GSBS Dissertations and Theses   Graduate School of Biomedical Sciences

1993-09-01

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

Penny E. Shockett
University of Massachusetts Medical School

Let us know how access to this document benefits you.

Follow this and additional works at: https://escholarship.umassmed.edu/gsbs_diss

Part of the Amino Acids, Peptides, and Proteins Commons, Carbohydrates Commons, Cells Commons, Enzymes and Coenzymes Commons, Genetic Phenomena Commons, Hemic and Lymphatic Diseases Commons, Immune System Diseases Commons, and the Neoplasms Commons

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:

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

SEPTEMBER 1993

IMMUNOLOGY

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

Approved as to style and content by:

Dr. Raymond Welsh, Chairman of Committee

Dr. Paul R. Dobner, member

Dr. Robert E. Humphreys, member

---   -----------------------------

Dr. Robert T. Woodland, member   **Dr. Janet Stavnezer, Thesis Advisor**

------------------------------------   -------------------------------

Dr. Naomi Rosenberg, member   **Dr. Thomas B. Miller, Jr., Dean of**
                                Graduate School of Biomedical
                                Sciences

      Program in Immunology and Virology
      September 1993

ACKNOWLEDGMENTS

I wish to thank Dr. David Parker for CTLL cells, purified 11B11 and S4B6 antibodies and cloned hybridomas producing anti-T-cell antibodies, Dr. Eva Severinson for the H-28 supernatant, H-28 cells, and 11B11 supernatant, Dr. Robert Woodland for purified 11B11 and AD8 antibodies, Dr. Dana Fowlkes for purified recombinant IL-6, Dr. Steve Gillis of Immunex Corp. and also the Sterling Research Group for recombinant IL-4, Dr. Mark Smulson for the PARP cDNA clone, Dr. Steve Klaus for the GAPDH probe, members of the Stavzezer lab for various α and ϵ-germline probes and helpful discussion, Jennifer Nieutepski for technical assistance, Drs. Tom Honeyman, James Dobson, Guy Poirier, Hans Küpper, Mark Smulson, David Parker and Robert Woodland for helpful discussions, Steven Baker for help with statistics, and Drs. Deborah Albrecht and Yi-Chaung Lin, and Ms. Victoria Yuschenkoff for critical reading of manuscripts.

I also thank extremely supportive close friends and family for encouragement and love during these years. I am grateful for the advice and encouragement from Dr. David Parker and Dr. Robert Woodland and the help and friendship of members of their labs. I am also grateful to members of the Department of MGM including students, faculty and secretaries who were always willing to answer questions. I am thankful to members of the Stavnezer lab, past and present, for technical help and inspiring discussions. I thank my thesis exam committee for meeting on short notice. Most of all, I thank my advisor Dr. Janet Stavnezer, who has been an extremely helpful and understanding mentor and friend and a great person with whom to brainstorm about science.

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 ($S\alpha$) sequences are elevated in clones of this line which switch well as compared to clones which switch less frequently. TGFβ_1 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 $S\alpha$-$C\alpha$ 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 ϵ-germline transcripts are not increased by TGFβ. IL-4, which modestly increases switching to IgA in I.29μ cells, slightly increases trancription 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 ϵ-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 IgG$_1$ 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 C_μ and C_α specific probes to genomic DNA fragments containing the expressed VDJ-C_μ and the unrearranged S_α-C_α segments, respectively, indicating that PARP inhibition increases rearrangment 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 C_α gene, although TGFβ is required for optimal induction by PARP inhibitors, consistent with a requirement for transcription of the unrearranged C_H gene. PARP inhibitors do not overcome the requirement for endogenously produced IL-4.

TABLE OF CONTENTS

LIST OF TABLES

x

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 (C_H), 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_μ), to be subsequently expressed with one of six downstream C_H genes, ordered in the mouse 5'-$\gamma 3$-$\gamma 1$-$\gamma 2b$-$\gamma 2a$-ϵ-α-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 consisiting of tandemly repetitive sequences located 5' of C_μ and every C region gene except C_δ (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_μ 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 S_μ 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 (T_H) 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; Severinson-Gronowicz et al. 1979; Shapira et al. 1992;

Spaulding and Griffin 1986).

Considerable evidence indicates that this general machinery is directed to a particular isotype, or C_H 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 C_H gene, hypomethylation and DNAse sensitivity of that C_H gene, and the steady state level of RNA transcripts initiating upstream of and proceeding though 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 C_H 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 C_H 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 α-germline transcript (Radcliffe et al. 1990). A minor 1.8 kb species of mouse α-germline transcripts contains an open reading frame consisting of approximately 30 bp of sequence of I_α exon spliced to C_α exons (Gaff and Gerondakis 1990). Likewise, the mouse γ_1 and γ_{2a} 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 γ_{2a} 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 T_H-cell line (or polyclonally activated membrane fractions from either T_H 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, T_H2-cells induce IgG_1 expression. T_H1-cells can support IgG1 expression if supplemented with T_H2-cell supernatant or IL-4 and/or anti-IFNγ antibody. (Coffman et al. 1988;

Hodgkin et al. 1991; Stevens et al. 1988). Similarly, activated TH_1-cell membranes supplemented with IL-4 and IL-5 or TH_2 cell supernatant can support IgG_1 responses (Hodgkin et al. 1991; Noelle et al. 1991). Additionally, murine splenic B-cells treated with LPS produce mainly IgG_3 and IgG_{2b}. When treated with IL-4 in addition to LPS they make increased levels of IgG_1 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-δ-dextran or *Nippostrongylus brasiliensis* (Finkelman et al. 1990). Moreover, IgE responses are absent and IgG_1 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 $\gamma1$ and ε-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 $\gamma1$ 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 β_1 ($TGF\beta$) is a pleiotropic cytokine generally antiproliferative for lymphocytes. Although it has not been directly detected in Peyers 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 S_μ to S_α 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 transcrips 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 TGFβ_1 be present, emphasizing the requirement for transcription of the unrearranged C_α 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 H_2O were: caffeine; 8-(4-chlorophenylthio)-adenosine 3': 5'-cyclic monophosphate sodium (cpt-cAMP); N^6, 2'-O-dibutyryl adenosine 3': 5'-cyclic monophosphate sodium (db-cAMP); 8-(6-aminohexyl)-amino-adenosine 3': 5'-cyclic monophosphate ($8-NH_2$-cAMP); N^2, 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 Sµ and S$_\alpha$ switch regions (Stavnezer et al. 1985). Clones 22A10 and 22D of I.29µ (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°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), 50µM 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 µg/ml. Platelet derived porcine TGFβ$_1$ (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 IFNγ 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 IgG$_1$) (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 IgG$_1$ anti-ars idiotype antibody (given by Robert Woodland, Worcester, MA).

Analysis of switching in I.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% NaN$_3$. 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/NaN$_3$ 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, C$_\mu$ 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), I$_\alpha$ 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 Sα3 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. C$_\alpha$ is a fragment from the cDNA clone pJ558[13] encoding Cα

sequences (Marcu et al. 1980; Stavnezer et al. 1988), Cε is a 2.1 kb BamHI-HindIII genomic DNA fragment encoding the 5' portion of the Cε 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 $C_κ$ is a 730 bp fragment containing cDNA sequences coding for $C_κ$ and a small portion of the V region of the MOPC384 κ chain isolated from the plasmid pM384κ 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_2O.   All solutions were treated with 0.1% diethyl pyrocarbonate (Sigma) prior to use.   RNA was quantitated spectrophotometrically, and either 1.5 to 3 μg poly(A)$^+$ (selected once on oligo(dT)- cellulose (Collaborative Research, Inc., Bedford, MA) or 10 to 20 μg 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

(Fisher Scientific, Fairlawn, NJ or Mallinckrodt, Paris, KY). 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 $NaHPO_4$ (pH7.8) (composed of 268.08g $Na_2HPO_4 \cdot 7 \, H_2O$ + 4 ml H_3PO_4 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° for aqueous hybridization. Autoradiography was performed at $^-70^{o}$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 [^{32}P]-UTP (800 Ci/mmole) or [^{32}P]-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 ^{32}P-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 H_2O (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 Boerhinger 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 0.25×10^6/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 [^{3}H]-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 (T_0) 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 (T_0) and at 5, 10, and 15 h post addition. RNA was analyzed by RNA blotting and hybridization with the I$_\alpha$ 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. % T_0 RNA remaining (Simple Curve Fit, Cricket Graph Version 1.3.2, Cricket Software, Malvern, PA).

Assay of IL-4 in supernatants from I.29μ cells. In the measurement of IL-4 activity in I.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 [^{3}H]-thymidine incorporation after incubation of CTLL cells with I.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 ^{3}H-thymidine/well for the last 4-7 h of a 24 h incubation period at 37°C and 5% CO_2.

Southern blot analysis: I.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 transfered 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 NH_4Cl, 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 ($2x10^7$/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 IgG_{2b} 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 $2x10^7$ cells/ml and incubated for 30 min on ice in a solution of MAR 18.5, a mouse IgG_{2a} 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

(Coligan et al. 1991). 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 $0.5-2.0 \times 10^6$/ml in 5 ml RPMI 1640 containing 10% fetal calf serum (as above except insulin was omitted and CO_2 was 5%). For induction of IgG_1 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 10mM 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 1×10^6 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-IgG_1-R-phycoerytherin (PE) (Southern Biotechnology Associates) diluted 1:200 and 1:120, respectively, in PBS/ 1.5% FCS/ 0.2% NaN_3. Cells were washed 3X in PBS/FCS/NaN_3 at 4°C and resuspended in 0.5ml PBS/FCS/NaN_3. Paraformaldehyde (4%) was added (0.2ml), and cells were vortexed and fixed 5 min. at room temperature. Cells were washed 2X in PBS/FCS/NaN_3 and resuspended in 0.4 ml PBS/FCS/NaN_3 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 I.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 C_α 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 1.29μ cells. To determine whether the increase in IgA switching with TGFβ treatment was preceeded 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 I_α 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_α gene segment, which detects smaller α-germline RNA species, showed that the 1.3kb α-germline transcript was also induced by TGFβ. This RNA is the same size as the major α-germline transcript induced by TGFβ 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 α-germline transcript resulting from a more 5' splice donor site (Gaff and Gerondakis 1990). The membrane forms of the α-germline transcript also appear to be induced by TGFβ (Fig. 4, upper). The 1.0-kb germline transcript was not induced. The induction of germline RNA by TGFβ is specific for the IgA isotype, because the 1.6kb ε-germline RNA was not induced (Fig. 4). In two experiments the ε germline RNA appeared to be reduced by TGFβ treatment in the absence of LPS.

Kinetics of induction of α-germline RNA. Optimal induction of germline α RNA in I.29μ cells occurred after 24 h of treatment with TGFβ. The mean induction of α-germline RNA observed after 22 to 26 h of treatment with 2ng/ml TGFβ in the absence of LPS was 8.8-fold (SE $\pm$ 2.1) (Table I). Twelve-hour treatment with TGFβ yielded smaller increases in α-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 α-germline transcripts by TGFβ, I.29μ cells were induced for 24 h with 2ng/ml TGFβ in the presence or absence of 50 μg/ml LPS. At 24 h, a portion of the cells were removed and analyzed for the presence of α-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 α-germline transcript was induced by TGFβ

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 I.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 I.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 Iα probe. As shown in Figure 3, this probe contains sequences corresponding to the Iα upstream exon of the 1.7kb α-germline RNA. Transcription of RNA hybridizing to the Sα3 (α-switch region) and Cα (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 I.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 occured after 12 h of treatment with TGFβ (Fig. 7 and Table II). The average induction by TGFβ of RNA hybridizing to the Iα and/or Sα3 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 $\pm$ 1.3 in the presence of LPS and 1.9 $\pm$ 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 + TGFβ_1-induced IgA expression was inhibited 46.9% (range $\pm$ 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 $\pm$ 7.2%) and 62.3% (SEM $\pm$ 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 ^{3}H-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 I.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 I.29μ cells. Because

the α-germline promoter from I.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 I.29μ cells. Addition of isobutyl-methyl-

xanthine (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, I.29μ cells were treated with

chlorophenylthio (cpt)-cAMP (a phosphodiesterase resistant cAMP analog),

dibutyryl (db)-cAMP, or 8-NH$_2$-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 cytophylic 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$^+$IgM$^+$ 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 C_μ and C_α 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 C_μ and C_α 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 *BglII* 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 C_μ. The largest *BglII* fragment (15.4kb) contains the Sμ region and segment of the expressed chromosome located 5' to C_μ. The intermediate sized band (6.0kb) represents the 3'C_μ *BglII* fragment present on both the expressed and nonexpressed chromosomes. The smallest band represents the 5'C_μ fragment of the nonexpressed chromosome. Hybridization of the same blot to a C_α probe

detects the 16.6kb unrearranged $BglII$ fragment of the expressed and nonexpressed chromosomes which contains both S_α and C_α. The C_κ probe detects a 2.75kb kappa light chain fragment that does not rearrange in I.29μ cells. To control for DNA loading and transfer, hybridization results have been normalized to those with the C_κ 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'C_μ fragment of the expressed chromosome containing S_μ is reduced in intensity after culture with inducers of switching. A 60% diminution is seen with LPS + TGFβ, and a 75% diminution is seen with LPS + TGFβ + nicotinamide. Diminution of the 3'μ fragment is intermediate as would be expected if the expressed μ gene undergoes deletion but the nonexpressed μ gene does not. In this clone of I.29μ cells the 5'C_μ $BglII$ fragment of the nonexpressed chromosome is 5.7 kb shorter than in the parental I.29μ 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 C_α fragment is also most reduced in the presence of nicotinamide. The very low intensity of hybridization signal with the C_α probe of DNA from the nicotinamide-treated cells precluded scanning of this band by densitometry.

Since the signals from the nonexpressed C_μ fragment and the C_κ 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 I.29μ cells. We
next determined whether inhibiting PARP increases IgA switching in I.29μ
cells by increasing transcription through the α-switch region.   I.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 I$_α$ 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. I.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 K_i 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 I.29μ cells. To determine whether endogenously produced IL-4 was required for switching to IgA in I.29μ cells in the presence of PARP inhibitors, I.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 I.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 IgG_1 and IgM expression by FACS analysis. Addition of nicotinamide to these cultures increased IgG_1 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 IgG_1 by LPS + IL-4, and one including experiments in which greater than 10% of cells were induced to express IgG_1 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 chomosome (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. IFN$_\gamma$ did not reduce levels of α-germline transcripts in the absence of LPS. Inhibition by IFN$_\gamma$ 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 IFN$_\gamma$ strongly suggests that IFNγ is acting early to inhibit switching, we have not determined whether IFN$_\gamma$ 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, C_ϵ and $C_{\gamma 1}$-germline transcripts, and reporter genes driven by C_ϵ and $C\gamma 1$-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 V_H 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 $I_{\gamma 2b}$ exon and promoter with a neomycin resistance (neo^r) gene expressed in reverse orientation (Zhang et al. 1993). In B cells from these mice, LPS-induced IgG$_{2b}$ 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 TGFß 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 α-germline transcription. Although TGFβ optimally increases α-germline transcripts in the absence of LPS, TGFβ 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.29μ cells contain detectable IL-4. Furthermore, LPS-induced switching to IgA is inhibited 67% by anti-IL-4 antibody. Since LPS does not induce α-germline transcription, and since anti-IL-4 does not inhibit levels of α-germline transcripts in the presence of LPS or LPS + TGFβ, endogenously produced IL-4 appears to increase IgA switching in I.29μ 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 α, γ2b and γ3 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/IgG1 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.29μ cell supernatants. In contrast to I.29μ 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 IgG2b in these cells was not significantly affected by inclusion of anti-IL-4 antibody, although only low amounts of antibody were used (2.3μg/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 $IgM^+IgA^-IgG_{2b}^-IgG_3^-$ 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_μ or S_α. Previously it has been demonstrated that switching to IgA in I.29μ cells occurs by a typical switch recombination event between S_μ and S_α sequences, deleting the C_μ gene and all other intervening C_H genes, resulting in the joining of the expressed VDJ segment to the C_α 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_μ and in S_α, 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_α fragment on Southern blots from cells treated with PARP inhibitors would be unlikely. A diminution in C_μ and C_α 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 + TGFβ, 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 + TGFβ induced switching to IgA in I.29μ 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. Conversly, 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^r) gene was increased 2 to 7-fold by induction of transcription of the neo^r 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$^+$, releasing nicotinamide as a byproduct. cADP-ribose has been shown to stimulate intracellular Ca^{++} mobilization (Galione 1993). While Ca^{++} 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$^+$ 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-δ-dextran ultimately result in the inhibition of PARP.

We cannot explain the differing abilities of PARP inhibitors to increase switching in I.29μ 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.29μ cells than in splenic B-cells, and thus PARP inhibitors have a greater effect in I.29μ 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 IgG$_1$ 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 unnable 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 G₁ (Ohtsuki and Massagué 1992). The tandem repeats found within the α-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 α-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 α-switch region, or the binding of TGFβ-inducible proteins to the α-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 (GCTGGTGG) 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 funtions 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_μ to S_α, 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[a]

	α-germline RNA accumulation[b]		α-germline RNA transcription[c]		Fold increase in %IgA+IgM+ cells at			Fold increase in %IgA+ cells at Day 10-11
	Hours of Treatment	Fold Increase	Hours of Treatment	Fold Increase	Day 3	Day 4	Day 5	
LPS[d]	23-26	0.7±0.2 (3)	18	0.5±0.2 (3)				
	46-48	0.9±0.1 (7)						
LPS + TGFβ[e,f]	23-26	5.4±1.7 (2)	18	5.2±0.9 (2)	>11.4 ± 2.2 (18) (p<0.0001)	6.9±2.7 (7) (p<0.001)	5.4±1.5 (4) (p<0.02)	2.1±0.2 (7) (p<0.001)
	47-48	5.6±1.7 (3)						
TGFβ[d,f]	22-26	8.8±2.1 (7)	12	5.6±1.0 (3)	1(1)	1(2)	1(1)	
	43-47	4.9±1.0 (3)						

[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).

[b] Determined by RNA blot analysis at the times indicated.

[c] Determined by nuclear run-on transcription analysis at the times indicated. Each experiment represents hybridization to the Iα or Sα3 probe.

[d] Values relative to uninduced control.

[e] Values relative to LPS alone.

[f] Fold increase in total % IgA+ 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

		Probe	
Exp #	Hours of TGFβ Treatment	Iα	Sα3
1	12	-	3.7[a]
2	12	7.1	6
3	4	2	1.6
	8	2.4	1.3
	18	2.9	3.7

a   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[a]

	Fold increase in α-germline RNA accumulation[b]		Fold increase in α-germline RNA transcription[c]		Fold increase in %IgA+ cells at day 10-11
	Hours of treatment	Fold increase	Hours of treatment	Fold increase	
IL4[d]	24 or 48	1.4 ± 0.1 (3)	12 hr	1.5 (1)	0.9 ± 0.2 (4)
IL4 + LPS[e]	48	1.6 ± 0.2 (2)		-	1.6 ± 0.2 (18)
TGFβ + IL4[d]	25	8.6 (1)[f]	12 hr	4.3 (1)[g]	-
LPS + TGFβ + IL4[e]		-			1.7 ± 0.2 (6)
IFNγ[d]	48	1.1 ± 0.1 (2)		-	1.1 ± 0.6 (3)
IFNγ + LPS[e]	48	0.4 ± 0.1 (4)		-	0.4 ± 0.1 (9)
IFNγ + IL4 + LPS[e]	48	0.7 ± 0.1 (2)		-	0.6 ± 0.1 (6)
IL5 + LPS[e]		-	18 hr	1[h]	0.6 ± 0.1 (12)
IL6 + LPS[e]		-	18 hr	1[h]	1.1 ± 04 (5)

[a] 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.
[b] Determined by RNA blot analysis at the times indicated.
[c] Determined by nuclear run-on transcription analysis at the times indicated.
[d] Values relative to uninduced control.
[e] Values relative to LPS alone.
[f] 1.1-fold relative to TGFβ alone. The same result was also obtained in another experiment where 4ng/ml TGFβ was used.
[g] 1.2-fold relative to TGFβ alone.
[h] Preliminary results.

TABLE IV: ABILITY OF VARIOUS COMPOUNDS TO INCREASE IgA SWITCHING IN I.29µ Cells[a]

Inducer	Modes of Action	Increased Switching in I.29µ cells	Fold Increase Relative to LPS[b]		Fold Increase Relative to LPS+TGFB	
			IgA+IgM+	IgA+	IgA+IgM+	IgA+
TGFß 2ng/ml	Increase α-germline transcription	YES YES YES	DAY 3 11.4±2.2(18) DAY 4 6.9± 2.7 (7) DAY 5 5.4± 1.5 (4)	3.8± 0.9(18) 3.6± 1.3(7) 2.6± 0.4(4)		
IBMX 1mM	Inhibit cAMP/cGMP phosphodiesterase	YES	5.5 ± 1.1(12)	2.9 ±0.3(12)	1.3 ± 0.3(9)	1.9 ± 0.4(9)
Caffeine 1mM	Adenosine R blockade, Inhibit DNA repair, Inhibit Topo I & Topo II, DNA intercalators, Stimulate Ca++ flux Inhibit poly (ADP-ribose) polymerase	YES	1.6 ± 0.3(3)	2.3 ± 0.4(3)	3.8 ± 1.9(5)[c]	3.0 ± 0.6(5)[c]
Forskolin 10µM,100µM	Stimulate adenylate cyclase (Increase cAMP)	NO	1.3 ± 0.4(3)	1.4 ± 0.2(3)	1.0 ± 0.0(2)	1.2 ± 0.2(2)
8-cpt-cAMP 6-db-cAMP 1mM 8-NH2cAMP 500µM	cAMP analogs	NO NO NO	1.3 ± 0.3 (7) 0.3 (1) 2.0 (1)	0.9 ± 0.1 (7) 0.6 (1) 1.3 (1)	1.4 ± 0.4 (7) 0.8 (1) 1.4 (1)	1.1 ± 0.3(7) 0.7 (1) 1.0 (1)
RO-20-1724 500µM	Non-xanthine cAMP phosphodiesterase inhibitor	NO	0.7 ± 0.2(2)	0.9 ± 0.1(2)	0.4 ± 0.2(2)	0.5 ± 0.2(2)
Dipyrid-amole* 10nM,100nM, 1µM	Non-xanthine cGMP phosphodiesterase inhibitor	NO	0.5 ± 0.2(3)	1.0 ± 0.2(3)	0.9 ± 0.04(3)	0.9 ± 0.1(3)
8-Br-cGMP 1mM, 3mM db-cGMP 1mM	cGMP analogs	NO NO	0.9 ± 0.1(2)[d] 1.3 (1)	0.8 ± 0.0(2)[d] 0.8 (1)	1.3 ± 0.1(2)[d] 0.4 (1)	1.0 ± 0.1(2)[d] 0.4 (1)
2-Cl-adenosine 1µM, 10µM	Adenosine deaminase-resistant adenosine analog	NO[e]	1.3 ± 0.0(2)	1.1 ± 0.0(2)	0.7 ± 0.0(2)	0.9 ± 0.2(2)
A23187 100; 250ng/ml Ionomycin 1.6µg/ml	Ca++ ionophores	NO	2.5 ± 1.2(4)[f]	1.3 ± 0.3(4)	1.2 ± 0.2(4) 1.7 (1)	1.2 ± 0.2(4) 1.6 (1)
BAPTA-AM 1µM	Intracellular Ca++ chelator	NO	1 ± 0.0(2)	1.8 ± 0.2(2)	0.9 ± 0.1(2)	0.8 ± 0.0(2)
Proflavin 0.1µM, 1µM	Topo II inhibitor, DNA intercalator	NO	0.3 ± 0.3(2)	0.5 ± 0.2(2)	0.2 (1)	0.3 (1)
Ara-C 0.5; 5; 10µM	Inhibit DNA repair	NO	0.5 ± 0.5(2)	0.4 ± 0.3(2)	0.4 ± 0.1(2)	0.5 ± 0.1(2)

(Table IV footnotes)

a   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 + TGFB are included in the cultures, as indicated by the column in which the results are found.

b   TGFB 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 + TGFB: 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 + TGFB: 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 ± TGFB. No blocking of IBMX effect by 2-Cl 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 I.29μ cells

	Fold over LPS	
Treatment [a]	IgA+IgM+ [b]	IgA+ [b]
3-Methoxybenzamide (2mM)	>5.3 +/- 0.6 (8)[c] $P<0.0001$ [d]	2.9 +/- 0.4 (8) $P<0.002$
M-anisic acid (2mM)	>2.1 +/- 0.6 (7) $P>0.05$	1.4 +/- 0.3 (7) $P>0.05$
Nicotinamide (10mM)	>6.8 +/- 1.7 (5) $P<0.02$	3.5 +/- 0.9 (5) $P<0.12$
Nicotinic Acid (10mM)	0.8 +/- 0.1 (4) $P>0.05$	0.7 +/- 0.2 (4) $P>0.05$

	Fold over LPS + TGFß	
Treatment [a]	IgA+IgM+	IgA+
3-Methoxybenzamide (2mM)	4.9 +/- 1.2 (7) $P<0.003$	4.6 +/- 0.5 (7) $P<0.0001$
M-anisic acid (2mM)	1.7 +/- 0.5 (5) $P>0.05$	1.6 +/- 0.2 (5) $P>0.05$
Nicotinamide (10mM)	5.0 +/- 0.7 (8) $P<0.0001$	5.4 +/- 0.4 (8) $P<0.0001$
Nicotinic Acid (10mM)	0.7 +/- 0.2 (6) $P>0.05$	0.8 +/- 0.1 (6) $P>0.05$

[a] Compounds listed were added in addition to LPS (upper) or LPS + TGFß (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 + TGFß alone (lower).

[d] Paired two-tailed students t test with Bonferroni adjustment. Comparisons to LPS (upper) or LPS + TGFß (lower) log10 (raw values).

Table VI. Induction of IgA Switching in I.29μ cells by 1,5-dihydroxy-isoquinoline

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

[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

Exps. Pooled:	% sIgG1+cells induced by LPS + IL-4	LPS + IL-4 + Nicotinamide (10mM)		LPS + IL-4 + Nicotinic Acid (10mM)	
		%IgG1+ cells	Fold [a]	%IgG1+ cells	Fold
<10% IgG1	5.1 ± 0.8 (n=7)	10.5 ± 2.0	2.1 ± 0.3 (P<0.008)	6.7 ± 1.4	1.3 ± 0.1 (P>0.05)[b]
>10% IgG1	15.5 ± 2.1 (n=6)	20.2 ± 2.0	1.3 ± 0.1 (P<0.007)	18.7 ± 2.5	1.2 ± 0.04 (P<0.02)[c]

[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.
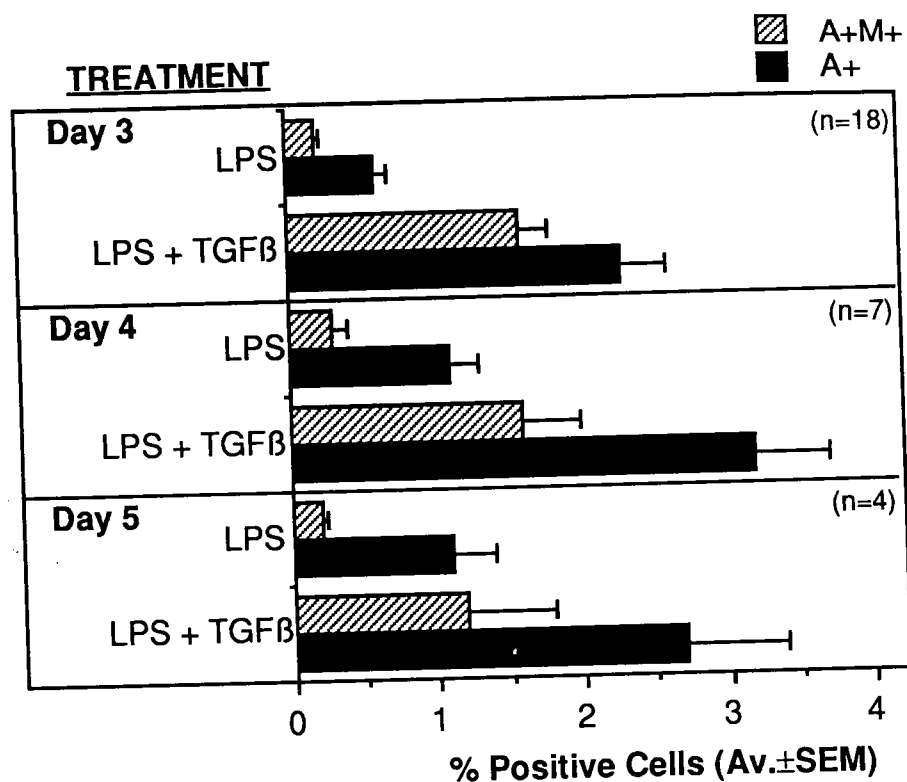
Figure 1. Raw values of TGFβ induced IgA switching in 1.29μ cells on day 3, 4, and 5. Cells were cultured in 50μg/ml LPS, added on day 0, with 2ng/ml TGFβ, added on days 0 and 1, or days 0,1, and 2. Inducers were diluted 6-fold on day 1 with fresh media and cultures were assayed for % IgA$^+$M$^+$ and 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.
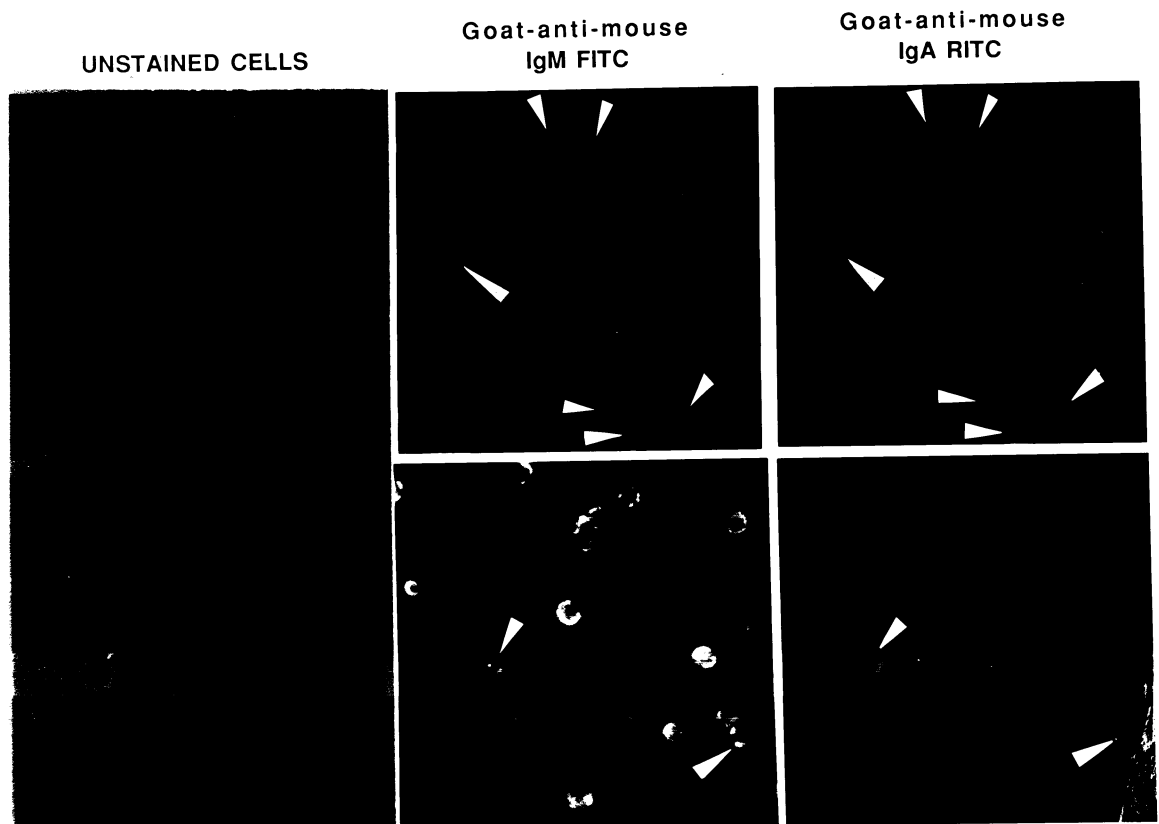
Figure 2. Immunofluorescence microscopy demonstrating double positive cells. For each row, *left*, unstained cells; *middle* and *right*, cells stained with anti-IgM FITC and anti-IgA RITC, respectively. White arrowheads, double positive cells.
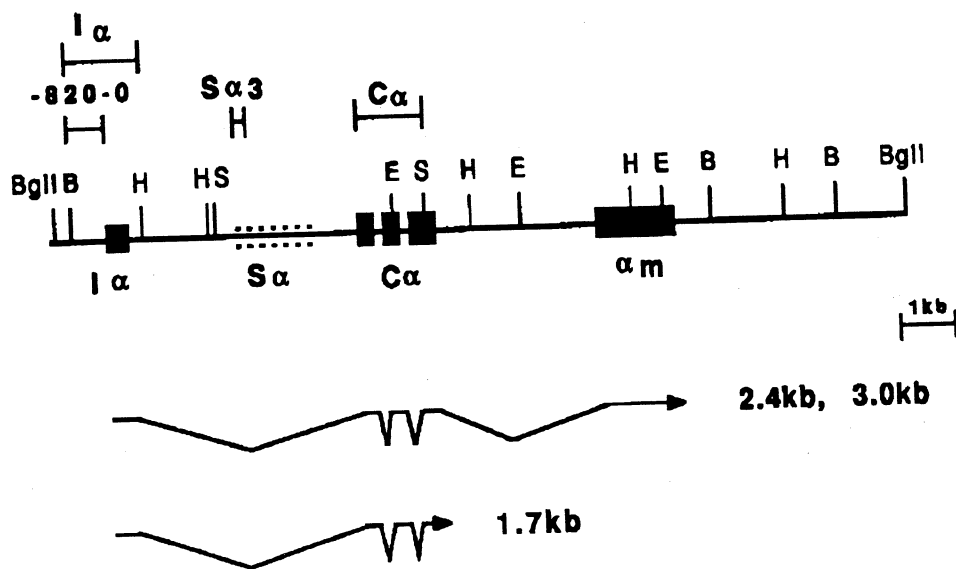
Figure 3. Map of unrearranged $C\alpha$ gene locus, α-germline transcripts, and probes. The 1.7kb transcript is the predominant α-germline RNA in I.29μ cells and contains the Iα exon spliced to $C\alpha$ exons. The 2.4kb and 3.0kb forms also encode α membrane domains (Stavnezer et al. 1988). Probes are described in detail in Materials and Methods and contain the following; Iα, start site(s) and upstream exon of α-germline RNA; Sα3, α switch sequences; Cα, α constant region gene sequences; -820-0, sequences 5' of start sites of α-germline transcript.
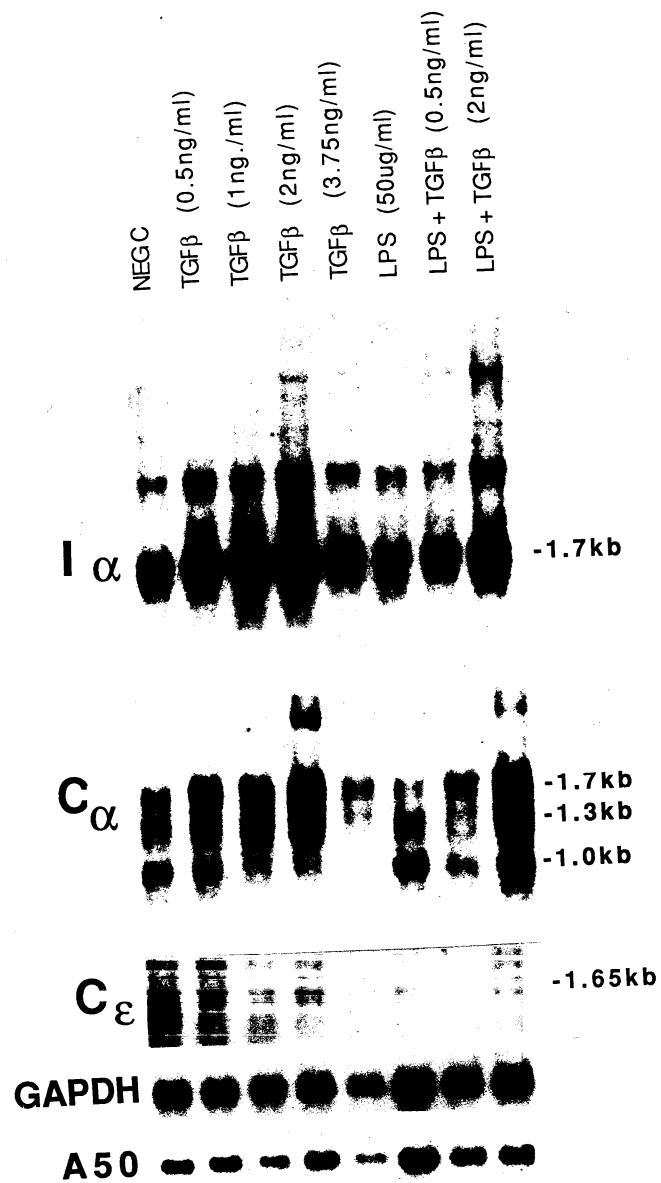
Figure 4. RNA blot demonstrating effects of TGFβ on the steady state level of germline transcripts in I.29μ cells. In this experiment, treatment with 2ng/ml TGFβ increased the level of 1.7kb α-germline transcript 3-fold in the absence of LPS and 4-fold in the presence of LPS. RNA was isolated from I.29μ 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.

22D6 24 hr. Induction

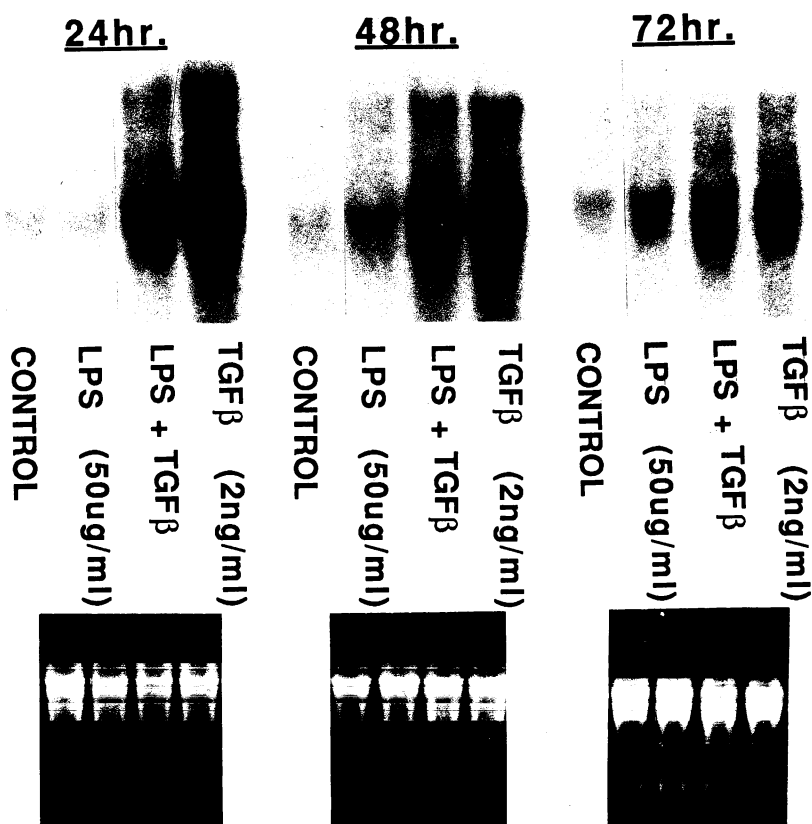## Time of RNA isolation:

24hr.   48hr.   72hr.

Figure 5. RNA blot analysis of RNA isolated from I.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 I_α probe detecting the predominant 1.7 kb α-germline transcript is shown. Below are photographs showing 18S ribosomal RNA (lower band) used for normalization.
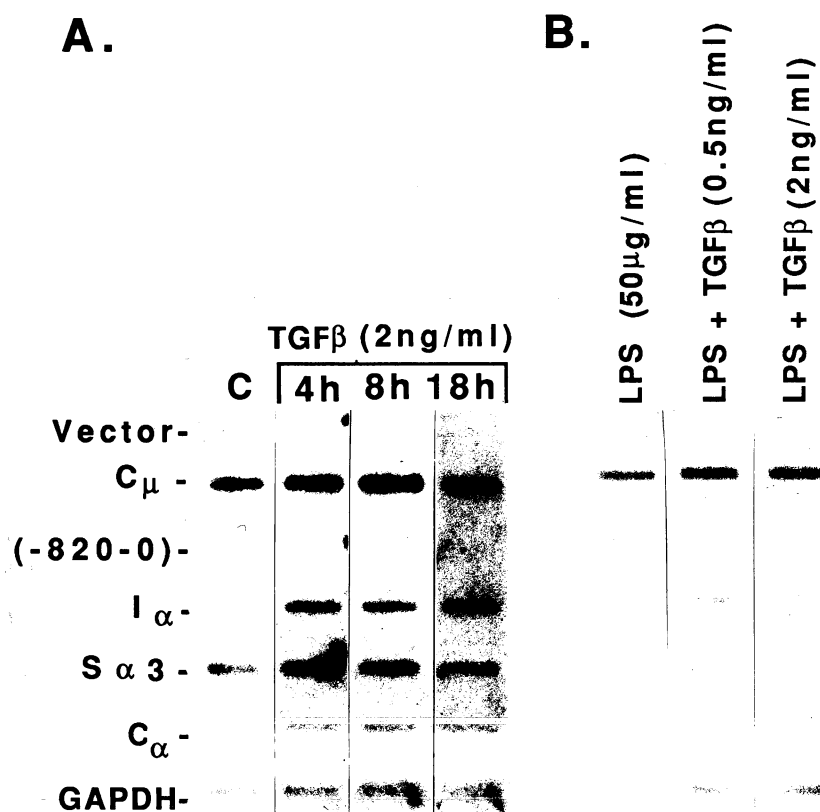
A.

B.

TGFβ (2ng/ml)

C | 4h 8h 18h

LPS (50μg/ml)

LPS + TGFβ (0.5ng/ml)

LPS + TGFβ (2ng/ml)

Vector-

Cμ -

(-820-0)-

I α -

S α 3 -

Cα -

GAPDH-

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).* ^{32}P-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.
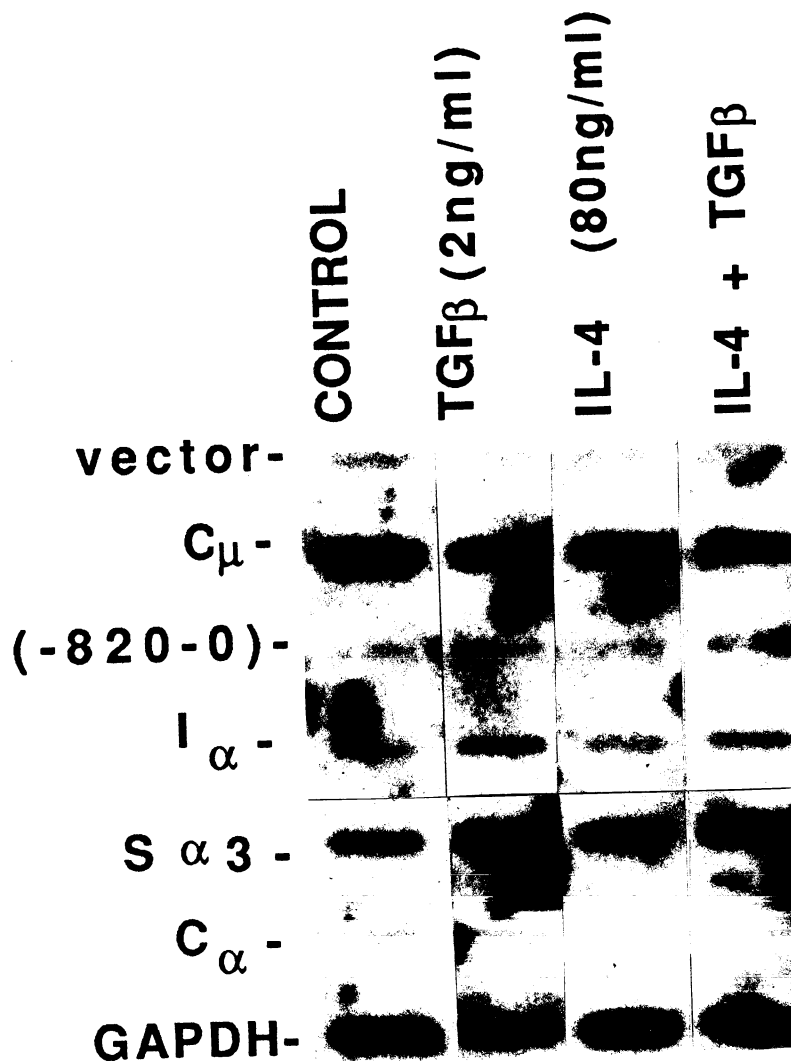
Figure 7. Run-on transcription assay in nuclei from I.29µ clone 22D induced for 12 h with TGFβ in the presence and absence of 80ng/ml (850U/ml) rIL-4. Note that in this experiment, hybridization to the $I\alpha$ probe did not exceed background hybridization to vector sequences in the control lane. Although induction by TGFβ was clearly visible, quantitation of labeled RNA hybridizing the $I\alpha$ probe could not be obtained. Quantitation was performed on RNA hybridizing to the $S\alpha3$ probe.

A.

36 h Induction

	t 1/2 (h)
■ LPS	7.6
O LPS + TGFB	5.4

B.

23 h Induction

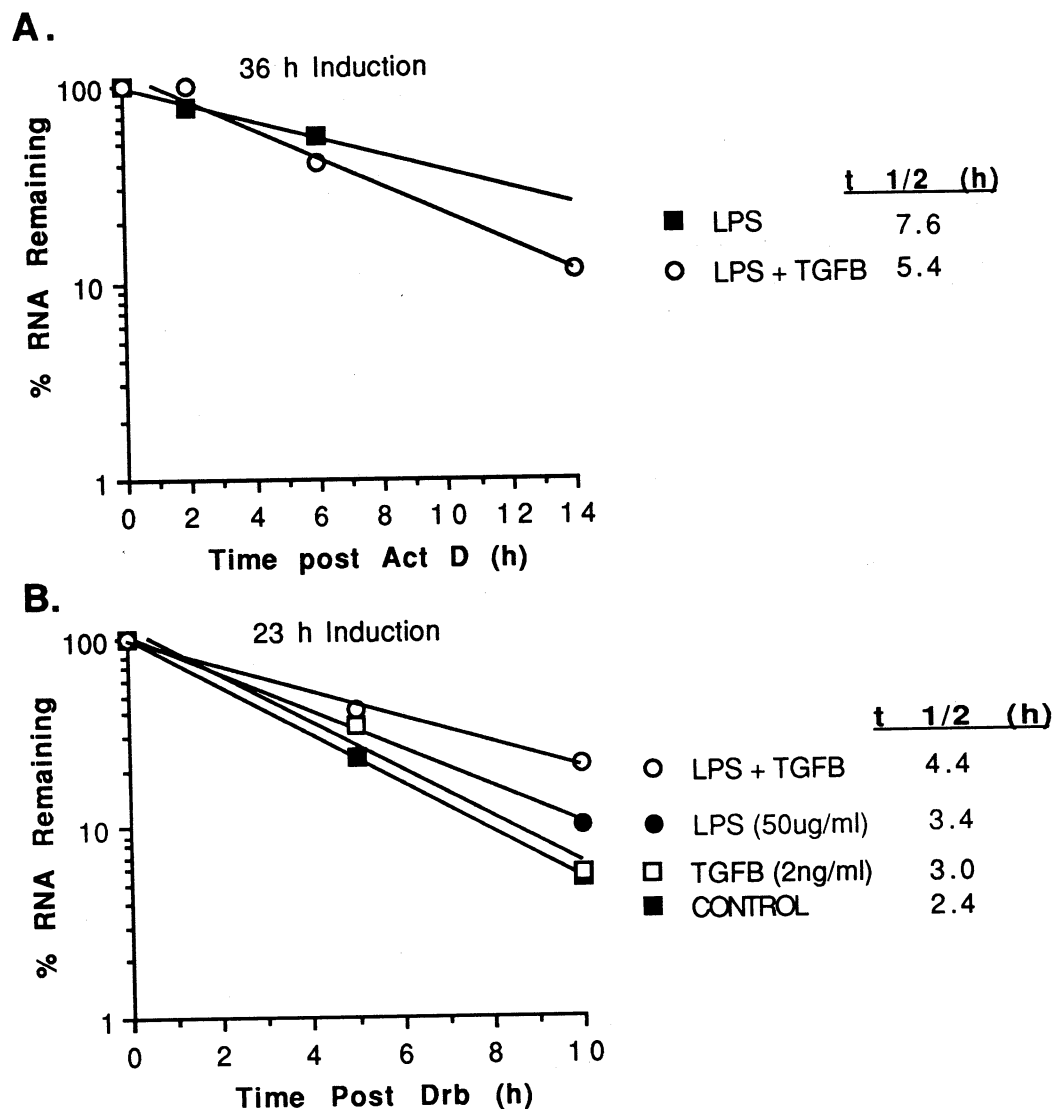|	t 1/2 (h)
O LPS + TGFB	4.4
● LPS (50ug/ml)	3.4
□ TGFB (2ng/ml)	3.0
■ CONTROL	2.4

Figure 8. *Measurements of α-germline RNA half-life in the presence of TGFβ.*
I.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
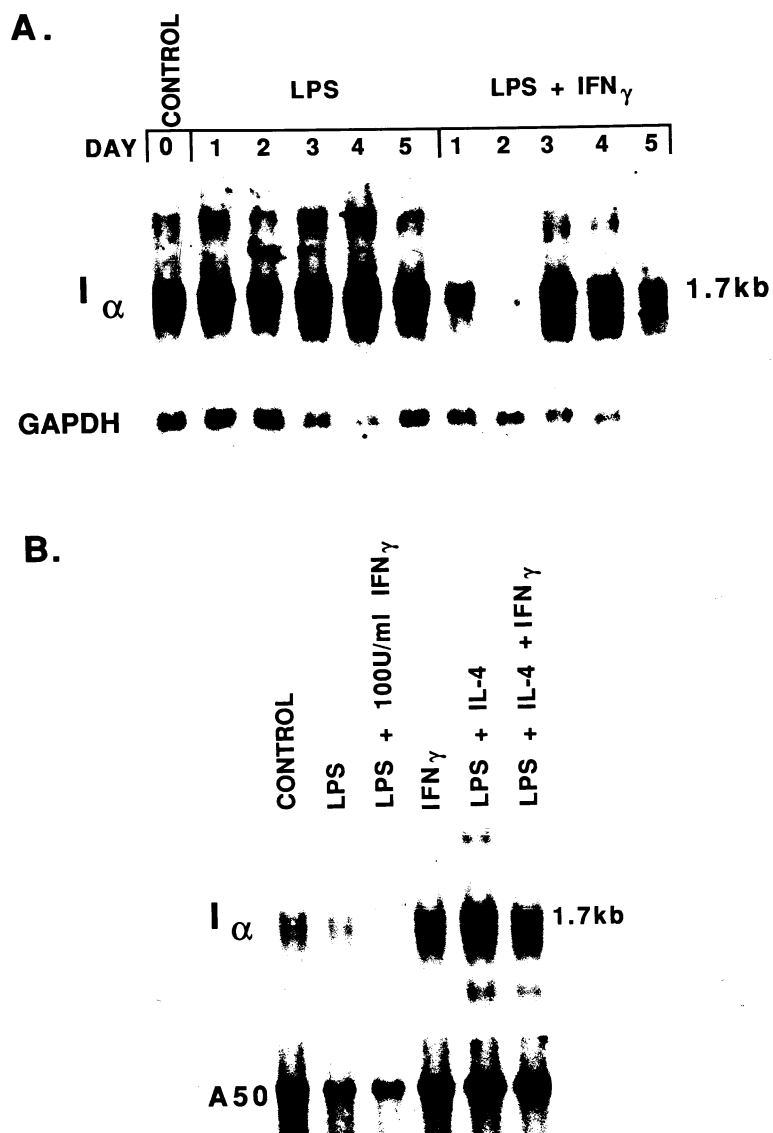linear regression.

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.*

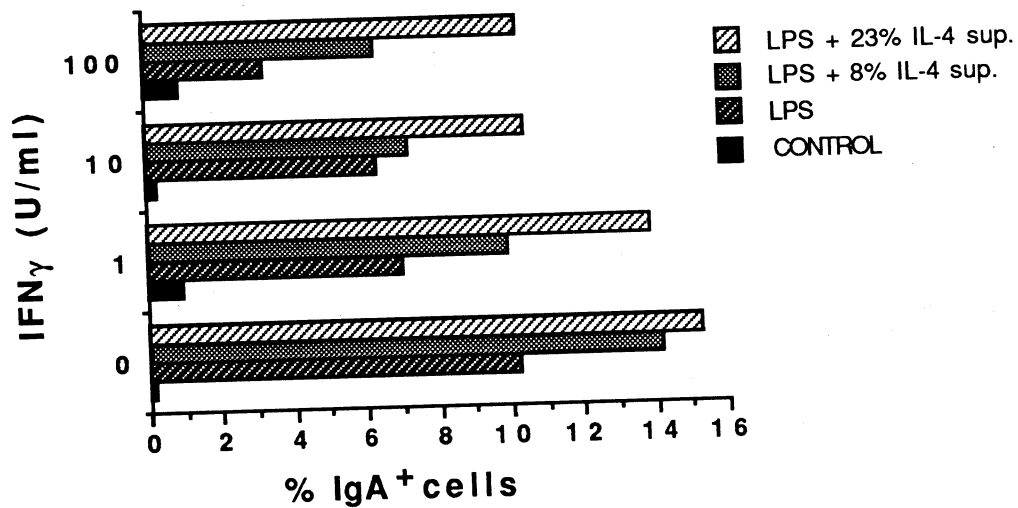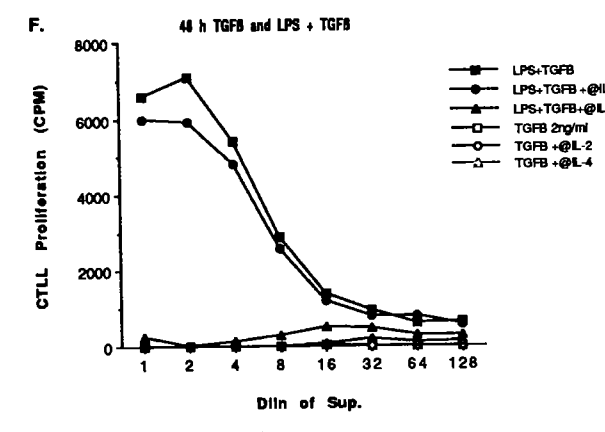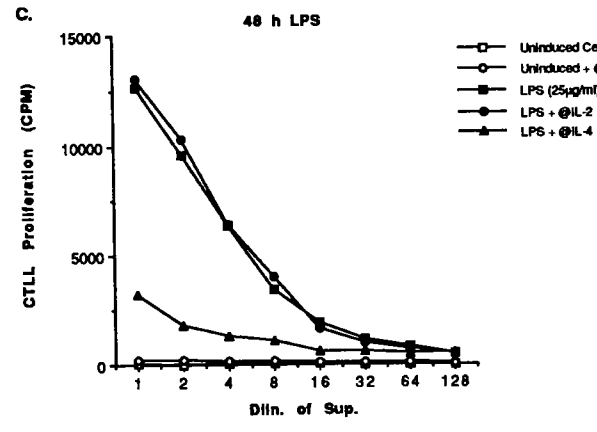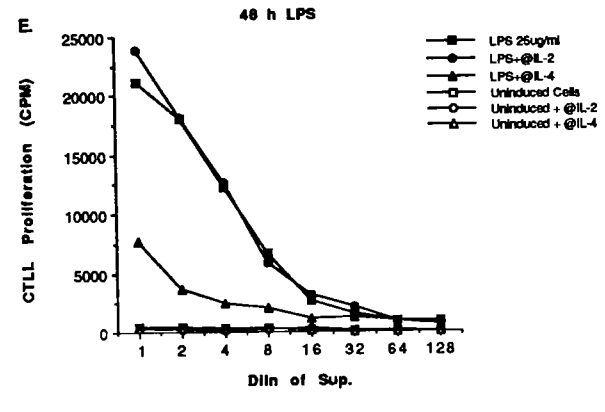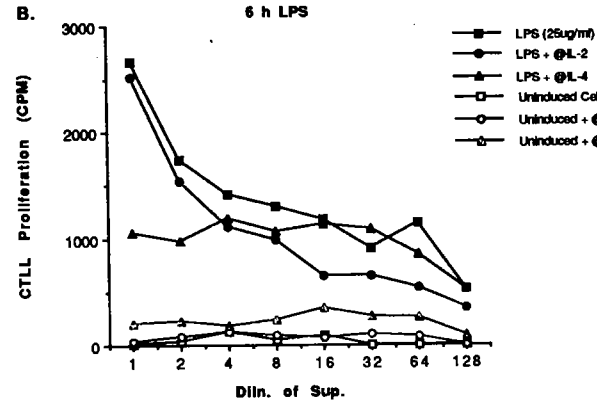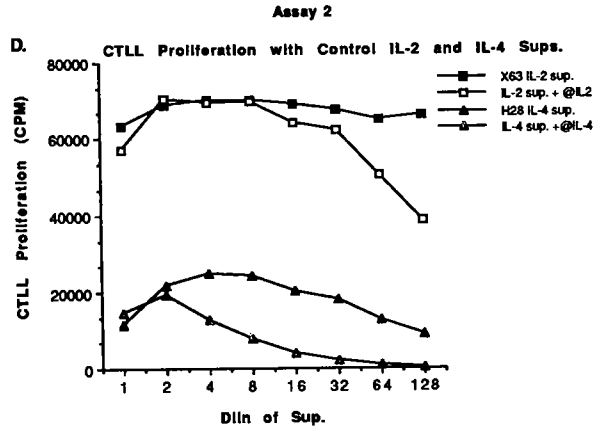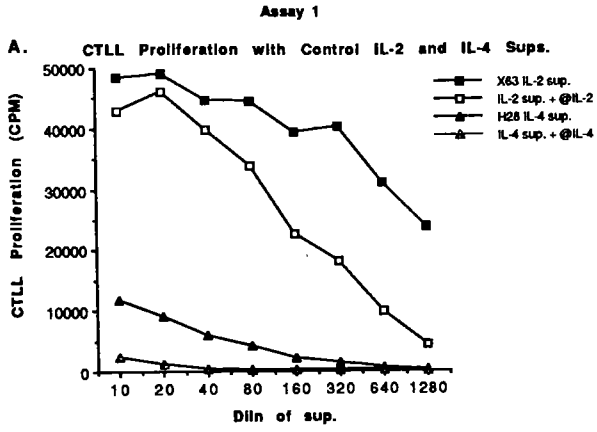Figure 10. Effect of IFNγ on expression of IgA in 1.29μ clone 22D in the presence and absence of IL-4 (H-28 supernatant). Cells were incubated with the indicated inducers for 4 days and assayed for the %IgA⁺ cells on day 10 by immunofluorescence microscopy.

Figure 11. I.29µ cells secrete IL-4 after treatment with LPS. I.29µ clone 22D was cultured at 1×10^6 cells/ml for 6 h or 0.25×10^6 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. *F*. 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).

Assay 1

A. CTLL Proliferation with Control IL-2 and IL-4 Sups.

X63 IL-2 sup.
IL-2 sup. + @IL-2
H28 IL-4 sup.
IL-4 sup. + @IL-4

Assay 2

D. CTLL Proliferation with Control IL-2 and IL-4 Sups.

X63 IL-2 sup.
IL-2 sup. + @IL2
H28 IL-4 sup.
IL-4 sup. +@IL-4

B. 6 h LPS

LPS (25ug/ml)
LPS + @IL-2
LPS + @IL-4
Uninduced Cells
Uninduced + @IL-2
Uninduced + @IL-4

E. 48 h LPS

LPS 25ug/ml
LPS+@IL-2
LPS+@IL-4
Uninduced Cells
Uninduced + @IL-2
Uninduced + @IL-4

C. 48 h LPS

Uninduced Cells
Uninduced + @IL-4
LPS (25ug/ml)
LPS + @IL-2
LPS + @IL-4

F. 48 h TGFB and LPS + TGFB

LPS+TGFB
LPS+TGFB +@IL-2
LPS+TGFB+@IL-4
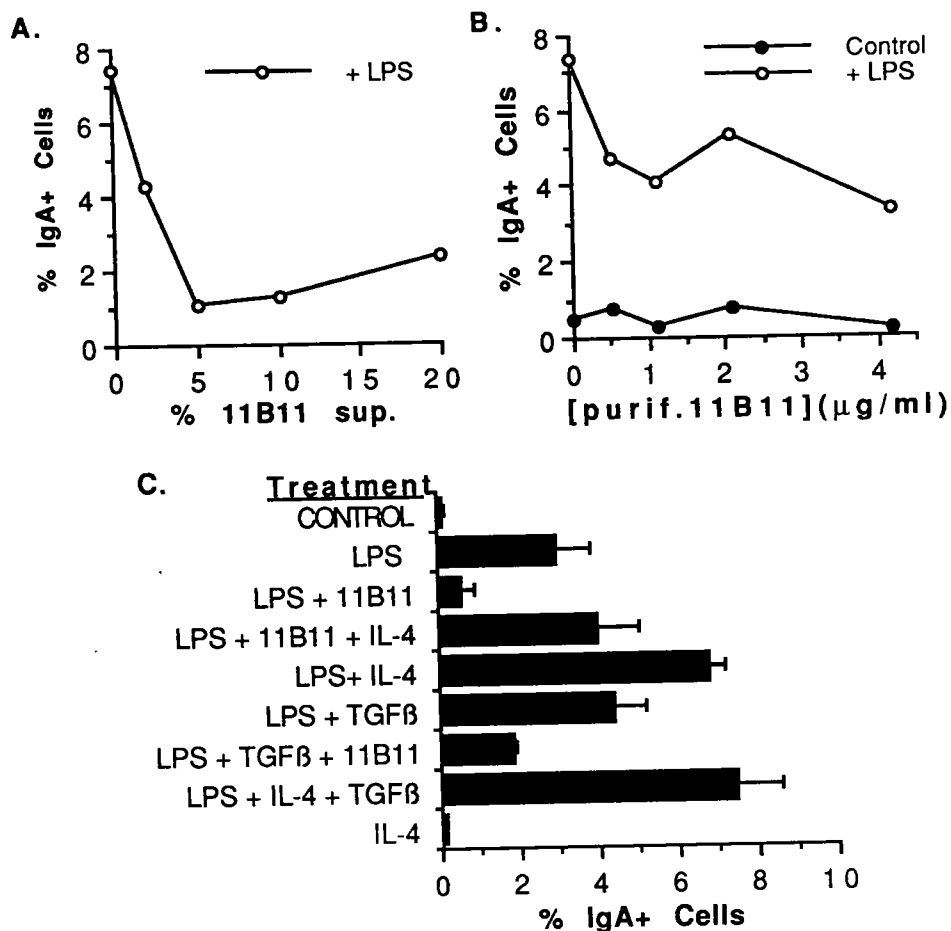TGFB 2ng/ml
TGFB +@IL-2
TGFB +@IL-4

A.

B.

C.

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. *C.* 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.
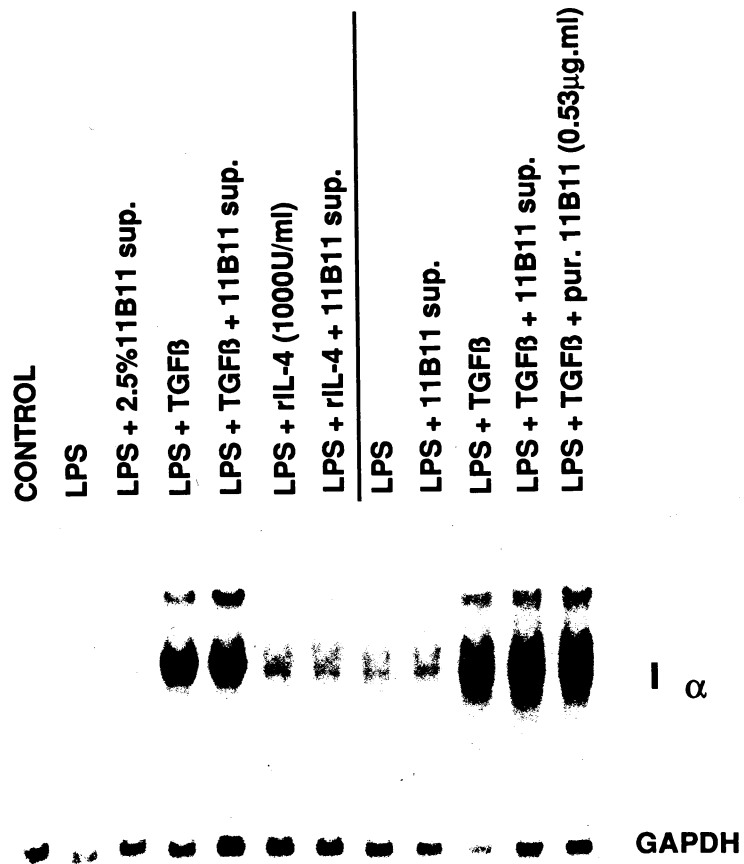
CONTROL
LPS
LPS + 2.5%11B11 sup.
LPS + TGFβ
LPS + TGFβ + 11B11 sup.
LPS + rIL-4 (1000U/ml)
LPS + rIL-4 + 11B11 sup.
LPS
LPS + 11B11 sup.
LPS + TGFβ
LPS + TGFβ + 11B11 sup.
LPS + TGFβ + pur. 11B11 (0.53µg.ml)

I α

GAPDH

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 I$_\alpha$ 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.
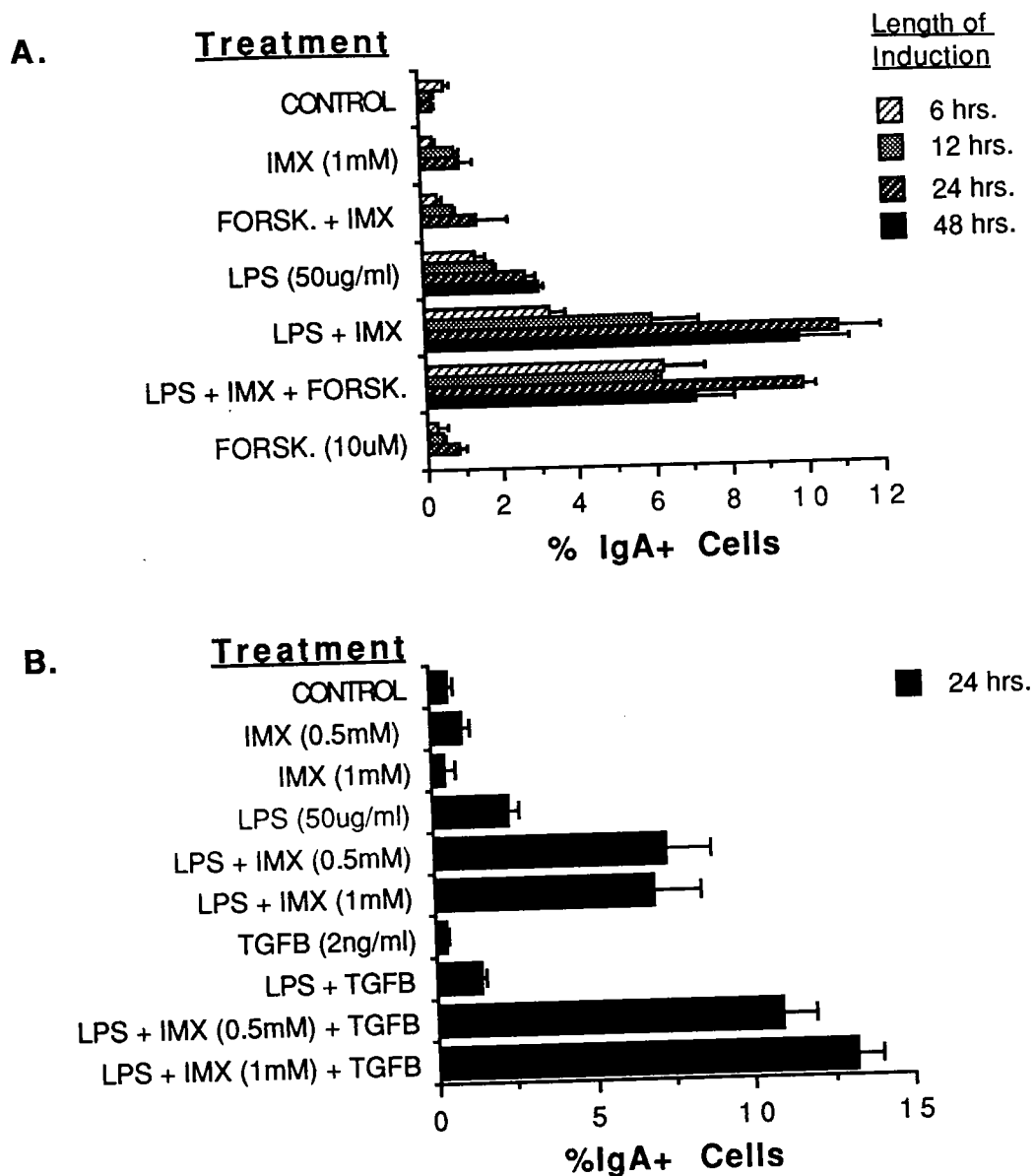
A.

B.

Figure 14. *IBMX increases IgA Expression on day 10.* I.29μ cells were cultured at 0.25×10^6/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.
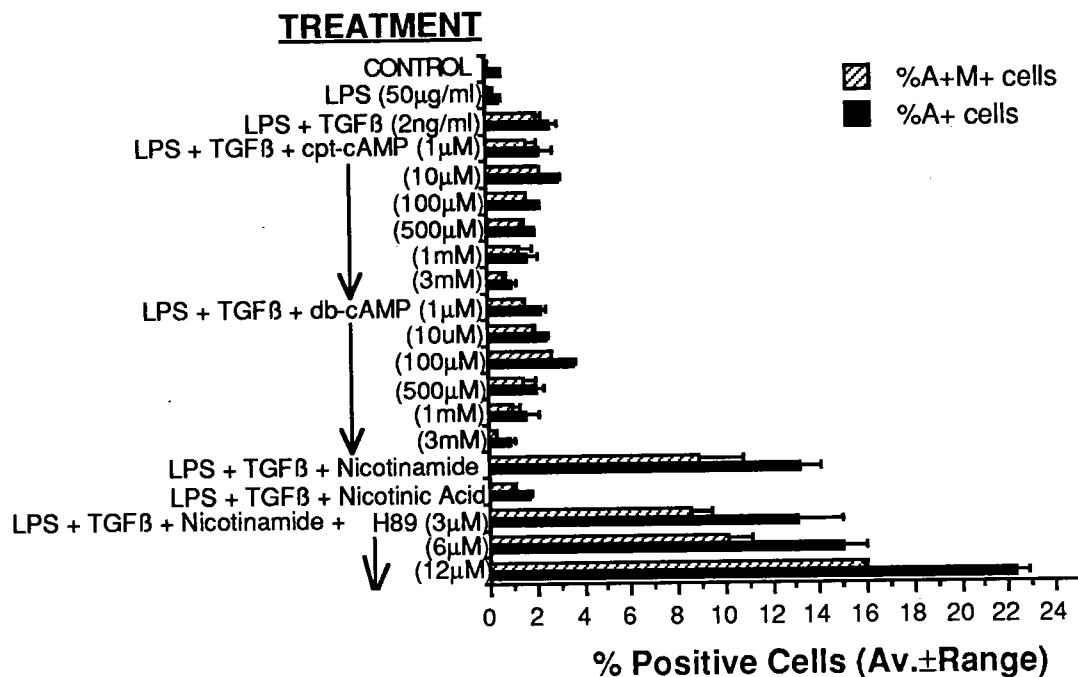
Figure 15. Switching to IgA in I.29μ cells is not increased by cAMP analogs or inhibited by inhibitors of protein kinase A (PKA). I.29μ cells were cultured at 0.15×10^6/ml with LPS (50μg/ml) added on day 0 and TGFβ (2ng/ml) added on days 0,1 and 2. Inducers were diluted 6-fold on day 1, and switching 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.

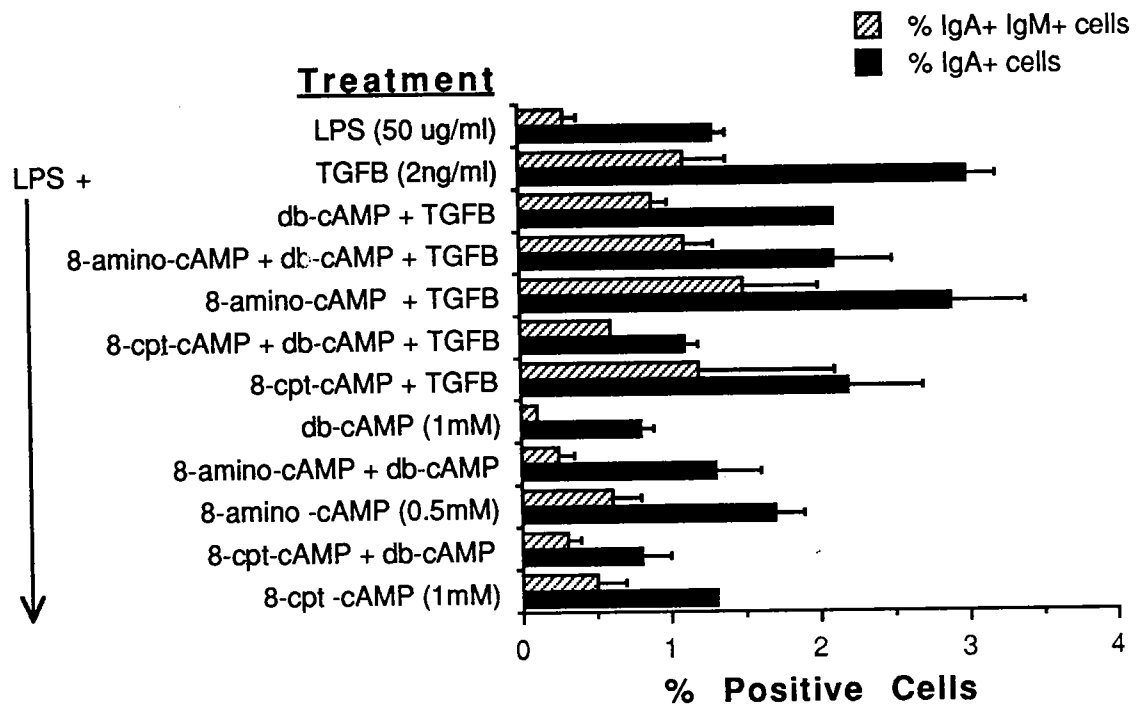*Figure 16.* *Failure of combinations of cAMP analogs to increase LPS-induced IgA Switching in I.29μ cells.* I.29μ cells were cultured at 0.25×10^6/ml with LPS (50μg/ml) ± TGFβ (2ng/ml)(added on days 0 and 1) and 8-Cl-phenyl thio-cAMP (1mM), db-cAMP (1mM) or 8-NH$_3$-cAMP (500μM) and various combinations thereof. Cultures were diluted 6-fold at 24 h and cells were analyzed by immunofluorescence microscopy on day 4.
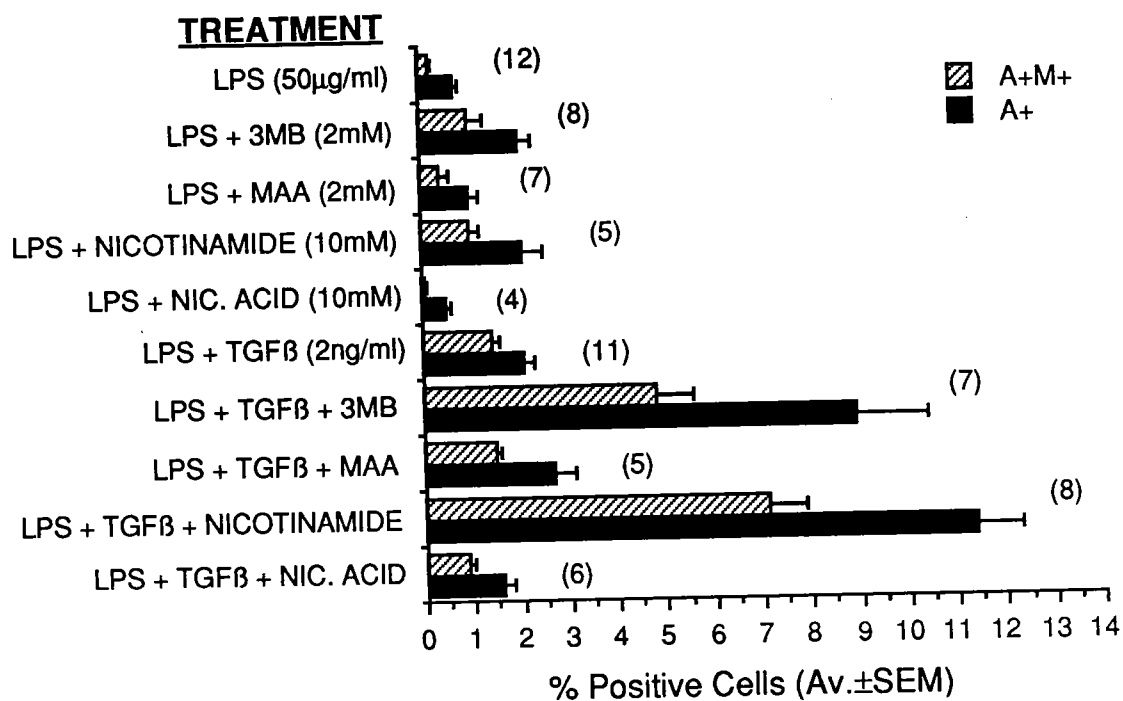
Figure 17. IgA⁺ cells induced on day 3 after treatment of 1.29μ cells with inhibitors of poly(ADP-ribose) polymerase (PARP). Cells were cultured at 0.15-0.4x10⁶/ml with LPS (50mg/ml) plus inhibitors (or inactive analogs) added on day 0 and TGFβ (2ng/ml) added on days 0, 1 and 2 or days 0 and 1 in a few 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.

ANTI-IgM-FITC   **ANTI-IgA-RITC**

A. LPS + TGFß

B. LPS + TGFß + NICOTINAMIDE

C. LPS + TGFß + NICOTINIC ACID
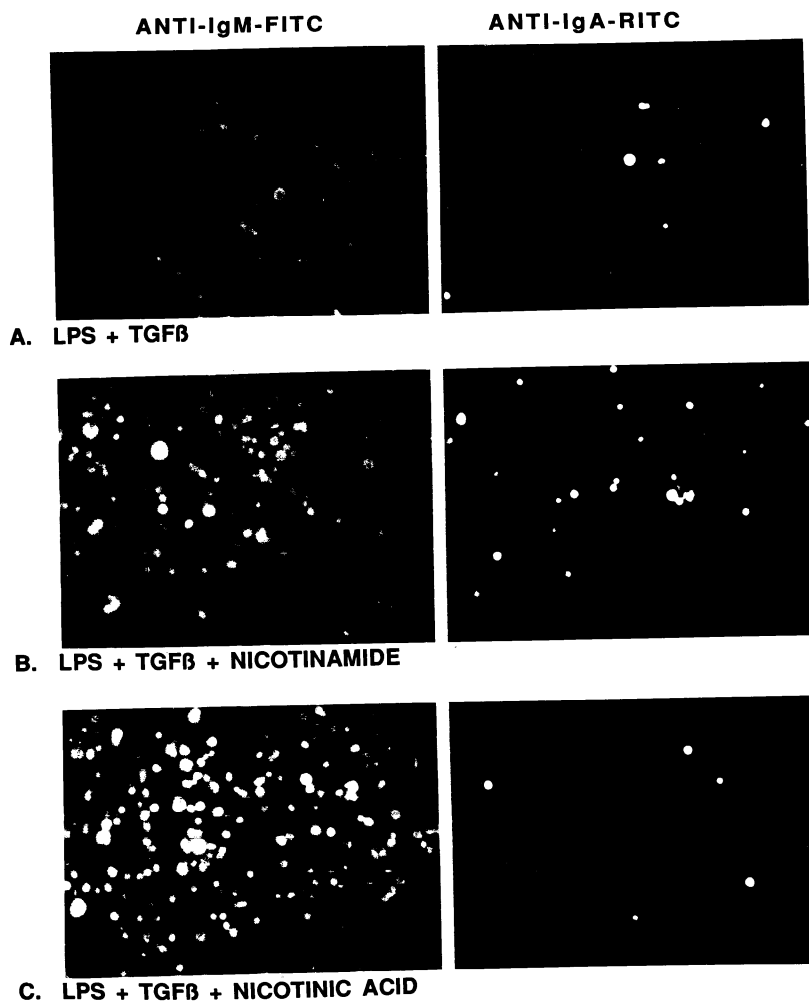
Figure 18. Photographs of representative fields observed by immunofluorescence microscopy after treatment with nicotinamide Cells were visualized under low magnification after treatment with *A.* LPS + TGFβ, *B.* LPS + TGFβ + nicotinamide, *C.* LPS + TGFβ + nicotinic acid. For each of A, B, and C: on 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.

A.

Treatment

B.
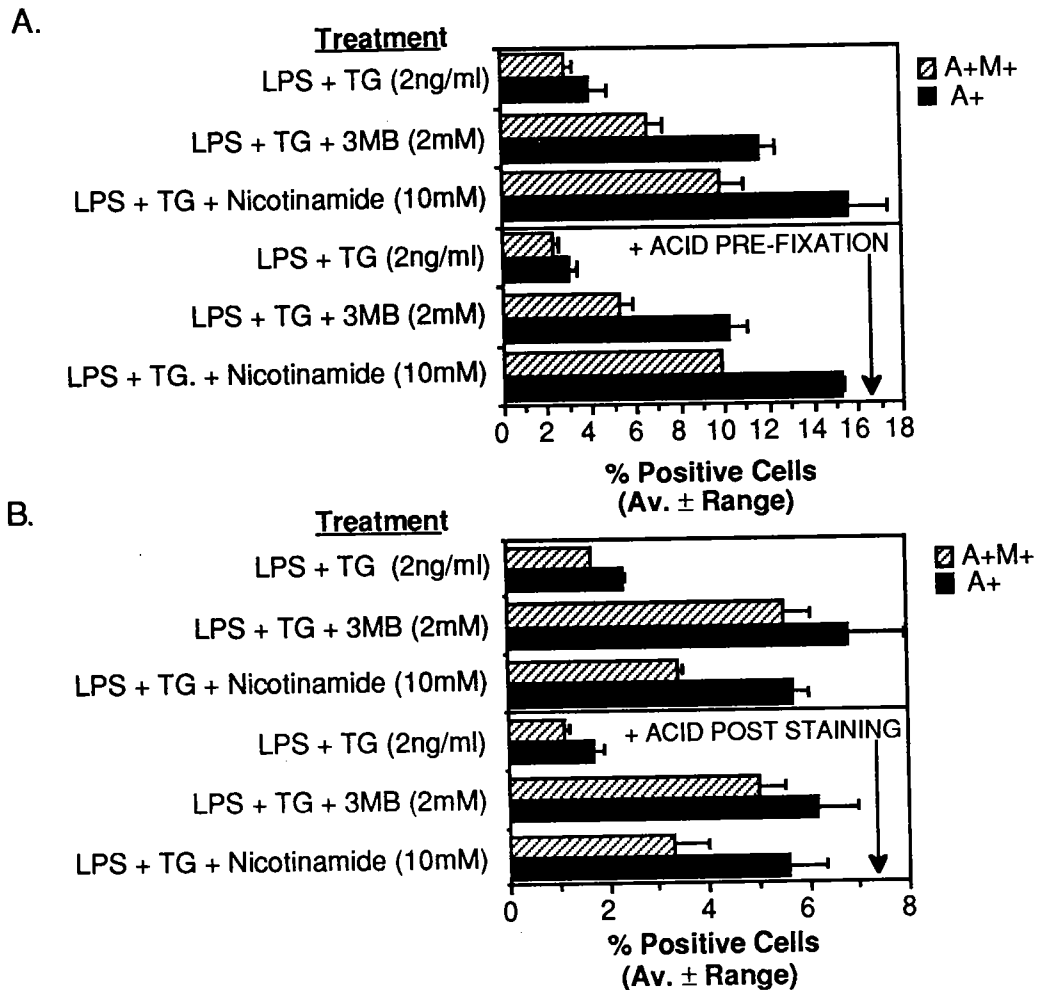
Treatment

Figure 19 Staining of I.29µ cells is resistant to acid treatment. I.29µ cells were cultured at 0.15×10^6/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.
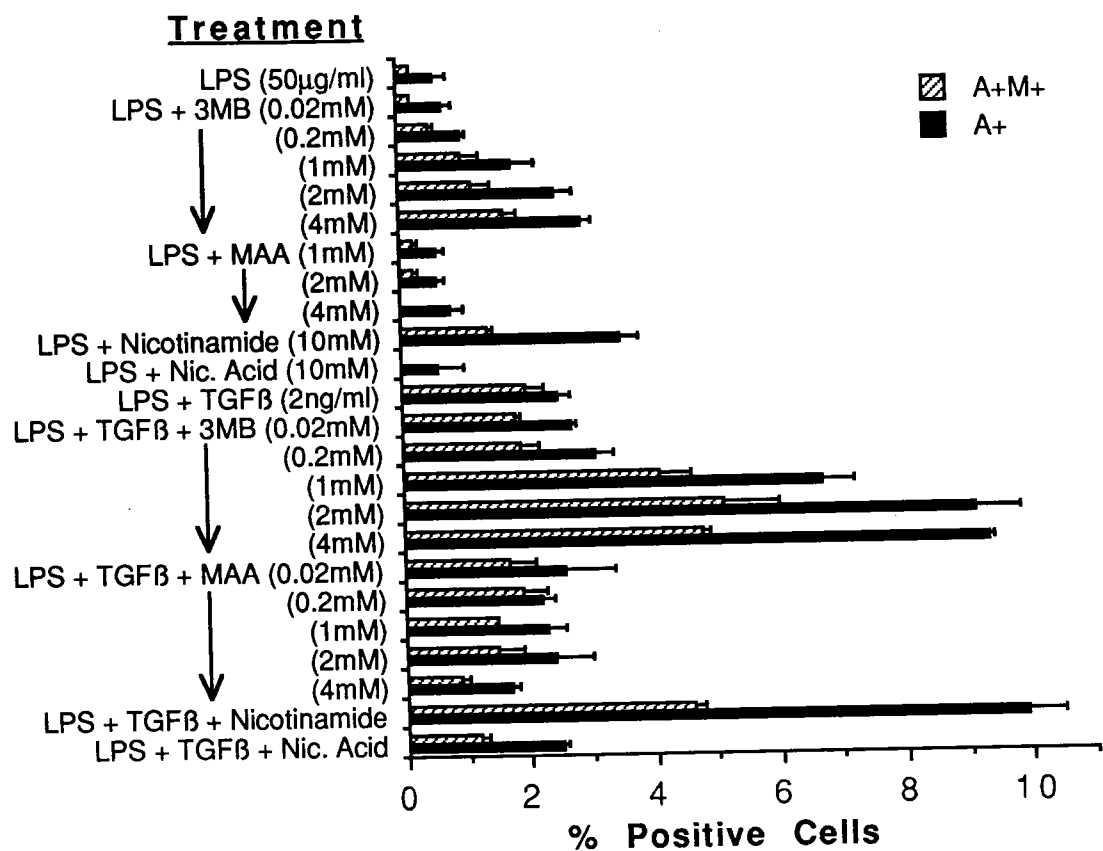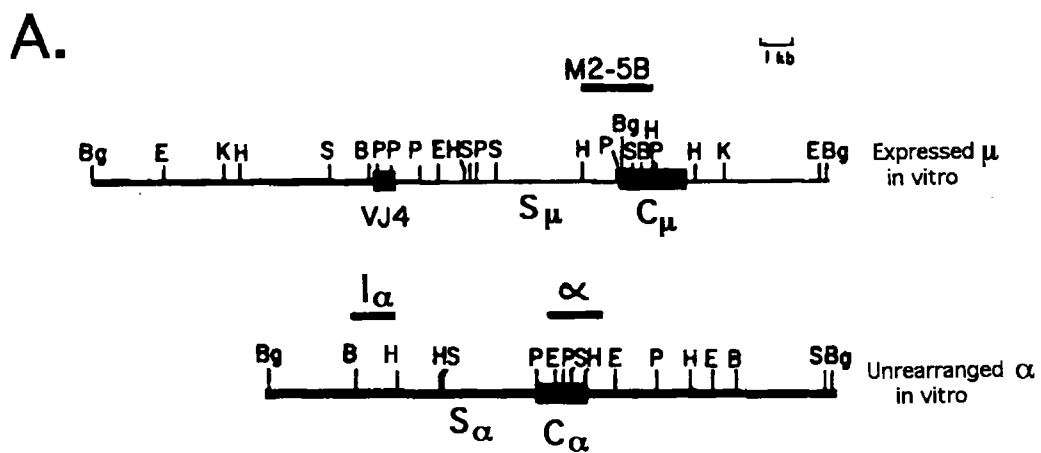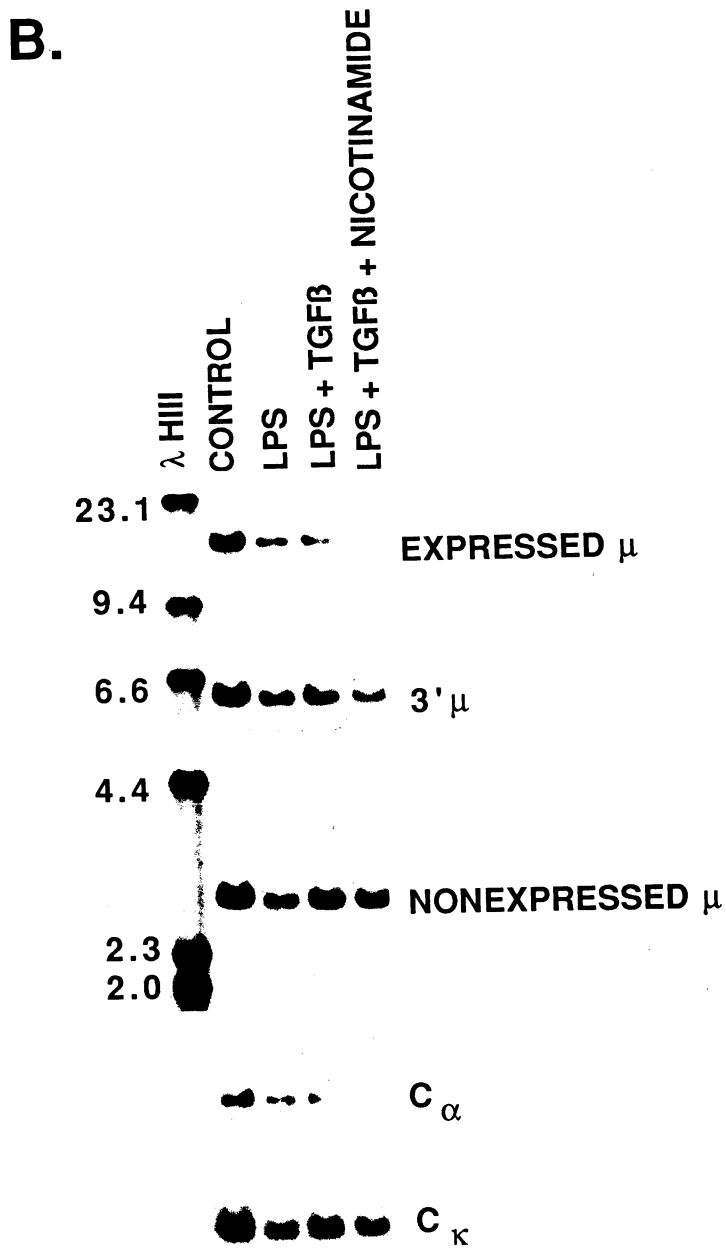
Figure 20. Experiment demonstrating dose responsiveness of IgA switch induction by 3MB. Cells were cultured with LPS (50μg/ml) and various doses of 3MB or other inducers diluted 6-fold on day 1. TGFβ (2ng/ml) was added on day 0 and 1. Cells were analyzed by immunofluorescence microscopy on day 3.

Figure 21. A. Map of the expressed μ gene and unrearranged α genes in IgM^+ I.29μ cells. Probes for C_μ (M2-5B), C_α (α), and I_α (upstream exon of α-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 BgIII DNA fragments containing μ and α switch regions.* DNA from I.29μ cells treated with the indicated inducers was digested with *Bgl*II. **Expressed** μ, denotes the unrearranged S_μ containing *Bgl*II fragment of the expressed chromosome; **3'μ**, denotes the *Bgl*II fragment containing most of C_μ and its 3' segment from the expressed and nonexpressed chromosomes; and **Nonexpressed** μ, denotes the 5'C_μ fragment of the nonexpressed chromosome. The unrearranged *Bgl*II fragment of the α locus is designated C_α and an unrearranged *Bgl*II fragment hybridizing with the C_κ probe is designated C_κ. *C. Densitometry of C_μ hybridization signal to blot in B. D. Results of switch assays from this experiment on days 3 and 7.*

FIGURE 21

B.

λ HIII
CONTROL
LPS
LPS + TGFß
LPS + TGFß + NICOTINAMIDE

23.1 ●   ●━━ EXPRESSED μ

9.4 ●

6.6 ● ●━●━●━━ 3'μ

4.4 ●

●━●━●━● NONEXPRESSED μ

2.3
2.0 ●

●━·━· C_α

●━●━●━● C_κ

C.
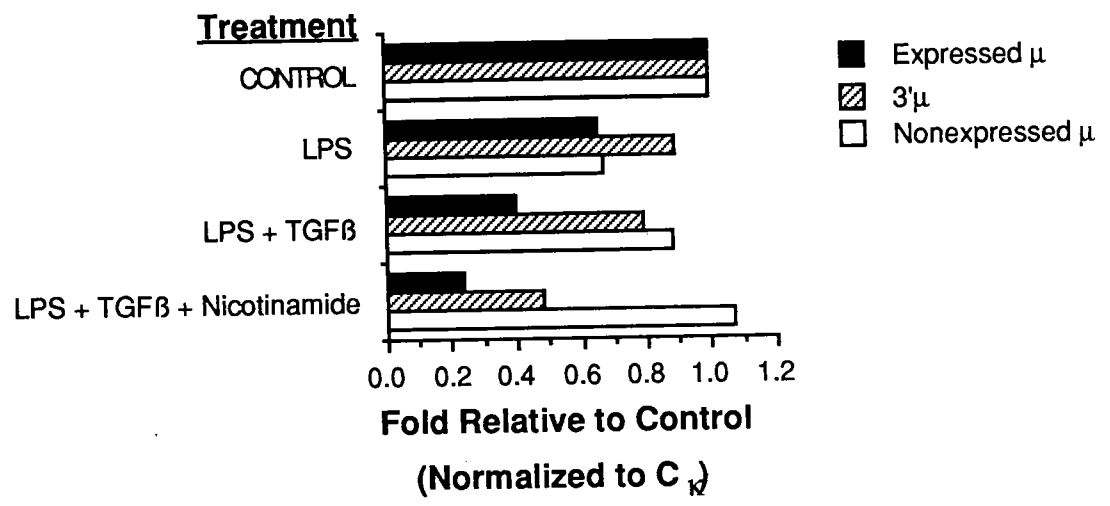
D.
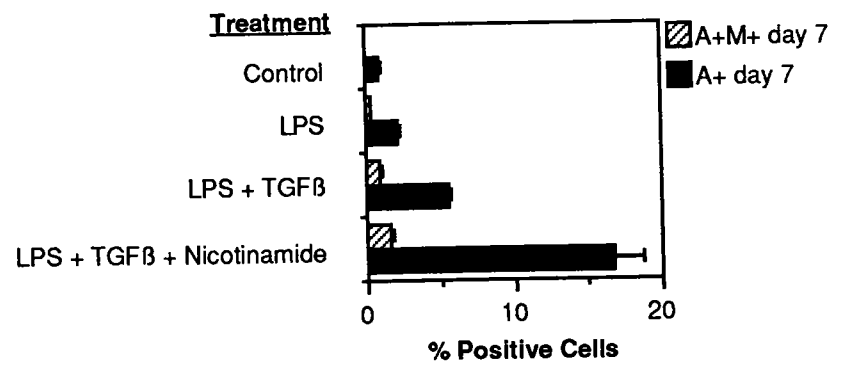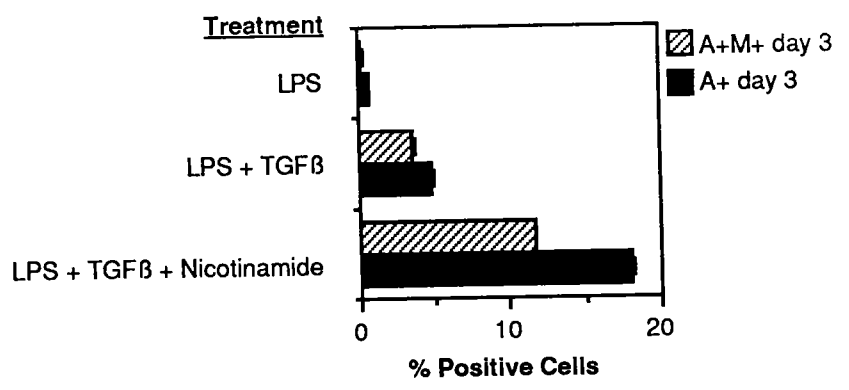
Figure 22. *A.* RNA *blot demonstrating that inhibitors of PARP do not increase* α*-germline transcripts in 1.29*μ *cells at 24 hours.* 10μg of total cell RNA was loaded per lane.   Blot was sequentially hybridized with probes specific for the germline exon (I$_\alpha$) of α-germline RNA (see Figs. 4 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.

I α   -1.7 kb

PARP   -3.8 kb

GAPDH   -1.3 kb

B.

TREATMENT

LPS (50ug/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

IgM+A+
IgA+

% Positive Cells
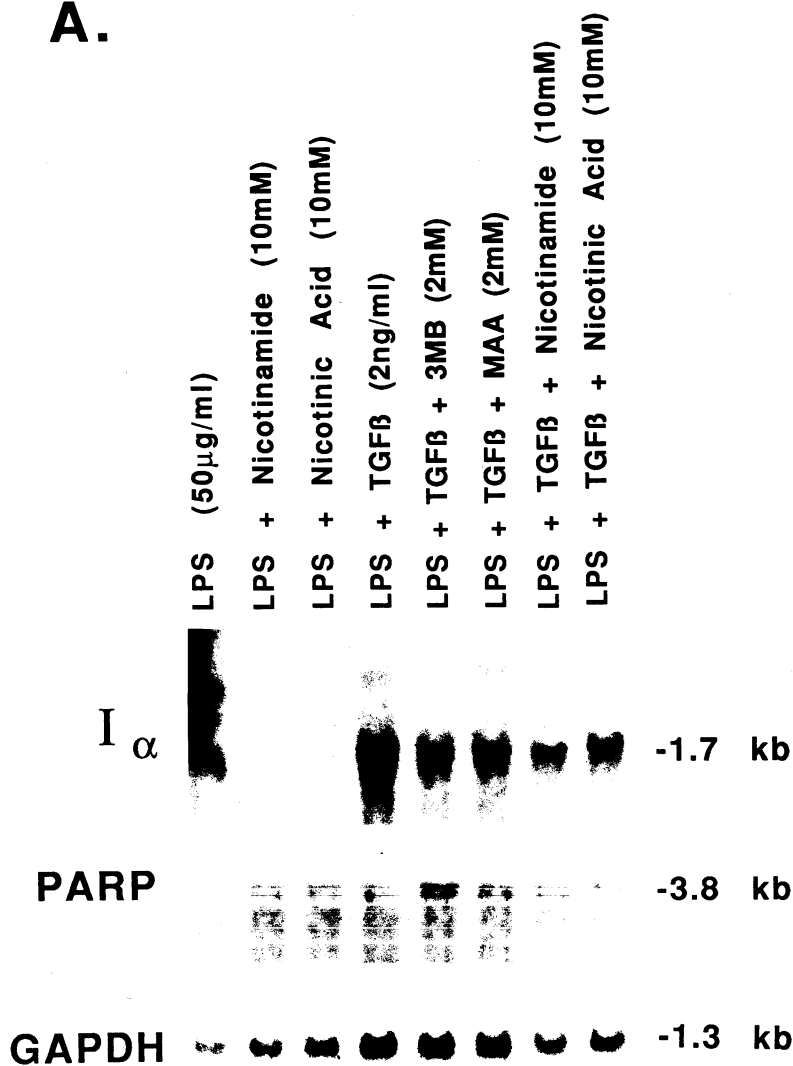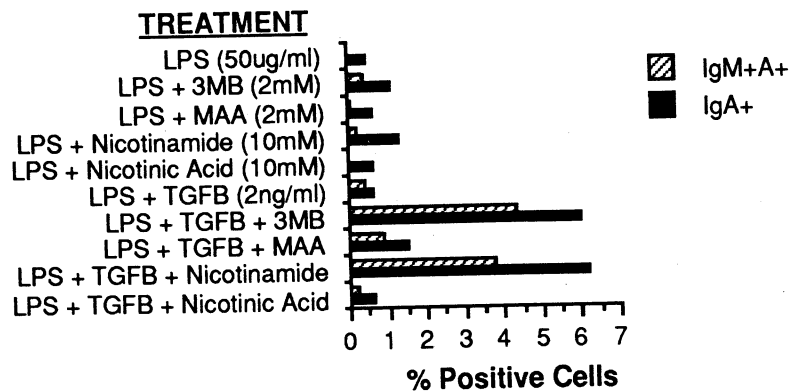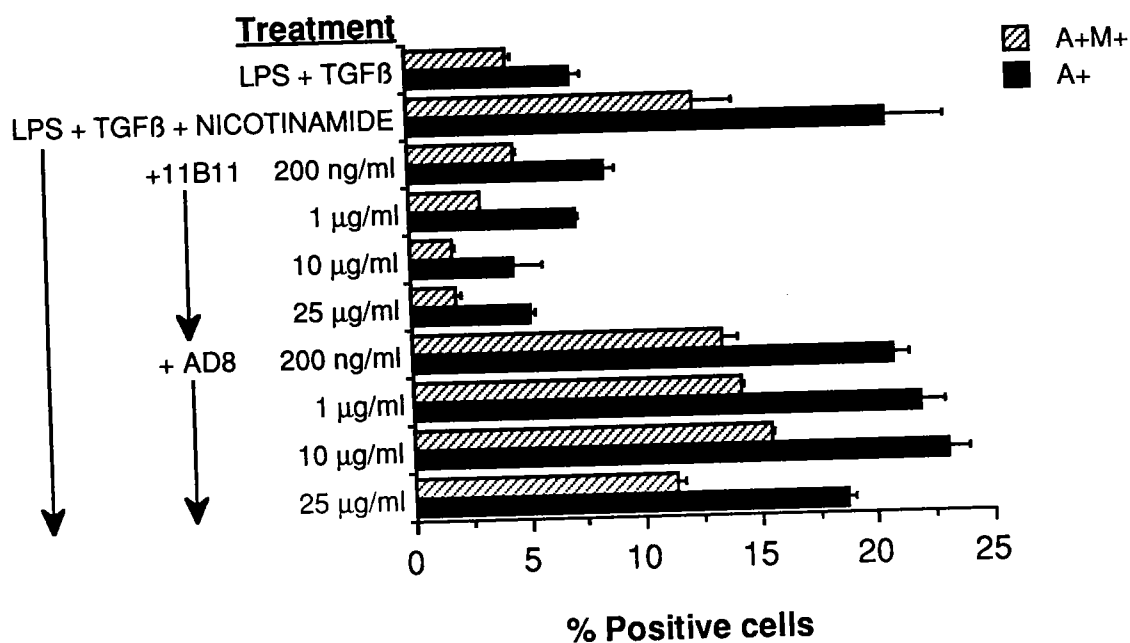
Figure 23. Nicotinamide cannot overcome the requirement for endogenous IL-4 in IgA switching by I.29μ cells. 0.15×10^6 I.29μ cells were stimulated with LPS (50μg/ml) + TGFβ (2ng/ml) ± Nicotinamide (10mM) ± purified 11B11 (or AD8) at the indicated doses. Inducers were diluted 6-fold on day 1. TGFβ was 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.

REFERENCES

Alberini, C., Biassoni, R., DeAmbrosis, S., Vismara, D. and Sitia, R. (1987). Differentiation in the murine B cell lymphoma I.29: Individual μ^+ clones may be induced by lipopolysaccharide to both IgM secretion and isotype switching. Eur. J. Immunol. *17*. 555-562.

Althaus, F. R. and Richter, C. (1987). ADP-ribosylation of proteins: Enzymology and biological significance. Molec. Biol. Biochem. Biophys. *37*. 3-125.

Banasik, M., Komura, H., Shimoyama, M. and Ueda, K. (1992). Specific inhibitors of poly(ADP-ribose) synthetase and mono(ADP-ribosyl)transferase. J. Biol. Chem. *267*. 1569-1575.

Barnard, J. A., Beauchamp, R. D., Coffey, R. J. and Moses, H. L. (1989). Regulation of intestinal epithelial cell growth by transforming growth factor type β. Proc. Natl. Acad. Sci., USA *86*. 1578-1582.

Beagley, K. W., Eldridge, J. H., Kiyono, H., Everson, M. P., Koopman, W. J., Honjo, T. and McGhee, J. R. (1988). Recombinant murine IL-5 induces high rate IgA synthesis in cycling IgA-positive Peyer's patch B cells. J. Immunol. *141*. 2035-2042.

Beaudoin, B. J., Tony, H.-P. and Parker, D. C. (1988). Mechanism of help for resting B cell growth. J. Immunol. *141*. 2230-2239.

Beebe, S. J., Holloway, R., Rannels, S. R. and Corbin, J. D. (1984). Two classes of cAMP analogs which are selective for the two different cAMP-binding sites of type II protein kinase demonstrate synergism when added together in intact adipocytes. J. Biol. Chem. *259*. 3539-3547.

Bergstedt-Lindqvist, S., Moon, H.-B., Persson, U., Moller, G., Heusser, C. and Severinson, E. (1988). Interleukin 4 instructs uncommitted B lymphocytes to switch to IgG1 and IgE. Eur. J. Immunol. *18*. 1073-1077.

Bergstedt-Lindqvist, S., Sideras, P., Macdonald, H. R. and Severinson, E. (1984). Regulation of Ig class secretion by soluble products of certain T-cell lines. Imm. Rev. *78*. 25-50.

Berton, M. T., Uhr, J. W. and Vitetta, E. (1989). Synthesis of germline γ1 immunoglobulin heavy-chain transcripts in resting B cells: Induction by interleukin 4 and inhibition by interferon γ. Proc. Natl. Acad. Sci. USA *86*. 2829-2833.

Berton, M. T. and Vitetta, E. S. (1990). Interleukin 4 induces changes in the chromatin structure of the γ1 switch region in resting B cells before switch recombination. J. Exp. Med. *172*. 375-378.

Bhatia, K., Kang, V. H., Stein, G. S., Bustin, M., Cherney, B. W., Notario, V., Haque, S. J., Huppi, K. and Smulson, M. E. (1990). Cell cycle regulation of an exogenous human poly(ADP-ribose) polymerase cDNA introduced into murine cells. J. Cell. Phys. *144*. 345-353.

Blackwell, T. K., Moore, M. W., Yancopoulos, G. D., Suh, H., Lutzker, S., Selsing, E. and Alt, F. W. (1986). Recombination between immunoglobulin variable region gene segments is enhanced by transcription. Nature *324*. 585-589.

Borsi, L., Castellani, P., Risso, A. M., Leprini, A. and Zardi, L. (1990). Transforming growth factor-β regulates the splicing pattern of fibronectin messenger RNA precursor. FEBS *261*. 175-178.

Buki, K. G., Bauer, P. I. and Kun, E. (1991). Inhibitory binding of adenosine diphosphoribosyl transferase to the DNA primer site of reverse transcriptase templates. Biochem. Biophys. Res. Comm. *180*. 496-503.

Buratowski, S. (1993). DNA repair and transcription: The helicase connection. Science *260*. 37-38.

Chen-Kiang, S. and   Lavery, D. J. (1989). Pulse Labeling of heterologous nuclear RNA in isolated nuclei. Methods Enzymol. *180*. 82-96.

Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. and Hidaka, H. (1990). Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J. Biol. Chem. *265*. 5267.

Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry *18*. 5294-5299.

Chou, C. L. and Morrison, S. L. (1993). A common sequence motif near nonhomologous recombination breakpoints involving Ig sequences. J. Immunol. *150*. 5350-5360.

Church, G. M. and Gilbert, W. (1984). Genomic sequencing. PNAS *81*. 1991-1995.

Coffman, R. L., Lebman, D. A. and Shrader, B. (1989). Transforming growth factor-β specifically enhances IgA production by lipopolysaccharide-stimulated murine B lymphocytes. J. Exp. Med. *170*. 1039-1044.

Coffman, R. L., Seymour, B. W. P., Lebman, D. A., Hiraki, D. D., Christiansen, J. A., Shrader, B., Cherwinski, H. M., Savelkoul, H. F. J., Finkelman, F. D., Bond, M. W. and Mossman, T. R. (1988). The role of helper T cell products in mouse B cell differentiation and isotype regulation. Immunol. Rev. *102*. 5.

Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M. and Strober, W. (1991). Current protocols in immunology. New York, John Wiley & Son. 3.8.11 supp. 2.

Collier, D. A., Griffin, J. A. and Wells, R. D. (1988). Non-B right-handed DNA conformations of homopurine-homopyrimidine sequences in the murine immunoglobulin C_α switch region. J. Biol. Chem. *263*. 7397-7405.

Collins, J. T. and Dunnick, W. A. (1993). Germline transcripts of the murine immunoglobulin γ_{2a} gene: structure and induction by IFN-γ. Int. Immunol. *5*. 885-891.

Cory, S. and Adams, J. (1980). Deletions are associated with somatic rearrangement of immunoglobulin heavy chain genes. Cell *19*. 37-51.

Darby, M. K., Schmidtt, B., Jongstra-Bilen, J. and Vosberg, H.-P. (1985). Inhibition of calf thymus type II DNA topoisomerase by poly(ADP-ribosylation). EMBO J. *4*. 2129-2134.

Defrance, T., Vanbervliet, B., Brière, F., Durand, I., Rousset, F. and Banchereau, J. (1992). Interleukin 10 and transforming growth factor β cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. J. Exp. Med. *175*. 671-682.

de Murcia, G., Huletsky, A. and Poirier, G. G. (1988). Review: Modulation of chromatin structure by poly(ADP-ribosylation). Biochem. Cell. Biol. *66*. 626-635.

de Murcia, G., de Murcia, J. M. and Schreiber, V. (1991). Poly(ADP-ribose) polymerase: Molecular biological aspects. BioEssays *13*. 455-462.

Ding, R., Pommier, Y., Kang, V. H. and Smulson, M. (1992). Depletion of poly(ADP-ribose) polymerase by antisense RNA expression results in a delay

in DNA strand break rejoining. J. Biol. Chem. *267*. 12804-12812.

Dunnick, W., Hertz, G. Z., Scappino, L. and Gritzmacher, C. (1993). DNA sequences at immunoglobulin switch region recombination sites. Nuc. Acids Res. *21*. 365-372.

Dunnick, W. and Stavnezer, J. (1990). Copy choice mechanism of immunoglobulin heavy chain switch recombination. Mol. Cell . Biol. *10*. 397-400.

Dunnick, W., Wilson, M. and Stavnezer, J. (1989). Mutations, duplication, and deletion of recombined switch regions suggest a role for DNA replication in the immunoglobulin heavy-chain switch. Mol. Cell. Biol. *9*. 1850-1856.

Ehrhardt, R. O., Strober, W. and Harriman, G. R. (1992). The effect of TGF-beta$_1$ on IgA isotype switch: TGF-beta$_1$ induces low-frequency switch to IgA regardless of the method of B cell activation. J. Immunol. *148*. 3830-3836.

Esser, C. and Radbruch, A. (1989). Rapid induction of transcription of unrearranged Sγ1 switch regions in activated murine B cells by interleukin 4. EMBO J. *8*. 483-488.

Farzaneh, F., Panayotou, G. N., Bowler, L. D., Hardas, B. D., Broom, T., Walther, C. and Shall, S. (1988). ADP-ribosylation is involved in the integration of foreign DNA into the mammalian cell genome. Nuc. Acid. Res. *16*. 11319-11326.

Ferro, A. M., Higgins, N. P. and Olivera, B. M. (1983). Poly(ADP-ribosylation) of a DNA topoisomerase. J. Biol. Chem. *258*. 6000-6003.

Ferro, A. M., McElwain, M. C. and Olivera, B. M. (1984). Poly(ADP-ribosylation) of DNA topoisomerase I: A nuclear response to DNA-strand interruptions. Cold. Spring Harbor Symp. Quant. Biol. *49*. 683-690.

Ferro, A. M. and Olivera, B. M. (1982). Poly(ADP-ribosylation) *in vitro*: Reaction parameters and enzyme mechanism. J. Biol. Chem. *257*. 7808-7813.

Finkelman, F. D., Holmes, J., Katona, I. M., Joseph F. Urban, J., Beckman, M. P., Park, L. S., Schooley, K., Coffman, R. L., Mosmann, T. R. and Paul, W. E. (1990). Lymphokine control of in vivo immunoglobulin isotype selection. Annu. Rev. Immunol. *8*. 303-333.

Fort, P., Marty, L., Piechaczyk, M., Sabrouty, S. E., Dani, C., Jeanteur, P. and Blanchard, J. M. (1985). Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucl. Acid. Res. *13*. 1431-1442.

Fukita, Y., Mizuta, T.-R., Shirozu, M., Ozawa, K., Shimizu, A. and Honjo, T. (1993). The human Sμbp-2, a DNA-binding protein specific to the single-stranded guanine-rich sequence related to the immunoglobulin μ chain switch region. J. Biol. Chem. *268*. 17463-17470.

Gaff, C. and Gerondakis, S. (1990). RNA splicing generates alternate forms of

germline α heavy chain transcripts. Int. Immunol. *2*. 1143-1148.

Galione, A. (1993). Cyclic ADP-ribose: A new way to control calcium. Science *259*. 325-326.

Galli, G., Guise, J. W., Mcdevitt, M. A., Tucker, P. W. and Nevins, J. R. (1987). Relative position and strengths of poly(A) sites as well as transcription are critical to membrane versus secreted μ-chain expression during B-cell development. Genes and Dev. *1*. 471-481.

Gascan, H., Gauchat, J.-F., Aversa, G., Vlasselaer, P. V. and de Vries, J. E. (1991). Anti-CD40 monoclonal antibodies or CD4$^+$ T cell clones and IL-4 induce IgG4 and IgE switching in purified human B cells via different signaling pathways. J. Immunol. *147*. 8-13.

Gauchat, J.-F., Lebmen, D. A., Coffman, R. L., Gascan, H. and de Vries, J. E. (1990). Structure and expression of germline ϵ transcripts in human B cells induced by interleukin 4 to switch to IgE production. J. Exp. Med. *172*. 463-473.

Gerondakis, S. (1990). Structure and expression of murine germ-line immunoglobulin ϵ heavy chain transcripts induced by interleukin 4. Proc. Natl. Acad. Sci., USA *87*. 1581-1585.

Goodman, D. J., Gaff, C. and Gerondakis, S. (1993). The IL-4 induced increase in the frequency of resting murine splenic B cells expressing germline Ig heavy chain $\gamma1$ transcripts correlates with subsequent switching to IgG$_1$. Int.

Immunol. *5*. 199-208.

Grabstein, K. H., Maliszewski, C. R., Shanebeck, K., Sato, T. A., Spriggs, M. K., Fanslow, W. C. and Armitage, R. (1993). The regulation of T cell-dependent antibody formation *in vitro* by CD40 ligand and IL-2. J. Immunol. *150*. 3141-3147.

Greenberg, M. E., Greene, L. A. and Ziff, E. B. (1985). Nerve growth factor and epidermal growth factor induce rapid transient changes in proto-oncogene transcription in PC12 cells. J. Biol. Chem. *260*. 14101-14110.

Gronowicz, E. S., Doss, C. and Schroder, J. (1979). Activation to Ig secretion by lipopolysaccharide requires several proliferation cycles. J. Immunol. *123*. 2057-2062.

Hanawalt and Mellon (1993). Stranded in an active gene. Curr. Biol. *3*. 67-69.

Harriman, G. R., Kunimoto, D. Y., Elliot, J. F., Paetkau, V. and Strober, W. (1988). The role of IL-5 in IgA B cell differentiation. J. Immunol *140*. 3033-3039.

Hodgkin, P., Yamashita, L. C., Coffman, R. and Kehry, M. R. (1990). Separation of events mediating B cell proliferation and Ig production by using T cell membranes and lymphokines. J. Immunol. *145*. 2025-2034.

Hodgkin, P. D., Yamashita, L. C., Seymour, B., Coffman, R. L. and Kehry, M. R. (1991). Membranes from both Th1 and Th2 cell clones stimulate B cell

proliferation and prepare B cells for lymphokine-induced differentiation to secrete IG. J. Immunol. *147*. 3696-3702.

Honjo, T. and Kataoka, T. (1978). Organization of immunoglobulin heavy chain genes and allelic deletion model. Proc. Natl. Acad. Sci. USA *75*. 2140-2144.

Howard, M., Santos-Argumendo, L., Grimaldi, C., Bazan, F., Harada, N. and Parkhouse, M. (1992). B cell triggering via CD38. J. Cell. Biochem. *Abs. Supp. 17B*. 159.

Huppi, K., Bhatia, K., Siwarski, D., Klinman, D., Cherney, B. and Smulson, M. (1989). Sequence and organization of the mouse poly (ADP-ribose) polymerase gene. Nucl. Acid. Res. *17*. 3387-3401.

Hurwitz, J. L. and Cebra, J. J. (1982). Rearrangements between the immunoglobulin heavy chain gene J_H and C_μ regions accompany normal B lymphocyte differentiation in vitro. Nature *299*. 742-744.

Islam, K. B., Nilsson, L., Sideras, P., Hammarström, L. and Smith, C. I. E. (1991). $TGF\beta_1$ induces germ-line transcripts of both IgA subclasses in human B lymphocytes. Int. Immunol. *3*. 1099-1106.

Iwasato, T., Arakawa, H., Shimizu, A., Honjo, T. and Yamagishi, H. (1992). Biased distribution of recombination sites within S regions upon immunoglobulin class switch recombination induced by transforming growth factor ß and lipopolysaccharide. J. Exp. Med. *175*. 1539-1546.

Iwasato, T., Shimizu, A., Honjo, T. and Yamagishi, H. (1990). Circular DNA is excised by immunoglobulin class switch recombination. Cell *62*. 143-149.

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

Jambou, R. C., Snouwaert, J. N., Bishop, G. A., Stebbins, J. R., Frelinger, J. A. and Fowlkes, D. M. (1988). High level expression of a bioengineered, cysteine-free hepatocyte-stimulating factor (interleukin 6)-like protein. PNAS *85*. 9426-9430.

Jongstra-Bilen, J., Ittel, M.-E., Niedergang, C., Vosberg, H.-P. and Mandel, P. (1983). DNA topoisomerase I from calf thymus is inhibited in vitro by poly(ADP-ribosylation). Eur. J. Biochem. *136*. 391-396.

Jung, S., Rajewsky, K. and Radbruch, A. (1993). Shutdown of class switch recombination by deletion of a switch region control element. Science *259*. 984-987.

Karasuyama, H. and Melchers, F. (1988). Establishment of mouse lines which constituitively secrete large quantities of interleukin 2, 3, 4, or 5 using modified cDNA expression vectors. Eur. J. Immunol. *18*. 97-104.

Kasid, U. N., Halligan, B., Liu, L. F., Dritschilo, A. and Smulson, M. (1989). Poly(ADP-ribose)-mediated post-translational modification of chromatin-

asssociated human topoisomerase I: Inhibitory effects on catalytic activity. J. Biol. Chem. *264*. 18687-18692.

Kearney, J. F. and Lawton, A. R. (1975). B lymphocyte differentiation induced by lipopolysaccharide. I. Generation of cells synthesizing four major immunoglobulin classes. J. Immunol. *115*. 671-676.

Kehrl, J. H., Roberts, A. B., Wakefield, L. M., Jakowlew, S., Sporn, M. B. and Fauci, A. S. (1986a). Transforming growth factor β is an important immunomodulatory protein for human B lymphocytes. J. Immunol. *137*. 3855-3860.

Kehrl, J. H., Wakefield, L. M., Roberts, A. B., Jakowlew, S., Alvarez-Mon, M., Derynck, R., Sporn, M. B. and Fauci, A. S. (1986b). Production of transforming growth factor β by human lymphocytes and its potential role in the regulation of T cell growth. J. Exp. Med. *163*. 1037-1050.

Kenter, A. L. and Watson, J. V. (1987). Cell cycle kinetics model of LPS-stimulated spleen cells correlates switch region rearrangements with S phase. J. Immunol. Methods *97*. 111-117.

Kim, P.-H. and Kagnoff, M. F. (1990). Transforming growth factor β1 increases IgA isotype switching at the clonal level. J. Immunol. *145*. 3773-3778.

Koyama, S.-y. and Podolsky, D. K. (1989). Differential expression of transforming growth factors α and β in rat intestinal epithelial cells. J. Clin.

Invest. *83*. 1768-1773.

Kühn, R., Rajewsky, K. and Müller, W. (1991). Generation and analysis of interleukin-4 deficient mice. Science *254*. 707-710.

Kumagai, K., Abo, T., Sekizawa, T. and Sasaki, M. (1975). Studies of surface immunoglobulins on human B lymphocytes: Dissociation of cell-bound immunoglobulins with acid pH or at 37°C. J. Immunol. *115*. 982-987.

Kunimoto, D. Y., Harriman, G. R. and Strober, W. (1988). Regulation of IgA differentiation in CH12LX cells by lymphokines. IL-4 induces membrane IgM-positive CH12LX cells to express membrane IgA and IL-5 induces membrane IgA-positive CH12LX cells to secrete IgA. J. Immunol. *141*. 713-720.

Küpper, J. H., de Murcia, G. and Bürkle, A. (1990). Inhibition of poly(ADP-ribosylation) by overexpressing the poly(ADP-ribose) polymerase DNA-binding domain in mamalian cells. J. Biol. Chem. *265*. 18721-18724.

Lars, N. and Paschalis, S. (1993). The human $I_\alpha 1$ and $I_\alpha 2$ germline promoter elements: proximal positive and distal negative elements may regulate the tissue specific expression of $C_\alpha 1$ and $C_\alpha 2$ germline transcripts. Int. Immunol. *5*. 271-282.

Lebman, D. and Coffman, R. (1988a). Interleukin 4 causes isotype switching to IgE in T cell-stimulated clonal B cell cultures. J. Exp. Med. *168*. 853-862.

Lebman, D. A. and Coffman, R. (1988b). The effects of IL-4 and Il-5 on the IgA response by murine peyer's patch B cell subpopulations. J. Immunol. *141*. 2050-2056.

Lebman, D. A., Lee, F. D. and Coffman, R. L. (1990a). Mechanism for transforming growth factor β and Il-2 enhancement of IgA expression in lipopolysaccharide-stimulated B cell cultures. J. Immunol. *144*. 952-959.

Lebman, D. A., Nomura, D. Y., Coffman, R. L. and Lee, F. D. (1990b). Molecular characterization of germ-line immunoglobulin A transcripts produced during transforming growth factor type β-induced isotype switching. Proc. Natl. Acad. Sci. USA *87*. 3962-3966.

Leonard, M. W. and Patient, R. K. (1991). Evidence for torsional stress in transcriptionally activated chromatin. Molec. Cell. Biol. *11*. 6128-6138.

Levi, V., Jacobson, E. L. and Jacobson, M. K. (1978). Inhibition of poly(ADP-ribose) polymerase by methylated xanthines and cytokinins. FEBS Lett. *88*. 144-146.

Liao, F., Giannini, S. L. and Birshstein, B. (1992). A nuclear DNA-binding protein expressed during early stages of B cell differentiation interacts with diverse segments within and 3' of the Ig H chain gene cluster. J. Immunol. *148*. 2909-2917.

Lin, H. Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R. A. and Lodish, H. F. (1992).

Expression cloning of the TGFβ type II receptor, a functional transmembrane serine/threonine kinase. Cell *68*. 775-785.

Lin, Y.-C. A. and Stavnezer, J. (1992). Regulation of transcription of the germline Igα constant region gene by an ATF element and by novel transforming growth factor-ß1-responsive elements. J. Immunol. *149*. 2914-2925.

Loetscher, P., Alvarez-Gonzalez, R. and Althaus, F. R. (1987). Poly(ADP-ribose) may signal changing metabolic conditions to the chromatin of mamalian cells. Proc. Natl. Acad. Sci., USA *84*. 1286-1289.

Louie, S. W., Ramirez, L. M., Krieg, A. M., Maliszewski, C. R. and Bishop, G. A. (1993). Endogenous secretion of IL-4 maintains growth and Thy-1 expression of a transformed B cell clone. J. Immunol. *150*. 399-406.

Lutzker, S. and Alt, F. W. (1988). Structure and Expression of germline immunoglobulin γ2b transcripts. Mol. Cell. Biol. *8*. 1849-1852.

Lutzker, S., Rothman, P., Pollock, R., Coffman, R. and Alt, F. W. (1988). Mitogen- and IL-4-regulated expression of germline Igγ2b transcripts: Evidence for directed heavy chain class switching. Cell *53*. 177-184.

Lycke, N. and Strober, W. (1989). Cholera toxin promotes B cell isotype differentiation. J. Immunol. *142*. 3781-3787.

Marcu, K. B., Banerji, J., Penncavage, N. A., Lang, R. and Arnheim, N. (1980). 5' flanking region of immunoglobulin heavy chain constant region genes displays length heterogeneity in germlines of inbred mouse strains. Cell 22. 187-196.

Marcu, K. B., Xu, L. and Kim, M. G. (1992). SαBP/BSAP/NF-Sμ, a murine and human B cell stage specific nuclear factor with DNA binding specificity implying roles in switch-recombination and transcription. Curr. Topp. Microbiol. Immunol. 182. 167-174.

Matsuoka, M., Yoshida, K., Maeda, T., Usuda, S. and Sakano, H. (1990). Switch circular DNA is formed in cytokine-treated mouse splenocytes: Evidence for intramolecular DNA deletion in immunoglobulin class switching. Cell 62. 135-142.

McIntire, T. M., Klinman, D. R., Rothman, P., Lugo, M., Dasch, J. R., Mond, J. J. and Snapper, C. M. (1993). Transforming growth factor β_1 selectively stimulates immunoglobulin G2b secretion by lipopolysaccharide-activated murine B cells. J. Exp. Med. 177. 1031-1037.

Mizuta, T.-R., Fukita, Y., Miyoshi, T., Shimizu, A. and Honjo, T. (1993). Isolation of cDNA encoding a binding protein specific to 5'-phosphorylated single-stranded DNA with G-rich sequences. Nucleic Acids Res. 21. 1761-1766.

Molinete, M., Vermeulen, W., Bürkle, A., de Murcia, J. M., Küpper, J. H., Hoeijmakers, J. H. J. and Murcia, G. d. (1993). Overproduction of the poly(ADP-

ribose) polymerase DNA-binding domain blocks alkylation-induced DNA repair synthesis in mamalian cells. EMBO J. *12*. 2109-2117.

Mossman, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A. and Coffman, R. L. (1986). Two types of murine T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J. Immunol. *136*. 2348-2357.

Murray, P. D., McKenzie, D. T., Swain, S. L. and Kagnoff, M. F. (1987). Interleukin 5 and interleukin 4 produced by Peyer's patch T cells selectively enhance immunoglobulin A expression. J. Immunol *139*. 2669-2674.

Nguyen, H. T., Medford, R. M. and Nadal-Ginard, B. (1983). Reversibility of muscle differentiation in the absence of commitment: Analysis of a myogenic cell line temperature-sensitive for commitment. Cell *34*. 281-293.

Nickoloff, J. A. (1992). Transcription enhances intrachromosomal homologous recombination in mamalian cells. Molec. cell. Biol. *12*. 5311-5318.

Nikaido, T., Nikai, S. and Honjo, T. (1981). Switch region of immunoglobulin Cμ gene is composed of simple tandem repetitive sequences. Nature *292*. 845-848.

Nikaido, T., Yamawaki-Kataoka, Y. and Honjo, T. (1982). Nucleotide sequences of switch regions of immunoglobulin Cϵ and Cγ genes and their comparison. J. Biol. Chem. *257*. 7322-7329.

Nilsson, L., Islam, K. B., Olafsson, O., Zalcberg, I., Samakovlis, C., Hammerstrom,

L., Smith, C. I. E. and Sideras, P. (1991). Structure of TGF-β1 induced human immunoglobulin Cα1 and Cα2 germ-line transcripts. Int. Immunol. .

Nishida, Y., Kataoka, T., Ishida, N., Nakai, S., Kishimoto, T., Bottcher, T. and Honjo, T. (1981). Cloning of mouse immunoglobulin ε gene and its location within the heavy chain gene cluster. PNAS 78. 1581-1585.

Noelle, R. J., Daum, J., Bartlett, W. C., McCann, J. and Shepherd, D. M. (1991). Cognate interactions between helper T cells and B cells. V. Reconstitution of T helper cell function using purified plasma membranes from activated Th1 and Th2 T helper cells and lymphokines. J. Immunol 146. 1118-1124.

O'Garra, A., Barbis, D., Harada, N., Lee, F. and Howard, M. (1989). Constituitive production of lymphokines by cloned murine B-cell lymphomas-CH12 B lymphoma produces interleukin-4. J. Mol. Cell. Immunol. 4. 149-159.

Ohara, J. and Paul, W. E. (1985). Production of a monoclonal antibody to and molecular charicterization of B-cell stimulatory factor-1. Nature 315. 333.

Ohtsuki, M. and Massagué, J. (1992). Evidence for the involvement of protein kinase activity in transforming growth factor-β signal transduction. Molec. Cell. Biol. 12. 261-265.

Oikawa, A., Tohda, H., Kanai, M., Miwa, M. and Sugimura, T. (1980). Inhibitors of poly(adenosine diphosphate ribose) polymerase induce sister chromatid exchanges. Biochem. Biophys. Res. Commun. 97. 1311-1316.

Parker, D. C. (1993). T cell-dependent B cell activation. Annu. Rev. Immunol. *11*. 331-360.

Pergola, F., Zdzienicka, M. Z. and Lieber, M. (1993). V(D)J recombination in mamalian cell mutants defective in DNA double-strand break repair. Molec. Cell. Biol. *13*. 3464-3471.

Preiss, J., Schlaeger, R. and Hilz, H. (1971). Specific inhibition of poly(ADP-ribose) polymerase by thymidine and nicotinamide in HeLa cells. FEBS Lett. *19*. 244-246.

Purkerson, J. M., Newberg, M., Wise, G., Lynch, K. R. and Isakson, P. C. (1988). Interleukin 5 and interleukin 2 cooperate with interleukin 4 to induce IgG1 secretion from anti-Ig-treated B cells. J. Exp. Med. *168*. 1175-1180.

Purnell, M. R. and Whish, W. J. (1980). Novel inhibitors of poly(ADP-ribose) synthetase. Biochem. J. *185*. 775-777.

Rabbits, T. H., Forster, A., Dunnick, W. and Bentley, D. L. (1980). The role of gene deletion in the immunoglobulin heavy chain switch. Nature *283*. 351-356.

Radcliffe, G., Lin, Y.-C., Julius, M., Marcu, K. and Stavnezer, J. (1990). Structure of germline immunoglobulin α heavy-chain RNA and its location on polysomes. Mol. Cell. Biol. *10*. 382-386.

Rankin, P. W., Jacobson, E. L., Benjamin, R. C., Moss, J. and Jacobson, M. K. (1989). Quantitative studies of inhibitors of ADP-ribosylation *in vitro* and *in vivo*. J. Biol. Chem. *264*. 4312-4317.

Reaban, M. E. and Griffin, J. A. (1990). Induction of RNA-stabilized DNA conformers by transcription of an immunoglobulin switch region. Nature *348*. 342-344.

Rothman, P., Chen, Y.-Y., Lutzker, S., Li, S. C., Stewart, V., Coffman, R. and Alt, F. W. (1990). Structure and expression of germline immunoglobulin heavy chain ε transcripts: Interleukin-4-plus lipopolysaccharide-directed switching to Cε. Mol. Cell. Biol. *10*. 1672-1679.

Rothman, P., Li, S. C., Gorham, B., Glimcher, L., Alt, F. and Boothby, M. (1991). Identification of a conserved LPS/IL-4 responsive element located at the promoter of germline ε transcripts. Molec. Cell. Biol. *11*. 5551-5561.

Rothman, P., Lutzker, S., Cook, W., Coffman, R. and Alt, F. W. (1988). Mitogen plus interleukin 4 induction of Cε transcripts in B lymphoid cells. J. Exp. Med. *168*. 2385-2389.

Rothman, P., Lutzker, S., Gorham, B., Stewart, V., Coffman, R. and Alt, F. W. (1990). Structure and expression of germline immunoglobulin γ3 heavy chain gene transcripts: Implications for mitogen and lymphokine directed class-switching. Int. Immunol. *2*. 621-627.

Sander, B., Anderson, J. and Anderson, U. (1991). Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. Immunol. Rev. *119*. 65-93.

Sastry, S. S., Buki, K. G. and Kun, E. (1989). Binding of adenosine diphosphoribosyltransferase to the termini and internal regions of linear DNAs. Biochem. *28*. 5670-5680.

Sastry, S. S. and Kun, E. (1990). The interaction of adenosine diphosphoribosyl transferase (ADPRT) with a cruciform DNA. Biochem. Biophys. Res. Comm. *167*. 842-847.

Satoh, M. S. and Lindahl, T. (1992). Role of poly(ADP-ribose) formation in DNA repair. Nature *356*. 356-358.

Satoh, M. S., Poirier, G. G. and Lindahl, T. (1993). NAD$^+$ dependent repair of damaged DNA by human cell extracts. J. Biol. Chem. *268*. 5480-5487.

Savelkoul, H. F. J., Lebman, D. A., Benner, R. and Coffman, R. (1988). Increase of precursor frequency and clonal size of murine IgE secreting cells by IL-4. J. Immunol. *141*. 749-755.

Schlissel, M. S. and Baltimore, D. (1989). Activation of immunoglobulin kappa gene rearrangement correlates with induction of germline kappa gene transcription. Cell *58*. 1001-1007.

Schmitz, J. and Radbruch, A. (1989). An interleukin 4-induced DNase I hypersensitive site indicates opening of the γ1 switch region prior to switch recombination. Int. Immunol. *1*. 570-575.

Schrader, C., George, A., Kerlin, R. L. and Cebra, J. J. (1990). Dendritic cells support production of IgA and other non-IgM isotypes in clonal microculture. Int. Immunol. *2*. 563-570.

Schultz, C. L., Elenich, L. A. and Dunnick, W. A. (1991). Nuclear protein binding to octamer motifs in the immunoglobulin γ1 switch region. Int. Immunol. *3*. 109-116.

Severinson, E., Fernandez, C. and Stavnezer, J. (1990). Induction of germ-line immunoglobulin heavy chain transcripts by mitogens and interleukins prior to switch recombination. Eur. J. Immunol. *20*. 1079-1084.

Severinson-Gronowicz, E., Doss, C., Assisi, F., Vitetta, E. S., Coffman, R. L. and Strober, S. (1979). Surface Ig isotypes on cells responding to lipopolysaccharide by IgM and IgG secretion. J. Immunol *123*. 2049-2056.

Shapira, S. K., Vercelli, D., Jabara, H. H., Fu, S. M. and Geha, R. S. (1992). Molecular analysis of the induction of immunoglobulin E synthesis in human B cells by interleukin 4 and engagement of CD40 antigen. J. Exp. Med. *175*. 289-292.

Shimizu, A., Takahashi, N., Yaoita, Y. and Honjo, T. (1982). Organization of the

constant-region gene family of the mouse immunoglobulin heavy chain. Cell *28*. 499-506.

Shimoyama, M., Kawai, M., Nasu, S., Shioji, K., Hoshi, Y. and Ueda, I. (1975). Inhibition of adenosine 3',5'-monophosphate phosphodiesterase by nicotinamide and its homologues *in vitro*. Physiol. Chem. & Physics *7*. 125-132.

Shockett, P. and Stavnezer, J. (1991). Effect of cytokines on switching to IgA and α germline transcripts in the B lymphoma I.29μ: Transforming growth factor-ß activates transcription of the unrearranged Cα gene. J. Immunol. *147*. 4374-4383.

Sideras, P., Mizuta, T.-R., Kanamori, H., Suzuki, N., Okamoto, M., Kuze, K., Ohno, H., Doi, S., Fukuhara, S., Hassan, M. S., Hammarstrom, L., Smith, E., Shimizu, A. and Honjo, T. (1989). Production of sterile transcripts of Cγ genes in an IgM-producing human neoplastic B cell line that switches to IgG-producing cells. Int. Immunol. *1*. 631-642.

Snapper, C. M. and Finkelman, F. D. (1993). Immunoglobulin class switching. Fundamental Immunology. New York, Raven Press. 3rd, ed.

Snapper, C. M., Finkelman, F. D. and Paul, W. E. (1988). Regulation of IgG1 and IgE production by interleukin 4. Immunol. Rev. *102*. 51-75.

Snapper, C. M. and Mond, J. J. (1993). Towards a comprehensive view of Ig class switching. Imm. Today *14*. 15-17.

Snapper, C. M. and Paul, W. E. (1987). Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science *236*. 944-947.

Sonoda, E., Matsumoto, R., Hitoshi, Y., Ishii, T., Sugimoto, M., Araki, S., Tominaga, A., Yamaguchi, N. and Takatsu, K. (1989). Transforming growth factor β induces IgA production and acts additively with interleukin 5 for IgA production. J. Exp. Med. *170*. 1415-1420.

Southern, E. M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. *98*. 503-517.

Spaulding, D. M. and Griffin, J. A. (1986). Different pathways of pre-B cell lines are induced by dendritic cells and T cells from different lymphoid tissues. Cell *44*. 507-515.

States, D., Walseth, T. and Lee, H. C. (1992). Similarities in amino acid sequences of aplysia ADP-ribosyl cyclase and human lymphocyte antigen CD38. Trends Biochem. Sci. *17*. 495.

Stavnezer, J., McGrath, J. P., Slavin, K. J., Li, R., Li, Y., Hertz, R. and Alhadeff, B. (1981). The numbers and rearrangements of genes in the T15 κ group. <u>ICN-UCLA Symposium on Molecular and Cellular Biology, XX</u>. New York, Academic Press, Inc.

Stavnezer, J., Radcliffe, G., Lin, Y.-C., Nieutupski, J., Berggren, L., Sitia, R. and

Severenson, E. (1988). Immunoglobulin heavy-chain switching may be directed by prior induction of transcripts from constant-region genes. Proc. Natl. Acad. Sci. USA *85*. 7704-7708.

Stavnezer, J., Shockett, P., Lin, Y.-C., Saikh, K. U. and Xu, M. (1990). The I.29 mouse B cell lymphoma: Regulation of the antibody class switch. <u>Cytokines</u>. Basel, Switzerland, S. Karger. 154-169.

Stavnezer, J., Sirlin, S. and Abbott, J. (1985). Induction of immunoglobulin isotype switching in cultured I.29 B lymphoma cells: Characterization of the accompanying rearrangements of heavy chain genes. J. Exp. Med. *161*. 577-601.

Stavnezer-Nordgren, J. and Sirlin, S. (1986). Specificity of immunoglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching. EMBO J. *5*. 95-102.

Stevens, T. L., Bossie, A., Sanders, V. M., Fernandez-Botran, R., Coffman, R., Mossman, T. R. and Vitetta, E. (1988). Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature *334*. 255-258.

Taccioli, G. E., Rathbun, G., Olz, E., Stamato, T., Jeggo, P. A. and Alt, F. W. (1993). Impairment of V(D)J recombination in double-strand break repair mutants. Science *260*. 207-210.

Thibeault, L., Hengartner, M., Lagueux, J., Poirier, G. G. and Muller, S. (1992).

Rearrangements of the nucleosome structure in chromatin by poly(ADP-ribose). Biochem. Biophys. Acta *1121*. 317-324.

Thomas, B. J. and Rothstein, R. (1989). Elevated recombination rates in transcriptionally active DNA. Cell *56*. 619-630.

Turaga, P. S. D., Berton, M. T. and Teale, J. M. (1993). Frequency of B cells expressing germ-line γ_1 transcripts upon IL-4 induction. J. Immunol. *151*. 1383-1390.

Vlasselaer, P. v., Punnonen, J. and de Vries, J. E. (1992). Transforming growth factor-β directs IgA switching in human B cells. J. Immunol. *148*. 2062-2067.

von Schwedler, U., Jack, H. M. and Wabl, M. (1990). Circular DNA is a product of the immunoglobulin class switch rearrangement. Nature *345*. 452-456.

Vos, J. C., Sasker, M. and Stunnenberg, H. G. (1991). Promoter melting by a stage-specific vaccinia virus transcription factor is independent of the presence of RNA polymerase. Cell *65*. 105-113.

Wakatsuki, Y. and Strober, W. (1993). Effect of downregulation of germline transcripts on immunoglobulin A isotype differentiation. J. Exp. Med. *178*. 129-138.

Waldman, A. S. and Waldman, B. C. (1991). Stimulation of intrachromosomal recombination in mammalian cells by an inhibitor of poly(ADP-ribosylation).

Nuc. Acid. Res. *19*. 5943-5947.

Waldman, B. C. and Waldman, A. S. (1990). Illegitimate and homologous recombination in mammalian cells: Differential sensitivity to an inhibitor of poly(ADP-ribosylation). Nuc. Acid. Res. *18*. 5981-5988.

Wang, L.-M., Keegan, A. D., Li, W., Leinhard, G. E., Pacini, S., Gutkind, J. S., Martin G. Meyers, J., Sun, X.-J., White, M. F., Aaronson, S. A., Paul, W. E. and Pierce, J. H. (1993a). Common elements in interleukin 4 and insulin signaling pathways in factor-dependent hematopoietic cells. Proc. Natl. Acad. Sci. USA *90*. 4032-4036.

Wang, L.-M., Martin G. Meyers, J., Sun, X.-J., Aaronson, S. A., White, M. and Pierce, J. H. (1993b). IRS-1: Essential for insulin- and Il-4-stimulated mitogenisis in hematopoietic cells. Science *261*. 1591-1594.

Waters, S. H., Saikh, K. U. and Stavnezer, J. (1989). A B-cell-specific nuclear protein that binds to DNA sites 5' to immunoglobulin Sα tandem repeats is regulated during differentiation. Mol. Cell. Biol. *9*. 5594-5601.

Weinstein, P. D. and Cebra, J. J. (1991). The preference for switching to IgA expression by peyers patch germinal center B cells is likely due to the intrinsic influence of their microenvironment. J. Immunol. *147*. 4126-4135.

Whitmore, A. C., Prowse, D. M., Haughton, G. and Arnold, L. W. (1991). Ig isotype switching in B lymphocytes. The effect of T cell-derived interleukins,

cytokines, cholera toxin, and antigen on isotype switch frequency of a cloned B cell lymphoma. Int. Immunol *3*. 95-103.

Williams, M. and Maizels, N. (1991). LR1, a lipopolysaccharide-responsive factor with binding sites in the immunoglobulin switch regions and heavy-chain enhancer. Genes Dev. *5*. 2353-2361.

Wuerffel, R., Jamieson, C. E., Morgan, L., Merkulov, G. V., Sen, R. and Kenter, A. L. (1992). Switch recombination breakpoints are strictly correlated with DNA recognition motifs for immunoglobulin sγ3 DNA-binding proteins. J. Exp. Med. *176*. 339-349.

Wuerffel, R. A. and Kenter, A. L. (1992). Protein recognition motifs of Sγ3 DNA are statistically correlated with switch recombination breakpoints. Curr. Top. Microbiol. Immunol. *182*. 149-156.

Wuerffel, R. A., Nathan, A. T. and Kenter, A. L. (1990). Detection of an immunoglobulin switch region-specific DNA binding protein in mitogen-stimulated mouse splenic B cells. Mol. Cell. Biol. *10*. 1714-1718.

Xu, L., Gorham, B., Li, S. C., Bottaro, A., Alt, F. W. and Rothman, P. (1993). Replacement of germ-line ε promoter by gene targeting alters control of immunoglobulin heavy chain class switching. PNAS *90*. 3705-3709.

Xu, L., Kim, M. G. and Marcu, K. B. (1992). Properties of B cell stage specific and ubiquitous factors binding to immunoglobulin heavy chain gene switch

regions. Int. Immunol. *4*. 875-887.

Xu, M. and Stavnezer, J. (1990). Structure of germline immunoglobulin heavy-chain γ1 transcripts in interleukin 4 treated mouse spleen cells. Dev. Immunol. *1*. 11-17.

Xu, M. and Stavnezer, J. (1992). Regulation of transcription of immunoglobulin germ-line γ1 RNA: analysis of the promoter/enhancer. EMBO J. *11*. 145-155.

Yoshihara, K., Hashida, T., Tanaka, Y., Matsunami, N., Yamaguchi, A. and Kamiya, T. (1981). Mode of enzyme-bound poly(ADP-ribose) synthesis and histone modification by reconstituted poly(ADP-ribose) polymerase-DNA-cellulose complex. J. Biol. Chem. *256*. 3471-3478.

Yoshihara, K., Itaya, A., Tanaka, Y., Ohashi, Y., Ito, K., Taraoka, H., Tsukada, K., Matsukage, A. and Kamiya, T. (1985). Inhibition of DNA polymerase α, DNA polymerase β, terminal deoxynucleotidyl transferase, and DNA ligase II by poly(ADP-ribosylation) reaction in vitro. Biochem. Biophys. Res. Comm. *128*. 61-67.

Zahradka, P. and Ebisuzaki, K. (1982). A shuttle mechanism for DNA-protein interactions: The regulation of poly(ADP-ribose) polymerase. Eur. J. Biochem. *127*. 579-585.

Zhang, J., Bottaro, A., Li, S., Stewart, V. and Alt, F. W. (1993). A selective defect in IgG_{2b} switching as a result of targeted mutation of the $I_{\gamma 2b}$ promoter and

exon. EMBO J. *12*. 3529-3537.