6-fold on day 1 with fresh media and cultures were assayed for % IgA+M+ and total % IgA+ cells by immunofluorescence microscopy on day 3, 4, or 5. The % A+ includes all cells expressing IgA, including double positive cells. The % A+M+ includes only cells expressing both IgA and IgM. Values shown are the average percent positive cells ± SEM. The number of experiments performed on each day is shown in parentheses. Increases in average %IgA+M+ cells with TGFβ were significant on days 3 and 4 with p<0.001 and p<0.02, respectively. On day 5, p>0.05 although the fold increase was significant (see Table I). P values were determined by a two-tailed students t test.
Figure 2. Immunofluorescence microscopy demonstrating double positive cells. For each row, left, unstained cells; middle and right, cells stained with anti-IgM FITC and anti-IgA RITC, respectively. White arrowheads, double positive cells.
Figure 3. Map of unrearranged $C_{\alpha}$ gene locus, $\alpha$-germline transcripts, and probes. The 1.7kb transcript is the predominant $\alpha$-germline RNA in I.29u cells and contains the $I_{\alpha}$ exon spliced to $C_{\alpha}$ exons. The 2.4kb and 3.0kb forms also encode $\alpha$ membrane domains (Stavnezer et al. 1988). Probes are described in detail in Materials and Methods and contain the following: $I_{\alpha}$, start site(s) and upstream exon of $\alpha$-germline RNA; $S_{\alpha}3$, $\alpha$ switch sequences; $C_{\alpha}$, $\alpha$ constant region gene sequences; -820-0, sequences 5' of start sites of $\alpha$-germline transcript.
Figure 4. RNA blot demonstrating effects of TGFβ on the steady state level of germline transcripts in I.29μ cells. In this experiment, treatment with 2ng/ml TGFβ increased the level of 1.7kb α-germline transcript 3-fold in the absence of LPS and 4-fold in the presence of LPS. RNA was isolated from I.29μ clone 22A10 cultured for 48 h in the presence of the indicated concentrations of inducers. 3.1μg poly(A)+ RNA was loaded per lane. Blots were hybridized to the indicated probes which are described in detail in Materials and Methods and/or shown in Fig. 3. NEG C refers to RNA obtained from cells cultured without inducers. The data were quantitated by normalization to the average of signals obtained for hybridization to probes specific for the uninduced genes GAPDH and A50.
Figure 5. RNA blot analysis of RNA isolated from 1.29μ clone 22D cultured for 24 h in the presence of the indicated inducers. At 24 h, inducers were removed, cells were diluted 2-fold, recultured, and RNA was isolated at the indicated times from the start of the cultures. 20μg of total cell RNA was loaded per lane. Hybridization of the Iα probe detecting the predominant 1.7 kb α-germline transcript is shown. Below are photographs showing 18S ribosomal RNA (lower band) used for normalization.
Figure 6. A. Run-on transcription analysis of nuclei isolated from 1.29μ clone 22D after the indicated times of incubation with TGFβ (2ng/ml). 32P-labeled RNA was hybridized to filters containing 5μg of immobilized plasmid DNA with the indicated inserts described in detail in Materials and Methods and/or Fig. 3. Data were normalized to values obtained for the uninduced gene GAPDH. B. Nuclear run-on analysis of nuclei isolated from 22A10 cells after 18 h of treatment with the indicated concentrations of TGFβ in the presence of LPS. Probes are the same as in 6A.
Figure 7. Run-on transcription assay in nuclei from 1.29μ clone 22D induced for 12 h with TGFβ in the presence and absence of 80ng/ml (850U/ml) rIL-4. Note that in this experiment, hybridization to the Iα probe did not exceed background hybridization to vector sequences in the control lane. Although induction by TGFβ was clearly visible, quantitation of labeled RNA hybridizing the Iα probe could not be obtained. Quantitation was performed on RNA hybridizing to the Sa3 probe.
Figure 8. Measurements of α-germline RNA half-life in the presence of TGFB.
1.29µ cells were cultured for A, 36 h in or B, 23 h with the indicated inducers at which time actinomycin D or DRB was added, respectively, to inhibit transcription. Total cell RNA was analyzed by RNA blot analysis and hybridization with the Iα probe (see Fig 3) at the indicated times after addition of transcription inhibitor. Half life of α germline RNA was determined by linear regression.
Figure 9. **A.** Blot analysis of RNA isolated from 1.29μ clone 22D after various days of treatment with 50μg/ml LPS and LPS + 100U/ml IFNγ. Cells were split 1:3 on days 2 and 4. LPS and IFNγ were added on day 0 and day 2. 2.4 μg of poly(A)+ RNA was loaded in each lane. Blots in A and B were hybridized with the Iα probe. Normalization to the GAPDH signal was used to correct for unequal loading. **B.** RNA blot analysis of 22D cells treated for 2 days ± 50μg/ml LPS ± 23% H-28 IL-4 sup. ± 100U/ml IFNγ. 3μg poly(A)+ RNA was loaded in each lane. Unequal loading was corrected by normalization to the A50 signal.
Figure 10. Effect of IFN\textsubscript{\gamma} on expression of IgA in 1.29\mu clone 22D in the presence and absence of IL-4 (H-28 supernatant). Cells were incubated with the indicated inducers for 4 days and assayed for the \%IgA\textsuperscript{+} cells on day 10 by immunofluorescence microscopy.
Figure 11. **1.29μ cells secrete IL-4 after treatment with LPS.** 1.29μ clone 22D was cultured at 1x10^6 cells/ml for 6 h or 0.25x10^6 cells /ml for 48 h with 25μg/ml LPS in the presence and absence of 2ng/ml TGFβ. Supernatants were harvested and assayed for the ability to support growth of the IL-2/IL-4-dependent cell line, CTLL as measured by [3H]-thymidine incorporation. Background proliferation of CTLL cells has been subtracted. Values are the average of triplicate wells. A and D. CTLL proliferation with IL-2 and IL-4 standards, B. CTLL proliferation in presence of sups from 22D cells cultured 6 h with LPS, and C and E. 48 h with LPS. E. CTLL proliferation in presence of sups from 22D cells cultured 48 h with TGFβ or LPS + TGFβ. Two assays were performed on sups from the same experiment. A, B, C, assay 1; D, E, F, assay 2. CTLL assays performed in the presence of anti-IL-2 or anti-IL-4-specific antibodies included, + @IL-2, + anti-IL-2 antibody (S4B6) and + @IL-4, + anti-IL-4 antibody (11B11) (described in Materials and Methods).
Figure 12. Anti-IL-4 inhibits LPS-induced IgA expression on day 10. I.29µ cells (clone 22D) were plated at 0.15x10⁶/ml in 1ml volumes with LPS (25µg/ml) and 11B11 sup (A) or purified 11B11 antibody (B). Cultures were split 2-3-fold on days 3, 4, and 7 with fresh media and analyzed by immunofluorescence microscopy on day 10. The averages of duplicate wells are shown. Ranges were ≤ ±0.5 for +LPS wells and ≤ ±0.8 for -LPS wells. Cells were cultured at 0.25x10⁶/ml with the indicated inducers. Inducers were removed on day 4 and cells were fed and split as necessary before analysis on day 10 by immunofluorescence microscopy. Concentrations of inducers were, LPS, 25µg/ml, 11B11 supernatant, 2.5%, rIL-4, 1000U/ml, TGFβ, 2ng/ml. Average (± range) of duplicate wells are plotted. 1000 cells were counted per well.
Figure 13. RNA Blot demonstrating that anti-IL-4 antibody does not inhibit α-germline transcripts in I.29μ cells. I.29μ cells were induced with LPS (50μg/ml) ± TGFβ (2ng/ml) ± 11B11 antibody (2.5% sup.) for 48 hr. Total cell RNA was isolated and 20μg loaded per lane. RNA was blotted and hybridized to the Iα probe (Fig. 3) and to the GAPDH probe. RNA from two separate experiments are shown on the left and right sides of the blot. In the experiment on the right, the quantity of 11B11 sup. used completely neutralized IL-4 activity in all the cultures in which it was added as assayed by CTLL assay.
Figure 14. IBMX increases IgA Expression on day 10. 1.29μ cells were cultured at 0.25x10^6/ml with the indicated inducers. Cells were diluted 16-fold at the indicated times in A and at 24h in B. Inducers were removed on day 4 (A) or 5 (B). Cells were fed and split as needed until day 10 and analyzed by immunofluorescence microscopy. In A, only wells containing LPS were analyzed at 48 h. The ranges of duplicate wells are indicated. 500-1000 cells/well in A and 1000 cells/well in B were counted.
**Figure 15.** Switching to IgA in I.29μ cells is not increased by cAMP analogs or inhibited by inhibitors of protein kinase A (PKA). I.29μ cells were cultured at 0.15x10^6/ml with LPS (50μg/ml) added on day 0 and TGFβ (2ng/ml) added on days 0, 1 and 2. Inducers were diluted 6-fold on day 1, and switching was assayed by immunofluorescence microscopy on day 3. %IgA+ cells includes all cells expressing IgA. IgA+IgM+ cells includes only those cells expressing both IgA and IgM. Results are the average ± range of two experiments in which cells were cultured in duplicate. 1000 cells were counted per well.
Figure 16. Failure of combinations of cAMP analogs to increase LPS-induced IgA Switching in I.29μ cells. I.29μ cells were cultured at 0.25x10^6/ml with LPS (50μg/ml) ± TGFB (2ng/ml)(added on days 0 and 1) and 8-Cl-phenyl thio-cAMP (1mM), db-cAMP (1mM) or 8-NH3-cAMP (500μM) and various combinations thereof. Cultures were diluted 6-fold at 24 h and cells were analyzed by immunofluorescence microscopy on day 4.
Figure 17. IgA+ cells induced on day 3 after treatment of 1.29μ cells with inhibitors of poly(ADP-ribose) polymerase (PARP). Cells were cultured at 0.15-0.4x10^6/ml with LPS (50μg/ml) plus inhibitors (or inactive analogs) added on day 0 and TGFβ (2ng/ml) added on days 0, 1 and 2 or days 0 and 1 in a few experiments. Cells were assayed by immunofluorescence microscopy on day 3. The average % total IgA+ or % IgA+IgM+ cells ± SEM is shown. The number of experiments is shown in parentheses. Differences in %IgA+M+ cells with 3MB and nicotinamide were significant with p<0.05 relative to controls without PARP inhibitors.
Figure 18. Photographs of representative fields observed by immunofluorescence microscopy after treatment with nicotinamide. Cells were visualized under low magnification after treatment with \( \text{A. LPS + TGF}\beta \), \( \text{B. LPS + TGF}\beta + \text{nicotinamide} \), \( \text{C. LPS + TGF}\beta + \text{nicotinic acid} \). For each of A, B, and C: on the left are cells stained with anti-IgM-FITC and on the right is the same field stained with anti-IgA-RITC. Photograph was taken from a representative experiment in which nicotinamide caused a 5-fold increase in double expressing and total IgA\(^+\) cells.
Figure 19 Staining of I.29μ cells is resistant to acid treatment. I.29μ cells were cultured at 0.15x10⁶/ml and treated with the indicated inducers which were diluted six-fold on day 1. A, TGFβ (TG) was added on day 0, 1, 2 and cells were analyzed by immunofluorescence microscopy on day 3. Prior to fixation, cells were treated for 1 min. at 4°C with pH 4.0 acetate buffer (described in Materials and Methods) to remove cytophilic Ig. B, TGFβ was added on days 0 and 1 and cells were analyzed on day 2. Cells were treated with pH 4.0 acetate buffer post staining to remove staining reagents associating by cytophilic attachment.
**Figure 20. Experiment demonstrating dose responsiveness of IgA switch induction by 3MB.** Cells were cultured with LPS (50μg/ml) and various doses of 3MB or other inducers diluted 6-fold on day 1. TGFβ (2ng/ml) was added on day 0 and 1. Cells were analyzed by immunofluorescence microscopy on day 3.
Figure 21. A. Map of the expressed \(\mu\) gene and unrearranged \(\alpha\) genes in IgM+ 1.29\(\mu\) cells. Probes for C\(_\mu\) (M2-5B), C\(\alpha\) (\(\alpha\)), and I\(\alpha\) (upstream exon of \(\alpha\)-germline transcripts) are indicated and are described in Materials and Methods. S denotes the tandemly repeated switch regions. B. Southern blot demonstrating that nicotinamide reduces the intensity of germline BglII DNA fragments containing \(\mu\) and \(\alpha\) switch regions. DNA from 1.29\(\mu\) cells treated with the indicated inducers was digested with BglII. Expressed \(\mu\), denotes the unrearranged S\(_\mu\) containing BglII fragment of the expressed chromosome; 3'\(\mu\), denotes the BglII fragment containing most of C\(_\mu\) and its 3' segment from the expressed and nonexpressed chromosomes; and Nonexpressed \(\mu\), denotes the 5'C\(_\mu\) fragment of the nonexpressed chromosome. The unrearranged BglII fragment of the \(\alpha\) locus is designated C\(\alpha\) and an unrearranged BglII fragment hybridizing with the C\(\kappa\) probe is designated C\(\kappa\). C. Densitometry of C\(_\mu\) hybridization signal to blot in B. D. Results of switch assays from this experiment on days 3 and 7.
FIGURE 21

A.

Expressed $\mu$ in vitro

Unrearranged $\alpha$ in vitro
C. Treatment

Control
LPS
LPS + TGFβ
LPS + TGFβ + Nicotinamide

Fold Relative to Control
(Normalized to Cγ)

Expressed μ
3′μ
Nonexpressed μ

D. Treatment

LPS
LPS + TGFβ
LPS + TGFβ + Nicotinamide

% Positive Cells

% Positive Cells

A+M+ day 3
A+ day 3

A+M+ day 7
A+ day 7
Figure 22. A. RNA blot demonstrating that inhibitors of PARP do not increase α-germline transcripts in 1.29μ cells at 24 hours. 10μg of total cell RNA was loaded per lane. Blot was sequentially hybridized with probes specific for the germline exon (Iα) of α-germline RNA (see Figs. 4 and 21A), the mouse PARP mRNA, and GAPDH mRNA. B. PARP inhibitors induce switching on day 2. Replicates from A were cultured for 2 days and assayed for IgA switching by immunofluorescence microscopy.
A.  

PARP  -3.8 kb  

GAPDH  -1.3 kb  

B.  

TREATMENT  
LPS (50 ug/ml)  
LPS + 3MB (2mM)  
LPS + MAA (2mM)  
LPS + Nicotinamide (10mM)  
LPS + Nicotinic Acid (10mM)  
LPS + TGFB (2ng/ml)  
LPS + TGFB + 3MB  
LPS + TGFB + MAA  
LPS + TGFB + Nicotinamide  
LPS + TGFB + Nicotinic Acid  

% Positive Cells  

IgM+A+  
IgA+
Figure 23. Nicotinamide cannot overcome the requirement for endogenous IL-4 in IgA switching by I.29μ cells. 0.15x10^6 I.29μ cells were stimulated with LPS (50μg/ml) + TGFβ (2ng/ml) ± Nicotinamide (10mM) ± purified 11B11 (or AD8) at the indicated doses. Inducers were diluted 6-fold on day 1. TGFβ was added on days 0, 1, and 2. 11B11 and AD8 were added on days 0 and 1. Cells were analyzed by immunofluorescence microscopy on day 3. At least 1000 cells were counted per well. The ranges of duplicate wells are shown.
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