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FACTOR-3

MECHANISMS OF IMMUNE MODULATION BY TRANSFORMING GROWTH FACTOR-β_1: A ROLE FOR SMAD3 AS AN INTRACELLULAR MEDIATOR

By

Susan C. McKarns

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2001

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ABSTRACT

MECHANISMS OF IMMUNE MODULATION BY TRANSFORMING GROWTH FACTOR-β_1: A ROLE FOR SMAD3 AS AN INTRACELLULAR MEDIATOR

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TGF-β_1 (Transforming growth factor-β_1) produces a diverse range of physiological immune modulatory effects. Targeted deletion of the TGF-β_1 gene exemplifies the importance of TGF-β_1 on maintaining immune homeostasis. TGF-$\beta_1^{-/-}$ mice develop a multifocal inflammatory disease with a T cell-dependent autoimmune process. A role for TGF-β_1 in maintaining T cell tolerance and demonstration that TGF-β_1 signaling in T cells is critical for maintaining B cell tolerance is illustrated by spontaneous T cell differentiation and autoimmunity in transgenic mice expressing a dominant negative TGF-β receptor under the control of a T cell-specific promoter. Mice homozygous for the TGF-β_1 receptor-activated Smad3 (Smad3$^{-/-}$) succumb to a deficiency in mucosal immunity and defective T cell responsiveness to TGF-β_1. In light of these observations, the overall objective of this research was to test the hypothesis that TGF-β_1 acts directly on T cells to regulate IL-2 (interleukin-2) expression through Smad-mediated signaling. RT-PCR and ELISA analyses demonstrated a concentration-dependent bifunctional augmentation and attenuation of IL-2 expression by TGF-β_1 in α-CD3 + α-CD28-activated T cells. In agreement, a concentration-dependent stimulation and suppression of immunoglobulin production by TGF-β_1 in antigen-stimulated B cells was also established. T cells obtained from Smad3$^{-/-}$ mice were refractory to inhibition of IL-2 mRNA and IL-2 protein secretion by TGF-β_1. Inhibition of α-CD3 + α-CD28-induced T

cell growth by TGF-β_1 was also attenuated in Smad3$^{-/-}$ T cells. Collectively, these results establish a role for Smad3 in regulating IL-2 production and T cell growth in response to TGF-β_1. Inhibition of LPS (lipopolysaccharide)-activated B cell growth was unaffected in Smad3$^{-/-}$ mice suggesting a cell-type specificity for Smad3 signaling. Demonstration of a reversal of TGF-β_1-induced T cell growth arrest by exogenous IL-2 suggested that Smad3 may modulate T cell growth through a direct effect on IL-2 production. These results provide a putative mechanism whereby TGF-β_1 may selectively and specifically target T cell growth. Smad3-null B cells were demonstrably less sensitive to the inhibitory effects of TGF-β_1 on IgM production *in vitro*. Moreover, a role for Smad3 in mediating IgA production by TGF-β_1 *in vitro* was evident by an inability of TGF-β_1 to augment IgA secretion in LPS-activated Smad3$^{-/-}$ splenic B cells. Five putative CAGA Smad3 response elements were identified in the IL-2 promoter. EMSA analyses demonstrated that CAGA sequences were critical for constitutive and α-CD3 + α-CD28-induced DNA binding activity at the CD28RE and proximal AP-1 (activating protein-1) site, but not the distal NFAT or NRE-A sites in the IL-2 promoter. Smad3 binding to the proximal AP-1 site was CAGA-independent suggesting that Smad3 may mediate its effects through protein•protein interactions. TGF-β_1 stimulated ERK MAP (extracellular regulated kinase mitogen activated protein) kinase activity in splenocytes. Blockade of ERK MAP kinase activity augmented Smad3 nuclear expression and attenuated inhibition of IL-2 by TGF-β_1 implicating Smad and MAP kinase regulatory cross talk. Taken together, these data substantiate a role for Smad3 signaling in the regulation of IL-2 production and provide a novel mechanism of immune homeostasis.

DEDICATION

To

Mike, Ellen, Michael, Angie, Tabitha, and Brian

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ACKNOWLEDGEMENTS

Foremost, I wish to express my sincere heartfelt appreciation to the members of my dissertation committee - Drs. Norbert E. Kaminski, Robert A. Roth, Michael P. Holsapple, James E. Trosko, and Lawrence J. Fischer for your insightful guidance, discussions, recommendations, support, and encouragement throughout my graduate studies. I had the distinct privilege of knowing many of you before I began my formal training and have come to respect you all. It was an honor to have shared my research with you. I have learned a great deal from each of you and sincerely thank you for your dedicated commitment to teach me.

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I sincerely thank Dr. John J. Letterio for providing me with the opportunity to test my hypothesis. I am grateful for the tremendous effort that you have put forth towards my training. Our collaborative research efforts have been educational, productive, and gratifying. Our conversations have been intellectually stimulating. I look forward to working with you for many years to come.

Dr. Gregory Fink, thank you for the expert statistical advice and guidance that you gave. Your comments were always critical and very much appreciated. Your contributions to my research have been significant.

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Dr. Stephanie Watts and the Watts' laboratory, thank you for sharing your reagents, resources, protocols, and technology. Collectively, you have 'turned mountains into mole hills' and I sincerely appreciate your efforts.

I express my gratitude to the faculty and staff of the Pharmacology and Toxicology Department, especially Drs. Kenneth Moore, Sue Barman, and Peter Cobbitt. Your support has been immense. It has been a pleasure to have been a part of your organization.

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The essence of this work can be traced back to my childhood. I thank my family for planting the seed of enthusiasm and determination in me. I thank God for giving me the courage and perseverance to finish, to move forward, and to give the world the things that I have to give. If I have ever doubted that we have the power to make choices, to give our lives new direction, I no longer do.

TABLE OF CONTENTS

V. T
A
B
C
D

VI. I

VII. R

VIII. O

IX. R

MATERIALS

I. A
II. C
III. T
IV. C
V. A
VI. C
VII. R
VIII. I
IX. A
X. E
A
B
C
D
E
F
G

XI. C

LIST OF TABLES

LIST OF FIGURES

ACM

AK

AKRI

ARC

ARCI

ARI

ARC

ARS

ATF

BCA

BCS

BMP

BNP

CAMK

CAMKII

CAMKIV

CAMKK

CBFA2

CBP

CC2

CD

CDKRE

LIST OF ABBREVIATIONS

α2M	α2-macroglobulin
Act	activin
ActRI	activin receptor type 1
AFC	antibody forming cell
AML2	acute myeloid leukemia-2
AP-1	activator protein-1
APC	antigen presenting cell
APS	ammonium persulfate
ATF	activating transcription factor
BCA	bicinchoninic acid
BCS	bovine calf serum
BMP	bone morphogenetic proteins
bZIP	basic/leucine zipper interacting protein
CaMK	calcium calmodulin kinase
CaMKII	calcium calmodulin kinase two
CaMKIV	calcium calmodulin kinase four
CaMKK	calcium calmodulin kinase kinase
CBFA3	Core Binding Factor subunit α3
CBP	CREB binding protein
CCl4	carbon tetrachloride
CD	cluster of differentiation
CD28RE	CD28 response element

CD4+

CD8+

Con A

co-SMAD

CRE

CREB

CSPD

CTF

ELISA

DPC

DNP

EBSS

ECM

EGF

ELISA

EMSA

ERK

ETS

FKBP

IM

GATA

GST

HBA

$CD4^+$	helper T cells
$CD8^+$	cytotoxic T cells
Con A	concanavalin A
co-SMAD	common-mediator Smad
CRE	cAMP response element
CREB	cAMP response element binding protein
CSPD	Clontech SEAP Detection
CTF	CAAT-binding transcription factor
dI/dC	polydeoxyinosine/polydeoxycysteine
DPC	deleted in pancreatic cancer
DNP	dinitrophenyl
EBSS	Earles' balanced salt solution
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal regulated kinase
ETS	ETS-motif binding protein
FKBP	FK506 binding immunophilin
fM	femtomolar
GATA	GATA-motif binding protein
GST	glutathione-S-transferase
HBx	hepatitis B virus

HDAC

HTLV-I

I-Smad

IFN

Ig

IκB

IL

iNOS

Ir

IS

I-SMAD

JNK

kD

LAP

Lat

LPS

LTBP

MAPase

MAD

MAPK

MAPKK

MAPKKK

MEF2C

HDAC	histone deacetylase
HTLV-1	human T cell leukemia virus type 1
I-Smad	inhibitory Smad
IFN	interferon
Ig	immunoglobulin
IκB	inhibitor of NF-κB
IL	interleukin
iNOS	inducible nitric oxide synthase
Io	ionomycin
IS	internal standard
I-SMAD	inhibitory Smad
JNK	c-Jun N-terminal kinase
kD	kilodalton
LAP	latency-associated peptide
Leu	leucine
LPS	lipopolysaccharide
LTBP	latent TGF-β binding protein
λ-ppase	lamba phosphatase
MAD	mothers against decapentaplegic
MAPK	mitogen activated protein kinase
MAPKK	mitogen activated protein kinase kinase
MAPKKK	mitogen activated protein kinase kinase kinase
MEF2C	myocyte enhancer factor 2C

MEK

MEKK

MG

MH

MHC

MHCI

MHCII

MIS

MKP

ML

MM

mRNA

NA

NC

NGF

NF-AT

NF-κB

NK

NLS

NO

NRE

MEK	MAP/ERK kinase
MEKK	MAP/ERK kinase kinase
mg	milligram
µg	microgram
MH	Mad homology
MHC	major histocompatability complex
MHCI	major histocompatability complex class I
MHCII	major histocompatability complex class II
MIS	mullerian inhibitory substance
MKP	MAP kinase phosphatase
mm	millimeter
mM	millimoloar
mRNA	messenger RNA
NA	naïve
ng	nanogram
NGF	nerve growth factor
NF-AT	nuclear factor of activated T cells
NF-κB	nuclear factor for immunoglobulin κ chain in B cells
NK	natural killer
NLS	nuclear localization signal
NO	nitric oxide
NRE	negative response element

CAP

DCT

PD98059

PAI-1

PDGF

PEA

PLC

PKA

PKC

pN

pM

PMA

PMA Io

Pro

PTK

R-SMAD

RASK

Rb

RCE

RE

RSK

RT-PCR

OAP	octamer-associated protein
OCT	octamer
PD98059	[2-(2'-amino-3'-methoxyphenyl)-oxonaphthalen-4-one]
PAI-I	plasminogen activator inhibitor-I
PDGF	platelet derived growth factor
PHA	phytohemagglutinin
PLC	phospholipase C
PKA	protein kinase A
PKC	protein kinase C
pKa	acid dissociation constant
pM	picomolar
PMA	phorbol-12-myristate-13 acetate
PMA/Io	phorbol ester plus calcium ionophore;
Pro	proline
PTK	protein tyrosine kinase
R-SMAD	receptor-regulated Smad
RASK	ribosome associated S6-kinase
Rb	retinoblastoma
RCE	Rb control element
RE	response element
RSK	ribosomal S6-kinase
RT-PCR	reverse-transcriptase polymerase chain reaction

SAD

SAPK

SARA

SB203580

SSE

SEAP

Sc

SE

SP-1

SF

Sc

SMAD

Sos

SNX

Sp1

SP1

SPLN

SRSC

SRE

SRF

STAT

TAK-1

SAD	Smad4 activation domain
SAPK	stress activated protein kinase
SARA	Smad anchor for receptor activation
SB203580	4-[4-fluorophenyl]-2-[4-methyl-sulfinylphenyl]-5-[4-pyridyl]-1-H-imidazole
SBE	Smad binding element
SEAP	secreted alkaline phosphatase
Ser	serine
SIE	sis-inducible enhancer
SIP-1	Smad interacting protein
SIF	sis-inducible factor
Ski	Sloan Kettering institute
SMAD	truncation of *C. elegans* Sma and *Drosophilia* Mad
Sno	ski related novel
SNX	sorting nexin
Sp1	specificity protein-1
SP1	simian-virus-40 protein-1
SPLN	splenocytes
sRBC	sheep red blood cells
SRE	serum response element
SRF	serum response factor
STAT	signal transducer and activator of transcription
TAK-1	TGF-β activated kinase-1

TAX

TER

TERI

TERII

TERIII

T-

TCR

TGIF

TGF-β

T.

T.1

T.2

TRMC

Tr

TS5

TNF

TRA

TRAP1

TRE

TRP

U1C6

VH

TAX	Tax transactivator protein of the HTLV-1 virus
TβR	transforming growth factor-beta receptor
TβRI	transforming growth factor-beta receptor type I
TβRII	transforming growth factor-beta receptor type II
TβRIII	transforming growth factor-beta receptor type III
T_C	T cytotoxic
TcR	T cell antigen receptor
TGIF	TG-interacting factor
TGF-β	transforming growth factor-beta
T_H	T helper
T_H1	T helper cell type I
T_H2	T helper cell type II
THMC	thymocytes
Thr	threonine
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TPA	12-O-tetradecanylphorbol 13-acetate
TRAP1	TGF-β receptor-associated protein 1
TRE	TPA response element
TRIP	TGF-β receptor interacting protein 1
U0126	1,4-diamino-2,3-dicyano-1,4 bis[2-aminnophenylthio]butadiene
VH	vehicle

Xbra2	Xenopus Brachury-2
ZEB	zinc finger E-box binding protein

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INTRODUCTION

I. Significance

The overall aim of this research is to acquire a mechanistic understanding of the signal transducing pathways underlying immune modulation by TGF-β_1 (transforming growth factor-β_1). This research is founded by a large clinical database correlating elevated plasma TGF-β_1 levels with liver injury and concomitant immune suppression. *In vivo* exposure of B6C3F1 mice to modest hepatotoxic doses of CCl_4 (carbon tetrachloride) induces potent immune suppression (Kaminski *et al.* 1990; Kaminski *et al.* 1989). Separation-crossover-reconstitution experiments with spleen cell subpopulations identified the T cell as the major immune cell population targeted by CCl_4 (Delaney *et al.* 1994). Similar to hepatotoxicity, metabolism of CCl_4 is essential for immunotoxicity (Kaminski *et al.* 1990). However, in contrast to hepatotoxicity, which is mediated through direct actions of metabolites, CCl_4-induced immunotoxicity is mediated through an indirect mechanism of action (Delaney and Kaminski 1993; Kaminski *et al.* 1990; Kaminski and Stevens 1992). Sera isolated from CCl_4-treated mice contain elevated levels of bioactive TGF-β_1 as determined by the mink lung cell line bioassay (Delaney *et al.* 1994). These results are consistent with other experimental and clinical evidence suggesting a role by TGF-β_1 in immune suppression following exposure to a wide range of chemicals and pharmacological agents that induce liver damage and/or diseases, for example, chemotherapeutic agents, immunosupressive drugs, alcohol, and acetaminophen (Ahuja *et al.* 1995; Gutierrez-Ruiz *et al.* 2001; He *et al.* 2000; Hori *et al.* 2000; Inoue *et al.* 2000; Lemmer *et al.* 1999; Neuman 2001; Simile *et al.* 2001; Szuster-Ciesielska *et al.*

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II. TGF-β Su

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2000; Vodovotz *et al.* 2000; Yin *et al.* 2001). In light of these observations, it is conceivable that TGF-β_1-induced systemic immune suppression may be a characteristic secondary effect of hepatotoxic compounds. The mechanisms underlying TGF-β_1-mediated immune suppression following CCl_4 exposure have not been identified. CCl_4 increases IL-2 (interleukin-2) production in Con A (concanavalin A)-activated splenic T cells (Delaney *et al.* 1994).

TGF-β_1 is well recognized for its ability to influence the production of and response to IL-2, a cytokine critical for cell- and humoral-mediated immune responses (Kehrl *et al.* 1986a; Kehrl *et al.* 1986b; Kehrl *et al.* 1986c; Stoeck *et al.* 1990; Stoeck *et al.* 1989a; Stoeck *et al.* 1989b). Recently, Smad proteins have been identified as TGF-β-specific signal transducing molecules (Massague 1998). The identification of elements that function downstream in the TGF-β_1 signaling pathway may provide mechanistic insight towards a better understanding of the diverse physiological immune regulatory properties of TGF-β_1. A principal focus of this research has been to elucidate the molecular mechanisms underlying modulation of IL-2 gene transcription by TGF-β_1.

II. TGF-β Superfamily

The TGF-β superfamily comprises more than 40 structurally related secreted proteins including TGF-βs, activins, inhibins, BMPs (bone morphogenic protein), MIS (mullerian inhibitory substance), and myostatin (Massague 1990; Wrana 1998). While each of these ligands has a broad range of biological activities, TGF-β and activin activate signaling through similar receptors, whereas BMP activates a distinctly different set of receptors (Massague 1992). Inhibin, MIS, and myostatin receptor signaling are not well defined.

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Flanders *et al.*

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Similar to many other growth factors, TGF-βs exists in multiple isoforms. To date, five isoforms have been identified: TGF-β_1, TGF-β_2, TGF-β_3, TGF-β_4, and TGF-β_5; each approximately 70-80% homologous to the others (Roberts 1998). Three of these isoforms, TGF-β_1, TGF-β_2, and TGF-β_3, are expressed in mammalian tissue and are greater than 98% conserved among species. The mammalian isoforms are localized on different chromosomes (human chromosomes 29q13, 1q41, and 14q24, respectively), encoded by distinct genes, expressed in a tissue-dependent manner, and are differentially regulated by numerous growth factors and hormones, including epidermal growth factor retinoic acid, dexamethosone, tamoxifen, phorbol esters, and TGF-βs themselves (Newfeld *et al.* 1999). The three mammalian TGF-β isoforms are interchangeable in most *in vitro* assays using primary cell cultures and established cell lines; however, the deletion of any one TGF-β isoform in transgenic animal models is not compensated for by any of the other isoforms and suggests that the TGF-β isoforms are not functionally redundant *in vivo* (Barone *et al.* 1998; Diebold *et al.* 1995; Foitzik *et al.* 1999; Guenard *et al.* 1995; Kulkarni *et al.* 1993; Li *et al.* 1999; Shull and Doetschman 1994).

In addition, immunohistochemical and *in situ* hybridization analyses have defined differential *in vivo* TGF-β isoform expression patterns. For example, within the nervous system, TGF-β_1 mRNA expression is confined to the meninges and choroid plexus. In contrast, TGF-β_2 and TGF-β_3 are co-expressed in glial cells and neuronal axons (Flanders *et al.* 1991; Heine *et al.* 1987). Accordingly, TGF-β expression *in vitro* is also differentially regulated. For example, human lung WI-38 and normal rat kidney fibroblasts predominantly secrete TGFβ_1; however, monkey kidney BSC-1 cells and human adenocarcinoma PC-3 cells primarily secrete TGFβ_2 (Danielpour *et al.* 1991;

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Jakowlew *et al.* 1992). TGF-β_1 isoforms have nine characteristic cysteine residues; seven of these are conserved in a defined spacing pattern in all members of the TGF-β superfamily. In addition, the mature form of each member of the TGF-β superfamily is a disulfide-linked dimer. In contrast to the sequence of the mature peptide, the pro-segments (discussed below) are only minimally conserved between isoforms (Gray and Mason 1990; Lopez *et al.* 1992).

The TGF-βs are characterized as 'prototypic multi-functional signaling molecules' as they function in autocrine, paracrine, and endocrine modes to control a wide variety of processes involving growth, differentiation, adhesion, and apoptosis in a host of cell types (Letterio and Roberts 1997, 1998; Piek *et al.* 1999; Roberts 1998; Roberts *et al.* 1988). Members of the TGF-β superfamily are also widely recognized for their regulatory role in formation of the ECM (extracellular matrix). For example, TGF-βs stimulate synthesis of collagens, fibronectin, vitronetin, tenascin, and proteoglycans (Bassols and Massague 1988; Ignotz and Massague 1986; Massague *et al.* 1992; Pearson *et al.* 1988), inhibit matrix degrading proteases which include plasminogen activators, collagenase, and stromelysin (Edwards *et al.* 1987; Kerr *et al.* 1990; Lund *et al.* 1987), and suppress activity of the protease inhibitors PAI-1 (plasminogen activator inhibitor-1) and TIMP-1 (tissue inhibitor of metalloproteinase-1) (Edwards *et al.* 1996; Edwards *et al.* 1987).

TGF-β_1 is the predominant isoform that has been implicated in regulating physiological immune and proliferative responses as well as a host of pathological disorders, including fibroproliferation, parasitic and autoimmune diseases, chronic allograft rejection, fibrotic glomerulopathies, and the progression of carcinogenesis

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1993; Kim et a

(Border 1994; Coupes *et al.* 2001; Hao *et al.* 1999; Jakubowiak *et al.* 2000; Letterio *et al.* 1996; Massague *et al.* 2000; Roberts 1998; Sporn and Roberts 1991).

III. TGF-β_1

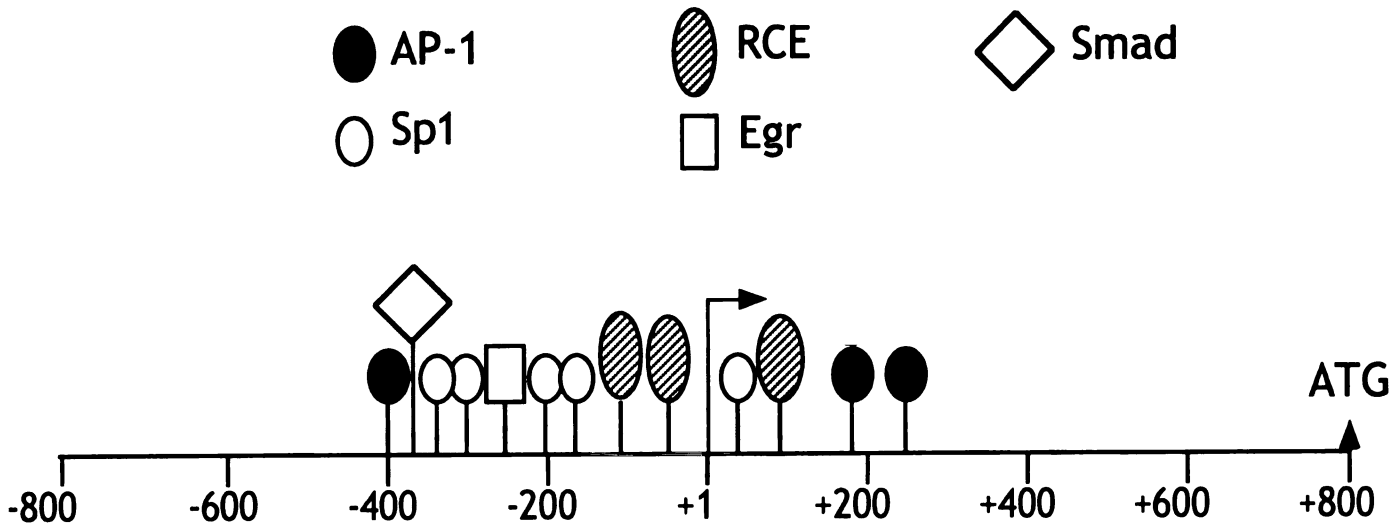
A. Nomenclature

TGF-β_1, the prototype of the TGF-β family, was discovered in 1978 (de Larco and Todaro 1978). TGF-β_1 was first isolated and purified from supernatants of Moloney MuSV-transformed mouse 3T3 fibroblasts, characterized as a growth-stimulating polypeptide, and termed 'sarcoma growth factor' (Roberts *et al.* 1980). The nomenclature 'transforming growth factor' was adopted shortly thereafter when it was demonstrated that TGF-β_1 stimulates anchorage independent growth of non-transformed rat kidney fibroblasts, a hallmark characteristic of neoplastic transformation (Roberts *et al.* 1980).

B. Transcriptional regulation of TGF-β_1 expression

The major regulatory domains of the human TGF-β_1 promoter are diagrammed in **Figure 1**. In contrast to TGF-β_2 and TGF-β_3, the TGF-β_1 gene lacks the classic TATA box and is characterized by a GC rich region containing several Sp1 (specificity protein-1) binding sites immediately upstream of the transcriptional start site. Expression of TGF-β_1 is up-regulated in response to EGF (epidermal growth factor), *jun*, *fos*, *src*, *abl*, *ras*, HTLV-1 (human T cell leukemia virus type 1), HMV (human cytomegalovirus), HBx (hepatitis B virus); tamoxifen, phorbol esters, TGF- β_1 and TGF-β_2 (Akhurst *et al.* 1988; Bascom *et al.* 1989; Colletta *et al.* 1990; Danielpour *et al.* 1991; Falanga *et al.* 1991; Kim *et al.* 1990a; Kim *et al.* 1989a; Kim *et al.* 1989b; Kim *et al.* 1989c; Kim *et al.*

Figure 1. Transcriptional regulation of TGF-β_1. A diagram of the major regulatory domains of the human TGF-β promoter. The *trans*-acting factors that bind to *cis*-acting elements to regulate TGF-β_1 gene transcription are illustrated.



Oncogenes	src, abl, ras, jun, fos, src, abl
Viral transactivators	HTLV-1 TAX CMV, IE2, HBx
growth factors	TGF-β, NGF

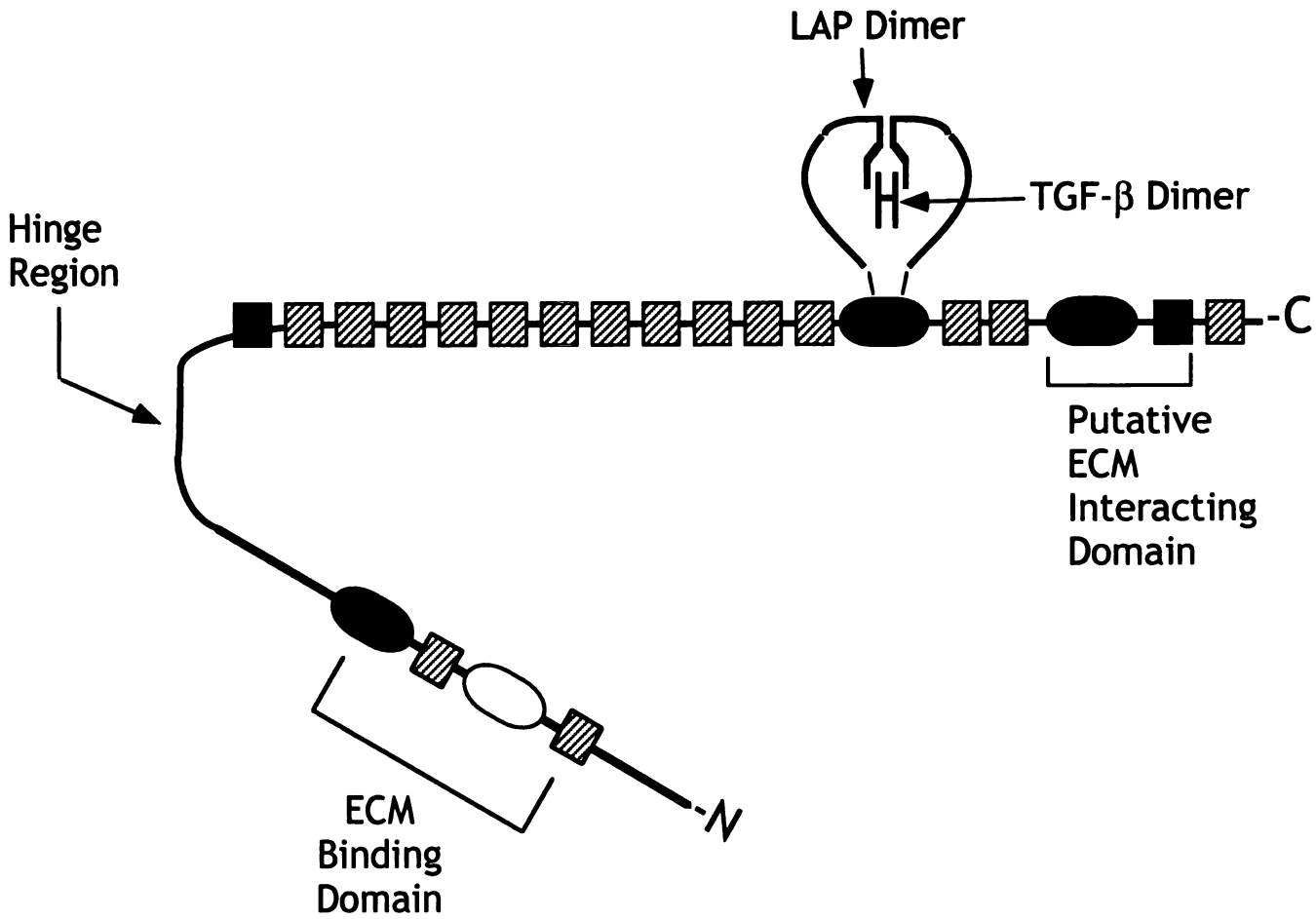
1990b).

Importantly, the ability of TGF-β_1 to induce its own expression allows for a sustained expression beyond the initiating stimulus, which has been demonstrated to be of particular significance in wound healing (Kim *et al.* 1990a). TGF-β_1 mRNA is also induced in response to tissue injury, examples include myocardial infarction (Thompson *et al.* 1988), hypoxia (Henrich-Noack *et al.* 1994; Khaliq *et al.* 1995; Klempt *et al.* 1992; McNeill *et al.* 1994; Scheid *et al.* 2000; Williams *et al.* 1995), hyperglycemia (Daniels *et al.* 2000; James *et al.* 2000; Weigert *et al.* 2000), bone repair (Joyce *et al.* 1990), liver regeneration (Fausto *et al.* 1986; Henrich-Noack *et al.* 1994; Khaliq *et al.* 1995; Klempt *et al.* 1992; McNeill *et al.* 1994; Scheid *et al.* 2000; Williams *et al.* 1995), hepatic schistosome infection (Czaja *et al.* 1989), carbon tetrachloride-induced liver injury (Armendariz-Borunda *et al.* 1990; Czaja *et al.* 1989; Nakatsukasa, 1990 #940; De Bleser*et al.* 1997; Delaney *et al.* 1994; Grasl-Kraupp *et al.* 1998; Greenwel *et al.* 1993; Jeon *et al.* 1997; Roth *et al.* 1998; Shimizu *et al.* 2001; Simile *et al.* 2001; Williams and Iredale 2000), acetaminophen-induced hepatotoxicity (Dalu and Mehendale 1996; Jeon *et al.* 1997; Miwa *et al.* 1997; Neuman 2001; Tygstrup *et al.* 1996) and hepatitis (Dudas *et al.* 2001; Flisiak and Prokopowicz 2000; Kim *et al.* 2000; Liu *et al.* 1999; Neuman 2001; Zhang *et al.* 1999b). Rb (retinoblastoma) protein binding to the RCE (retinoblastoma control element) attenuates TGF-β_1 gene expression (Udvadia *et al.* 1992).

C. Secretion of TGF-β_1 as a latent complex

TGF-β_1 is synthesized as a biologically inactive 390-amino acid dimeric precursor complex containing the C-terminal mature TGF-β and its N-terminal pro-domain, LTBP (latent TGF-β binding protein) (**Figure 2**). The LTBPs are members of the

Figure 2. Diagram of the biologically inert latent precursor complex of TGF-β_1. TGF-β_1 is synthesized as a biologically inactive 390-amino acid dimeric precursor protein. The mature form of TGF-β_1 is located within the carboxy-terminal domain. The latent TGF-β binding protein is contained within the amino-terminal domain. Activation to the mature biologically active form of TGF-β_1 requires multiple sequential proteolytic cleavages.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LTBP/fibrillin family of ECM glycoproteins and are important for the folding and secretion of TGF-β_1 as well as for the sequestration of latent TGF-β_1 complexes to the ECM (Lawrence 1991).

The TGF-β_1 precursor protein is a pre-pro-peptide and undergoes a two-step process to generate bioactive TGF-β_1 which is able to associate with the TGF-β signaling receptors (Lawrence 1991; Munger *et al.* 1997; Nunes *et al.* 1998) (**Figure 3**). The first step of this process is a proteolytic cleavage that eliminates the N-terminal hydrophobic LTBP peptide (amino acid residues 1 to 29) to generate a pro-peptide molecule. The second step is a subsequent proteolytic cleavage that removes the LAP (latency-associated peptide) (amino acid residues 30 to 278) to generate a mature active peptide (amino acid residues 279 to 390). The 74 kD LAP must be cleaved prior to TGF-β_1 binding to the TβR (TGF-β receptor), and thus is critical for TGF-β_1 activity. LTBP (125 to 160 kD), on the other hand, permits association with the ECM and functions to regulate localized storage of the inactive complex within the ECM (Munger *et al.* 1998; Munger *et al.* 1997; Saharinen *et al.* 1999; Taipale *et al.* 1996).

Four highly repetitive splice variants of LTBP (1-4) have been isolated. TGF-β_1 bioavailability may be differentially regulated in a tissue-specific manner, in part, by the formation of variable pre-pro-precursor complexes. For example, LTBP-1 is predominantly expressed in the heart, placenta, lung, spleen, and kidney. LTBP-2 is preferentially expressed in the lung, skeletal muscle, liver, and placenta. LTBP-3 and LTBP-4 are found primarily in the heart, small intestine, and ovaries. Interestingly, LTBP-1 additionally functions as a chemoattractant (Saharinen *et al.* 1999; Taipale *et al.* 1996).

Figure 3. Stepwise activation of latent TGF-β_1 precursor to its biologically active form. Illustrated are the multiple sequential post-translational regulatory steps associated with the activation of TGF-β_1 *in vivo.*

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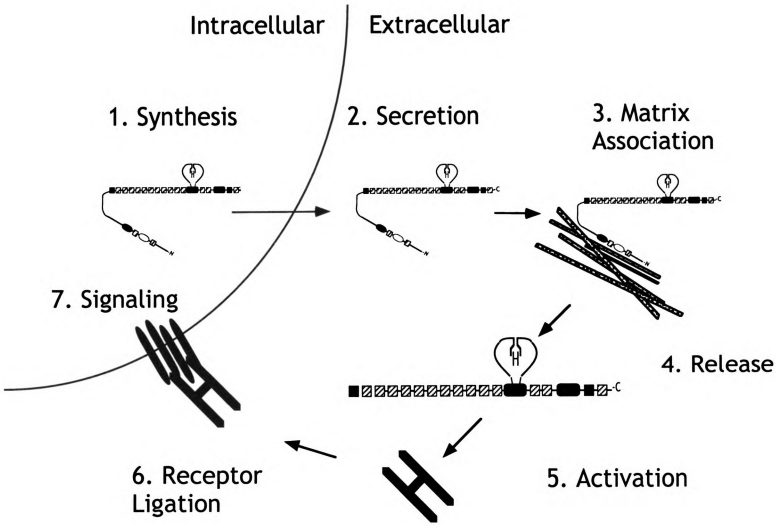
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In addition to associating with LAP and LTBP, active TGF-β_1 can readily form complexes with α2M (α2-macroglobulin), biglycan, decorin, type IV collagen, fibronectin, and thrombospondin. α2M is probably the most critical carrier of circulating TGF-β_1 and plays an important role in rapidly clearing TGF-β_1 from the bloodstream via binding to the α2M receptor (Munger *et al.* 1997). The most concentrated source of TGF-β_1 is the platelet alpha granules (Centrella *et al.* 1986). Osteoblasts and other bone cells represent additional major storage sites for TGF-β_1. Activated lymphocytes, macrophages, and neutrophils also secrete an abundance of TGF-β_1 (Assoian *et al.* 1984).

D. Activation of latent TGF-β_1

Activation of the latent complex is tightly controlled and represents a critical regulatory step in TGF-β_1 activity (Koli *et al.* 2001). A number of factors, including acidic pH, nitric oxide, plasmin, cathepsin, thrombospondins, and subtilisin-like pro-protein convertases function to activate latent TGF-β_1 (Barcellos-Hoff 1996; Chenevix-Trench *et al.* 1992; Godar *et al.* 1999; Harpel *et al.* 2001; Hugo *et al.* 1999; Letterio and Roberts 1998; Quan *et al.* 2001; Roberts *et al.* 1992; Vodovotz *et al.* 1999). The bioactive form of TGF-β_1 is a 25 kD disulfide-linked polypeptide dimer (Massague 1990). TGF-β_1 is usually produced as a homodimer, ie., TGF-β_1•TGF-β_1; however, TGFβ_1•TGF-β_2 heterodimers have been identified (Ogawa *et al.* 1992). A differential function of heterodimers relative to homodimers has not been established.

1. Physiochemical activation

The association between TGF-β_1 and LAP is via an electrostatic interaction and is readily disrupted *in vitro* by acid, high temperature, or chaotropic treatment (Brown *et al.* 1990; Lawrence 2001; Lawrence *et al.* 1985). Physiologically, it

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has been speculated that acidic microenvironments that occur within the bone and at sites of tissue repair may provide localized sites for TGF-β_1 activation *in vivo* (Salo *et al.* 1997). Interestingly, irradiation increases the concentration of active TGF-β_1 in mouse mammary tumors (Barcellos-Hoff *et al.* 1994). Although not confirmed, it has been hypothesized that reactive oxygen species generated during irradiation induce redox-mediated activation of latent TGF-β_1 (Barcellos-Hoff *et al.* 1994; Barcellos-Hoff and Dix 1996; Reiss and Barcellos-Hoff 1997; Vodovotz *et al.* 1999).

2. Enzymatic activation and protein interactions

Proteolysis targets the degradation of the LAP propeptide and the subsequent release of active TGF-β_1. Appropriate protease inhibitors impair TGF-β_1 activation *in vitro* (Antonelli-Orlidge *et al.* 1989; Huber *et al.* 1992). Numerous proteolytic enzymes, including mast cell chymase, leukocyte elastase, and plasmin are able to cleave LTBPs and release latent TGF-β_1 from the ECM (Olofsson *et al.* 1995; Taipale *et al.* 1994). TGF-β_1 also undergoes proteolytic activation via deglycosylation of LAP (Miyazono and Heldin 1989). Additionally, in certain cells, i.e., monocytes, endothelial, hepatocytes, smooth muscle cells, and pericytes, secreted latent TGF-β_1 can also be activated at the cell surface via direct binding to the mannose-6-phosphate receptor, which in turn induces cleavage of the LAP (Ariazi *et al.* 1999; Godar *et al.* 1999; Rudd *et al.* 2000). Importantly, the proteolytic release and activation of stored TGF-β_1 can generate a rapid signal important for repair of tissue damage and localized activation of the immune system in the absence of gene transcription (Koli *et al.* 2001; Lyons *et al.* 1990; Saharinen *et al.* 1999; Taipale *et al.* 1996).

3. Hormone-induced activation

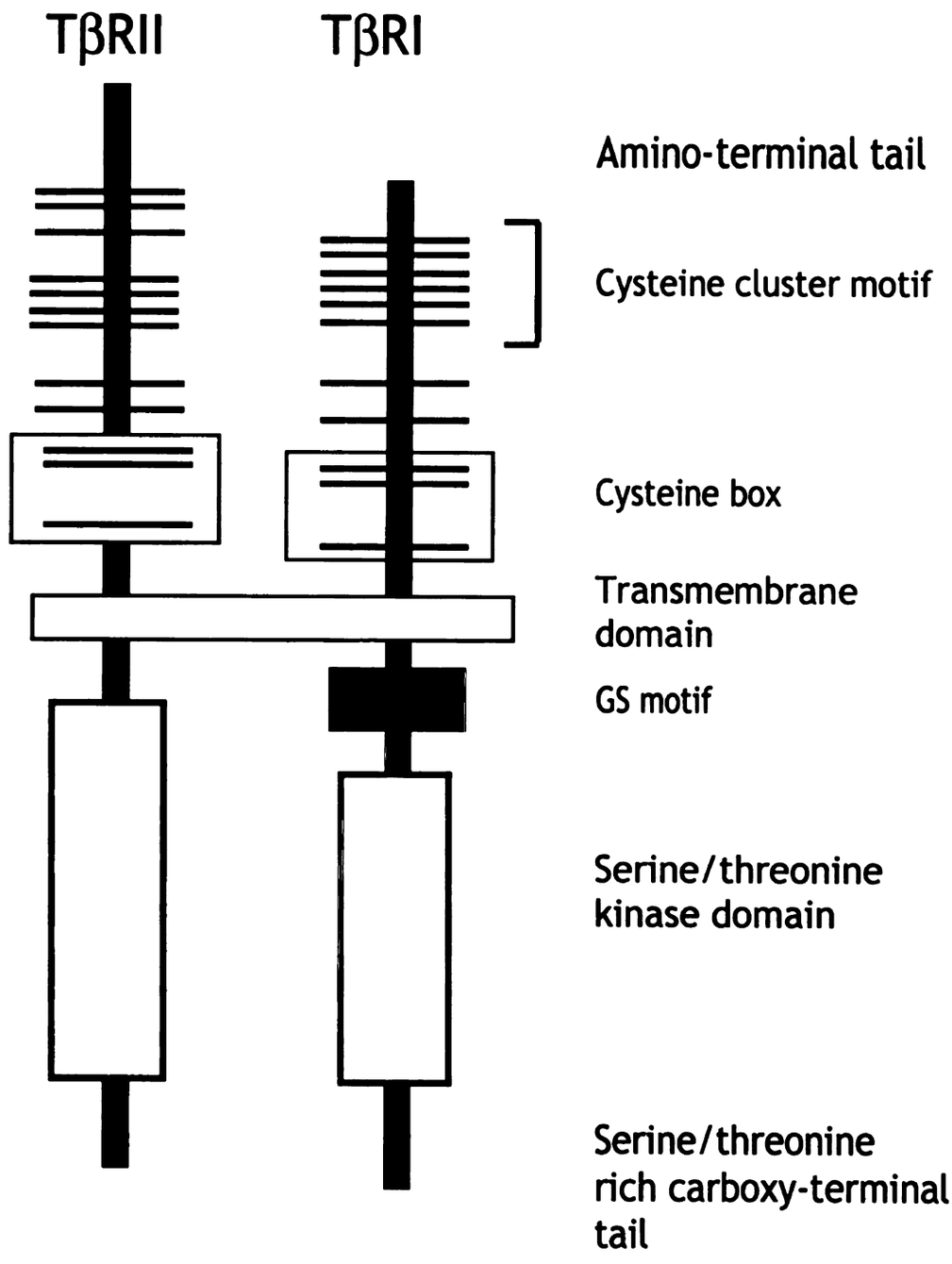
Steriod hormone superfamily members, including estrogens, anti-estrogens, retinoids, androgens and synthetic progestins, have been demonstrated to effectively activate TGF-βs in a number of *in vitro* cell models (Boulanger *et al.* 1995; Colletta *et al.* 1990; Djurovic *et al.* 2000; Fisher *et al.* 1992; Glick *et al.* 1991; Harpel *et al.* 2001; Knabbe *et al.* 1987; Koli and Keski-Oja 1993). The biochemical mechanism(s) by which TGF-β is activated by hormones is unknown and is a current topic of intense research.

E. TGF-β_1 Receptors

In contrast to most growth factors, which signal through tyrosine kinase receptors, TGF-β_1 binds to multimeric receptor complexes with intrinsic serine/threonine kinase activity. TGF-β signaling is mediated through ligand-induced hetero-oligomeric receptor complex formation, presumably between two molecules each of type I and type II receptors. Constitutively active transmembrane TβRII (TGF-β receptor type II) homodimers recruit and phosphorylate transmembrane glycoprotein TβRI (TGF-β receptor Type I) homodimers at the GS domain located upstream of the serine/threonine kinase domain (**Figure 4**). Activated TβRI is responsible for propagating the signaling via phosphorylating downstream cytoplasmic molecules, including Smads, MAPKs (Mitogen Activated Protein Kinases), PLC (Phospholipase C), and PKA (Protein Kinase A).

TβRs (TGF-β receptors) were initially identified via crosslinking of ^{125}I-TGF-β to cell surface proteins. Based upon differing electrophoretic mobility of ^{125}I-TGF-β to crosslinked complexes on non-denaturing polyacrylamide gels, a total of nine TβRs (type

Figure 4. Schematic representation of the type I and type II TGF-β receptors. The conserved GS motif with its SGSGSGLP sequence is characteristic of the TβRI and structurally precedes the kinase domain.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I, II, III, IV, V, VI, VII, VIII, and IX) have been identified; these receptors are now known to be cell type- and ligand-specific (Attisano *et al.* 1994; Wrana 1998). Based upon their abundance, wide distribution, high affinity binding, and correlation between receptor presence and TGF-β responsiveness, three of these receptors, i.e. TβRI, TβRII, and TβRIII were originally identified as putative signaling molecules. *In vitro* somatic cell hybrid complementation analyses of TβR mutant cells have confirmed that both co-expression of TβRI and TβRII is essential for TGF-β_1-induced signaling; in contrast, TβRIII is devoid of signaling by TGF-β (Letterio and Bottinger 1998). However, more recent *in vivo* studies employing a dominant negative TβRII transgenic mouse model have demonstrated that TGF-β_1 may actually mediate some biological responses through direct activation of either TβRI or TβRIII (Bottinger *et al.* 1997). Additional studies are needed to clarify TβRIII signaling properties.

Several highly homologous, yet different subtypes of TβRI and TβRII exist, and their ligand binding properties are highly dependent upon the TGF-β superfamily member to which they bind. Distinct receptor-associated signaling pathways may mediate separate TGF-β responses. In addition, the sequential activation of TβRII complexes prior to TβRI complexes allows for combinatorial diversity; i.e. different type II receptors can pair with different type I receptors, so that a given ligand may conceptually generate varied biological responses (Massague 1996). TGF-β_1 forms a high affinity heteromeric complex with only one type of TβRII and seven types of TβRI and TβRIII. A brief structural and functional overview of the mammalian TβRs is provided below.

1. TβRII (TGF-β Receptor Type II)

TβRI and TβRII mediate TGF-β_1 signaling. Each of these receptors are transmembrane serine/threonine kinases with a single transmembrane domain (**Figure 4**). Structurally, TβRII is a 75- to 85-kD glycoprotein composed of a 136-residue hydrophobic N-glycolsylated extracellular domain, a single transmembrane domain, and a 376-amino acid cytoplasmic domain comprised of a serine/threonine kinase domain and the carboxy-terminal tail (Lawler *et al.* 1994; Lin *et al.* 1992). Multiple extracellular cysteine residues are indicative of extensive disulfide bridge formation and folding that is required for ligand binding. In the absence of TβRI, TGF-β_1 binds TβRII with high affinity. Heteromeric complex formation between TβRI and TβRII does not demonstrably alter TGF-β_1 binding affinity for TβRII. TGFs-β are the only known ligands that bind to TβRII. Independent of ligand binding, TβRII constitutively resides as a homodimer; homomeric interactions are partly due to ability of the extra- and intra-cellular domains to interact with each other (Chen and Derynck 1994; Henis *et al.* 1994). Auto-phoshorylation of TβRII has been localized to Ser-549 and Ser-551 (located in the carboxy-terminus) and Ser-223, Ser-226, and Ser-227 (located in the juxtamembrane domain) (Souchelnytskyi *et al.* 1996). TβRIIs auto-phosphorylate on serine residues *in vivo*, but on serine and threonine residues *in vitro* (Lin *et al.* 1992). The physiological significance of this differential TβRII phosphorylation pattern is not known.

2. TβRI (TGF-β Receptor Type I)

Similar to TβRII, TβRI is a transmembrane serine/threonine kinase receptor, and resembles TβRII in sequence homology and structure. TGF-β binds to seven subtypes of TβRI, i.e., TβRI, ALK-1, ALK-5, ActRI, TSR-1, TSR3, and TSR4.

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TβRI is a 50 to 60 kD protein that is comprised of a 101-amino acid hydrophilic extracellular domain and a 355-amino acid cytosolic serine/threonine kinase containing motif. In contrast to TβRII, TβRI has a shorter extracellular domain, a truncated intracellular carboxy-terminal extension following the kinase domain, and the highly conserved GS domain immediately proceeds the serine/threonine kinase motif (**Figure 4**).

TβRI does not bind TGF-β in the absence of TβRII hetero-dimerization; however, the presence of TβRI is essential for TGF-β responsiveness, thus reflecting its critical role in signal transduction. The three-dimensional inactive conformation of the TβRI is maintained by interactions of multiple cytoplasmic motifs involving the GS domain, the N-terminal tail associated with ATP-binding, and the activation loop. TβRI is converted to its active confirmation via phosphorylation within the GS domain by TβRII. The L45 loop of the amino terminus of TβRI protrudes out from the kinase domain allowing for direct interaction with intracellular substrates, e.g. Smads.

3. TβRIII (TGF-β_1 binding proteins Type III)

TβRIII is a 280 to 330 kD transmembrane proteoglycan that binds TGF-β_1 with high affinity. TβRIII is presumably devoid of intrinsic signaling capabilities and is thought to function primarily to recruit TGF-β_1 to TβRII at the cell surface; however, as discussed earlier, these results are somewhat controversial.

F. TGF-β_1 receptor binding proteins

The FK506-binding immunophilin, FKBP12 binds to TβRI via a Leu-Pro sequence located in the GS domain and functions to inhibit spontaneous, ligand-independent activation by TβRI. TRAP1 (TGF-β receptor-associated protein 1)

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associates with inactive heteromeric TβR complexes and is released upon activation of ligand binding (Verrecchia *et al.* 2001a). TRAP1 also interacts with the common mediator, Smad4, in a ligand-dependent manner (Wurthner *et al.* 2001). It appears that TRAP1 only modestly stimulates TGF-β signaling in functional assays *in vitro*; however, deletion of TRAP1 diminishes the interaction of Smad4 with Smad2 and inhibits TGF-β signaling in reporter gene assays (Wurthner *et al.* 2001). These data suggest a model in which TRAP1 permits sequestration of Smad4 into the vicinity of the receptor complex and facilitates its transfer to the receptor-activated Smad proteins.

SNX6 (sorting nexins 6) a member of the SNX superfamily has also been shown to form heteromeric complexes with TβRs and plays a role in receptor trafficking (Parks *et al.* 2001). While the functional significance of this protein•protein interaction is not yet understood, other members of the SNX superfamily play a role in trafficking a host of receptors including EGF (epidermal growth factor) and PDGF (platelet derived growth factor).

G. Biological Functions of TGF-β_1

TGF-β_1 plays a critical regulatory role in a host of physiological and pathological events, including cell proliferation and differentiation, angiogenesis, extracellular matrix remodeling, wound repair, bone formation, inflammatory processes, and immune homeostasis (Bonewald 1999; Hata *et al.* 1998; Letterio and Roberts 1997; Massague *et al.* 2000). The precise nature of the response of a particular cell to TGF-β_1 is highly dependent upon cell type, differentiation, activation status, cell cycle, and the cytokine/growth factor/hormonal milieu in the surrounding micro-environment (McCartney-Francis *et al.* 1998; Wahl *et al.* 2000). TGF-β_1 also plays an important role

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in a host of pathological disorders, including autoimmunity, carcinogenesis, neurodegeneration, impaired wound healing, parasitic diseases, and fibrosis (Branton and Kopp 1999; Connor *et al.* 1989; Dudas *et al.* 2001; Fausto *et al.* 1991; Lawrence 1996; Prud'homme 2000; Scheid *et al.* 2000).

H. TGF-β_1 and Immune Modulation

TGF-β_1 plays an essential role in maintaining immune homeostasis (Letterio and Roberts 1998; Wahl *et al.* 2000). TGF-β_1 is secreted by all leukocytes and TβRs have been identified on all lymphoid cells (Letterio and Roberts 1998; McCartney-Francis *et al.* 1998). The immune modulatory effects of TGF-β_1 are multiple and target hematopoesis, proliferation, differentiation, and function of all classes of mature leukocytes (Letterio and Roberts 1998; McCartney-Francis *et al.* 1998; Wahl *et al.* 2000). For example, TGF-β_1 modulates the production and activity of numerous cytokines which include TNF-α (tumor necrosis factor-α), IFN-γ (interferon-γ), IL-1, IL-2, and IL-6. Additionally, TGF-β_1 regulates macrophage-induced production of superoxide and nitric oxide, adhesion molecule expression, Ig (immunoglobulin) synthesis, monocyte and neutrophil chemotaxis, and activation of lymphoid cells and their progression through the cell cycle. TGF-β_1 modulates proinflammatory as well as immunosuppressive responses in response to tissue injury. Femtomolar concentrations of TGF-β_1 that are present at the onset of an inflammatory response, act as a chemoattractant to elicit proinflammatory responses (Wahl *et al.* 1987). As inflammatory responses progress, circulating levels of bioactive TGF-β_1 rise. Substantially elevated plasma levels of bioactive TGF-β_1 present at the resolution phase of the inflammatory response act to suppress the function of

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numerous immune cells, including lymphocytes, macrophages, monocytes, and neutrophils (McCartney-Francis *et al.* 1998; Wahl 1992; Wahl *et al.* 1993).

1. The TGF-β_1 null mouse model

The TGF-β_1 null mouse probably best exemplifies the importance of TGF-β_1 in maintaining immunological balance. Targeted disruption of TGF-β_1 on a C57BL/6 X SV129 background produces an autoimmune-like phenotype that is characterized by enhanced circulating pathogenic IgG autoantibodies to nuclear antigens and elevated glomerular deposition of immune complexes. Increased expression of MHC class I and II molecules, elevated numbers of circulating immature granulocytes, monocytes, and platelets, and a decreased number of $B220^+$ B cells in peripheral lymphoid organs and bone marrow are also observed in TGF-β_1 null mice (Christ *et al.* 1994; Geiser *et al.* 1993; Kobayashi *et al.* 1999; Kulkarni *et al.* 1993; Letterio *et al.* 1996). TGF-β_1 null mice succumb within four weeks of age to a rapid, progressive multi-organ infiltration of lymphocytes. Neither thymic cellularity nor the relative distribution of cells within thymic cell subpopulations are altered in asymptomatic newborn mice; however, a significant reduction in double positive $CD4^+$, $CD8^+$ T cell pregenitors is evident as systemic inflammation progresses (Letterio *et al.* 1996).

2. T cells

The role of TGF-β_1 in the regulation of T cell responses has been perplexing, possibly because it is dependent on the type of T cell being regulated and the cytokine milieu in the surrounding microenvironment. A comprehensive picture entailing the effects of TGF-β_1 on T cells is complex because TGF-β_1 also indirectly targets T cells by regulating the function of antigen-presenting cells. Evidence

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establishing a critical role for T lymphocytes in TGF-β_1-induced immune homeostasis stems from the observation that the early onset of multi-organ inflammation in TGF-β_1 null mice is lymphocyte-mediated (Diebold *et al.* 1995). In addition, inflammatory cell infiltration and animal lethality are concomitantly reduced with *in vivo* depletion of $CD4^+$ T lymphocytes in TGF-β_1 null mice. Although T cells express a lower number of TGF-β receptors relative to most cells studied, the affinity for TGF-β_1 binding to the TGF-β receptor is five to ten fold higher in T cells (Kehrl *et al.* 1986c; Massague and Like 1985). Additionally, T cell activation increases the number and affinity of TGF-β receptors by approximately six- and three-fold, respectively. TGF-β_1 modulates T cell activation (discussed below), attenuates T cell apoptosis (Cerwenka *et al.* 1996; Cerwenka and Swain 1999; Genestier *et al.* 1999a; Genestier *et al.* 1999b), and induces G_1/S phase cell cycle arrest (Saltis 1996).

Transgenic mice expressing a truncated dominant negative TβRII under the control of a CD4 promoter construct lacking the CD8 silencer have been developed as a model to investigate the direct effects of TGF-β_1 on T cells *in vivo* (Gorelik and Flavell 2000). These mice survive beyond five months of age, but develop a phenotype with many features overlapping TGF-$\beta_1^{-/-}$ mice, including a multi-organ perivascular infiltration of mononuclear cells, circulating autoantibodies, and renal glomeruli immune complex deposition. T cells from these transgenic mice are refractory to inhibition by TGF-β_1 and spontaneously differentiate into type 1 or type 2 cytokine secreting cells, with $CD4^+$ T cells capable of secreting IFN-γ and/or IL-4 *in vitro* (Gorelik and Flavell 2000). These results demonstrate the importance of TGF-β in maintaining tolerance in T cells. In addition, these results also illustrate that the maintenance of B cell tolerance to

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self-antigens is dependent on normal TGF-β signaling in T cells. Investigation of the molecular mechanisms of how TGF-β_1 either stimulates or inhibits T cell function is needed in order to understand these modulatory effects at the cellular level.

3. B cells

TGF-β_1 elicits a broad range of effects on B cells, including inhibition of cell proliferation, antibody secretion, antigen receptor and MHC class II molecule expression, and Ig isotype class-switching (Letterio and Roberts 1998). In addition, systemic autoreactivity, B cell hyper-responsiveness, IgA-deficiency, and elevated serum IgG levels in transgenic mice lacking a functional B cell TGF-β type II receptor (Cazac and Roes 2000) provides strong evidence that TGF-β_1 also controls B cell homeostasis in a T cell-independent manner.

During the course of B cell differentiation, B cells undergo a process called isotype class switching, in which the initial synthesis of IgM antibody is converted to IgD, IgG, IgE, or IgA. During this isotype switching process, the immunoglobulin heavy chain constant region undergoes rearrangement, while the immunoglobulin light chain and the variable heavy chain remain unchanged, to generate a change of B cell effector function. Antigen specificity is not altered with isotype class switching. Class switching occurs via an intrachromosomal recombination event that joins variable region genes with a gene for a constant region to form a functional heavy chain gene for the IgD, IgE, IgG, or IgA immunoblogulin. This process appears to be directed by transcription of un-rearranged or germline transcript of the heavy chain constant region gene before switch recombination.

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TGF-β_1 induces transcription of the germline Igα constant region gene and directs IgA isotype class switching (Cazac and Roes 2000; Pardali *et al.* 2000a; Sonoda *et al.* 1992). Mechanistically, TGF-β_1 augments binding of the transcription factor AML2 (acute myeloid leukemia-2) to the regulatory region of the IgA germline promoter and induction of 'sterile' transcription of the locus that is known to play a critical role in immunoglobulin class switching (Hein *et al.* 1998; Lorenz and Radbruch 1997). Recently, it has been demonstrated that Smad3 and Smad4 directly bind to the TGF-β receptor response element within the IgA promoter and cooperate with AML proteins to stimulate germline IgA transcription (Pardali *et al.* 2000a; Park *et al.* 2001; Zhang and Derynck 2000).

4. T_H1/T_H2 development

The exact regulatory role of TGF-β_1 in T_H1/T_H2 development remains somewhat controversial. Dependent upon the experimental system, TGF-β_1 has been shown to favor selectively either T_H1 or T_H2 development (Gorham *et al.* 1998; Heath *et al.* 2000; King *et al.* 1998; Lee and Rich 1993; Ludviksson *et al.* 2000; Nagelkerken *et al.* 1993; Schmitt *et al.* 1994a; Schmitt *et al.* 1994b; Swain *et al.* 1991a; Swain *et al.* 1991b; Taylor *et al.* 2000; Thorbecke *et al.* 2000). In addition, a difference in T_H1/T_H2 development among mouse strains has been proposed to account for somewhat varying phenotypes of TGF-$\beta_1^{-/-}$ mice on different strain backgrounds (Gorham *et al.* 2001).

5. TGF and IL-2

One of the first demonstrated immune modulatory effects of TGF-β_1 on isolated lymphoid tissue inhibition of T cell growth. TGF-β_1 attenuates the production of

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IL-2, down-regulates the high affinity IL-2 receptor (Gorham *et al.* 1998; Ruegemer *et al.* 1990), and disrupts IL-2 signaling downstream of the IL-2 receptor (Bright *et al.* 1997). On-the-other-hand, under the appropriate experimental conditions, TGF-β_1 also enhances IL-2 production (Cerwenka *et al.* 1994).

IV. Transcriptional regulation of the IL-2 gene following T cell activation through the T cell antigen receptor

IL-2 is a 15 kD glycoprotein that functions as a critical autocrine/paracrine growth factor for a variety of immune cells including T cells, B cells, NK cells, and macrophages (Frey *et al.* 1987; Lozano Polo *et al.* 1990; Nelson *et al.* 1992; Smith 1992). IL-2 is produced predominantly but not exclusively by activated helper T cells. Activated $CD4^+$ T cells is the primary source of IL-2. NK (natural killer) cells also produce modest amounts of IL-2 (Lozano Polo *et al.* 1990). IL-2 functions as a major growth factor for T cells, thus its regulation is a central control mechanism for T cell clonal expansion and subsequent cell-mediated and humoral immune responses (Belardelli 1995; Gomez *et al.* 1998; Swain *et al.* 1991a; Waldmann *et al.* 1998).

A. Control of IL-2 Transcription

IL-2 mRNA transcripts are undetected in naïve resting T cells, but are up-regulated within 30 minutes of $CD4^+$ T cell activation (Paul and Seder 1994; Seder *et al.* 1994). *In vitro* nuclear run-on assays, DNase I hypersensitivity assays, and *in vivo* footprinting analyses are consistent in implicating a transcriptional mechanism for IL-2 mRNA expression. Steady-state levels of IL-2 mRNA in activated T cells are maintained between a balance of gene transcription and mRNA degradation. Following T cell

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stimulation, maximal mRNA levels are achieved approximately four to eight hours following T cell activation and return to background levels within 24 hours. The rate of decline of IL-2 mRNA expression is normally greater than the rate of decline of IL-2 gene transcription implicating a putative protein-dependent mechanism of IL-2 mRNA degradation. In support of such a mechanism, IL-2 mRNA accumulation in the absence of a change in IL-2 transcription is observed when activated T cells are treated with cyclohexamide to inhibit protein synthesis (Jain *et al.* 1995; Oldham *et al.* 1989). Furthermore, actinomycin D treatment prolongs IL-2 mRNA stabilization suggesting that mRNA degradation is regulated at the level of RNA synthesis (Jain *et al.* 1995).

Continual T cell stimulation is required to maintain IL-2 transcription, and a long-standing question of how long IL-2 transcription proceeds under conditions of continual T cell stimulation remains unanswered. *In vivo* footprinting analyses suggest protein binding at transcriptional regulatory elements for up to eleven hours following T cell stimulation (Jain *et al.* 1995). Based on this, it is tempting to speculate that transcription may proceed as long as antigen or appropriate stimulation is present, and that the decline in transcription may reflect a diminution in the level of activated transcription factor binding through secondary mechanisms e.g., recruitment of negative regulatory proteins and/or chromatin remodeling).

B. Signaling cascades through the TcR that regulate IL-2 gene expression

The intracellular signaling events that mediate IL-2 transcription have been studied extensively and implicate activation of multiple signaling cascades within

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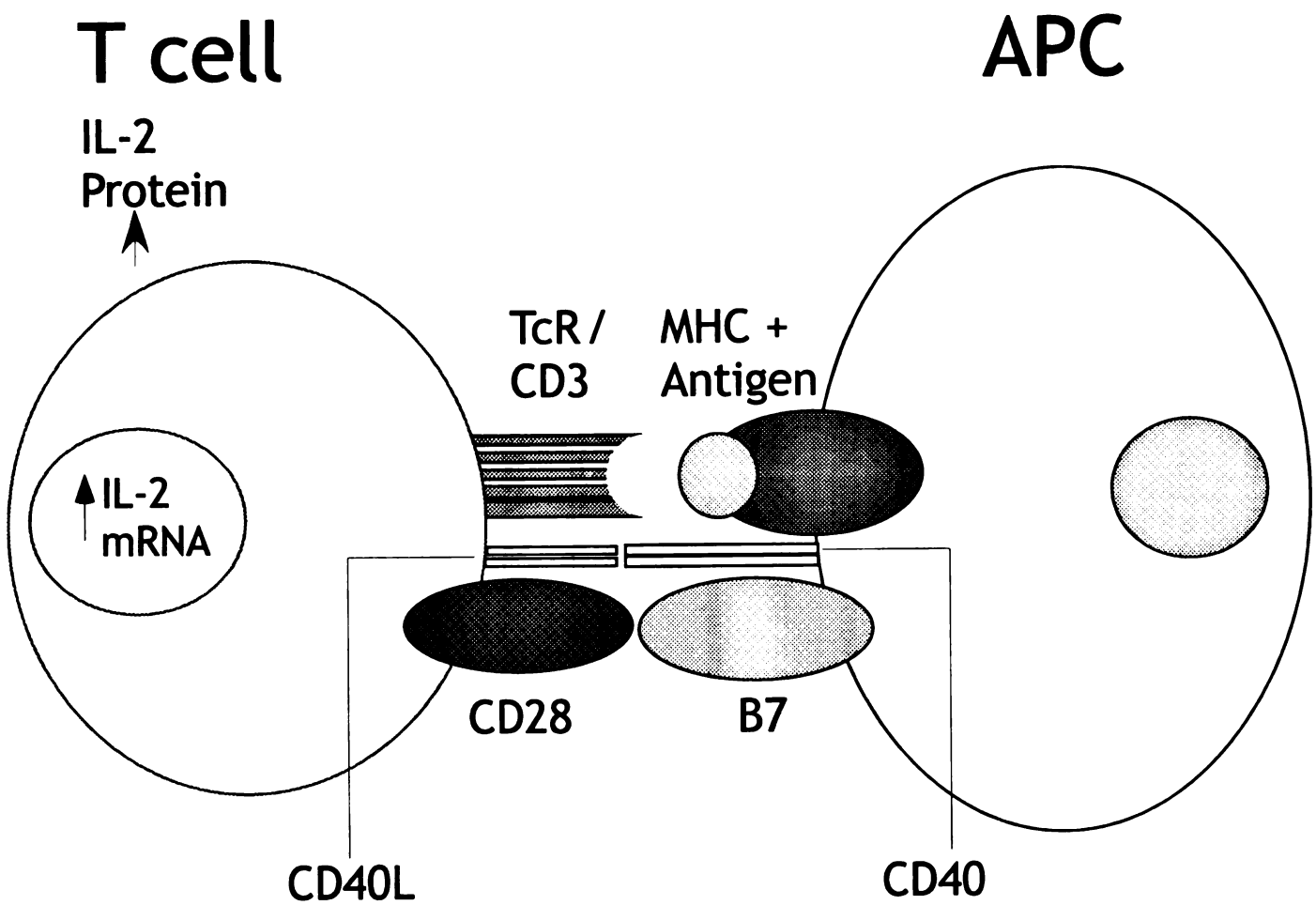
seconds of ligation at the TcR (T cell antigen receptor) complex. The majority of mature T cells express a transmembrane disulfide-linked α,β TcR heterodimer. Complete T cell activation requires a two-signal stimulation (Peterson and Koretzky 1999) (**Figure 5**). The first stimulus is provided by presentation of a processed antigenic peptide by an APC (antigen presenting cell) to a T helper cell through the TcR in context with an MHC class II molecule (Davis *et al.* 1989; Evavold *et al.* 1993; Farber 1998; Kane *et al.* 2000; O'Shea 2000; Saito *et al.* 1995; Sette *et al.* 1995; Shores and Love 1997; von Boehmer *et al.* 1989; Wange and Samelson 1996; Wilson and Garcia 1997).

The most critical second costimulatory signal is provided through the interaction of members of the B7 family, B7-1 (CD80) and B7-2 (CD86), found on the surface of antigen presenting cells (i.e. B cells, dendritic cells, and macrophages) with the CD28 molecule located on the surface of the helper T cell (Chiang *et al.* 2000; Holdorf *et al.* 2000; Hombach *et al.* 2001; Powell *et al.* 1998; Seder *et al.* 1994). The cytoplasmic tails of the TcR are devoid of signaling motifs. Therefore T cell activation through the TcR is dependent on translocation of cytosolic tyrosine kinases to the TcR complex upon ligand binding to initiate intracellular signaling (Clavreul *et* al. 2000; Hennecke and Wiley 2001; Kim *et a*l. 2001). TcR ligation activates the Src family PTKs (protein tyrosine kinase) lck and/or fyn, which associate with the CD3 and TcRζ subunits to initiate a rapid phosphorylation and activation of the Syk family PTK-ZAP-70. The end result of TcR-coupled tyrosine kinase activation is the initiation of numerous signaling pathways within the cell including, intracellular Ca^{2+}, PKC (protein kinase C), PKA (protein kinase A), and phosphatases. These activated cascades ultimately culminate in the activation of

Figure 5. Two-signal model for T cell activation. IL-2 protein synthesis requires a two-signal interaction between a T cell and an APC. Signal one is mediated through the interaction of the TcR/CD3 complex with the peptide antigen/MHC complex that leads to increased T cell surface expression of CD40L. CD40L interacts CD40 on the APC to induce expression of B7 on the surface of the APC. B7 interacts with CD28, a co-stimulatory receptor on the T cell surface. TcR/CD3 and CD28 signals are essential for sufficient IL-2 gene expression for complete T cell activation and prevention of T cell anergy (Modified from Weiss, 1999).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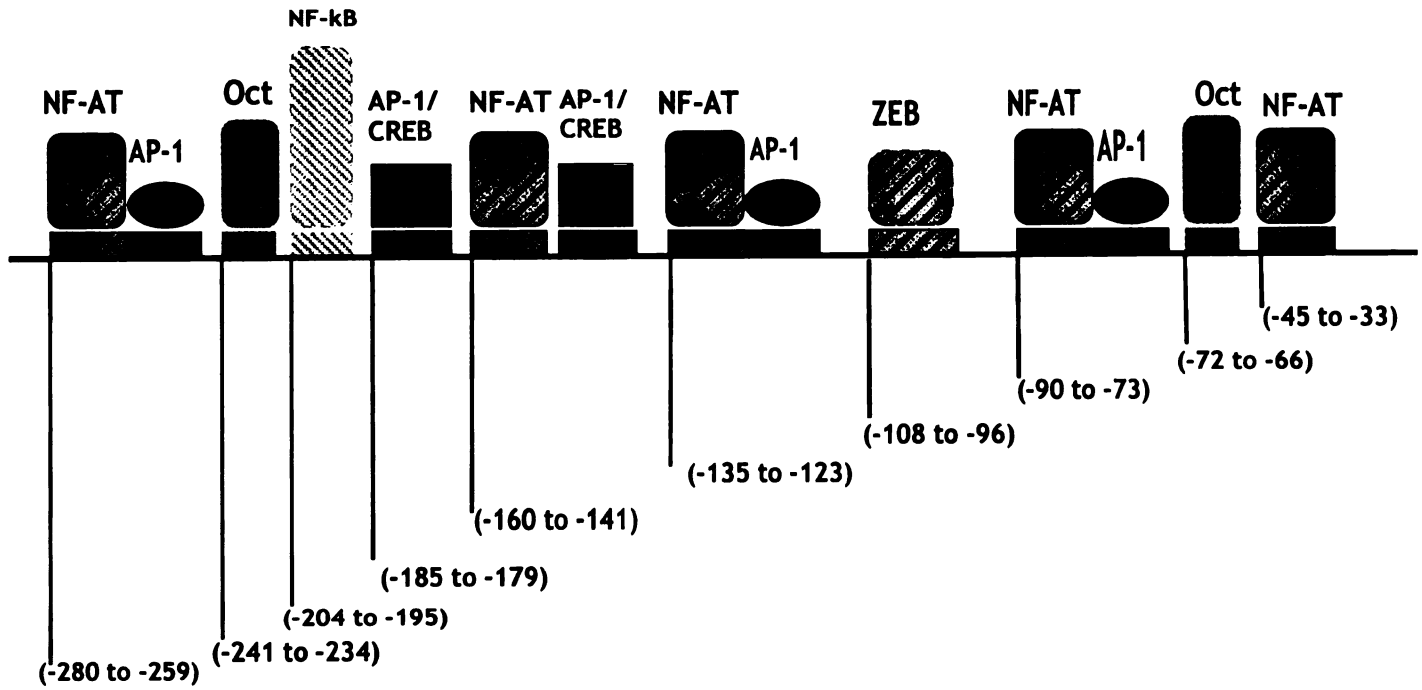
C. Regulatory elements of the IL-2 gene

IL-2 gene expression is stringently regulated at the transcriptional level via binding of several nuclear *trans*-acting factors to *cis*-acting elements within the regulatory region of the IL-2 promoter. Post-translational modifications including glycosylation and phosphorylation also regulate IL-2 stabilization and secretion, respectively (Robb *et al.* 1984; Rooney *et al.* 1995). The first 300 bp region directly upstream of the transcription start site of the IL-2 promoter is essential for regulation of IL-2 gene transcription. (Rooney *et al.* 1995; Serfling *et al.* 1995; Serfling *et al.* 1989). The mouse and human IL-2 genes share close sequence homology of this promoter region (Novak *et al.* 1990).

As shown in **Figure 6**, the minimal essential regulatory region of the IL-2 promoter/enhancer contains multiple DNA binding motifs for several critical TcR-inducible *trans*-acting factors important for transcriptional regulation of the IL-2 gene. These *trans*-acting factors include AP-1 (activator protein-1), NFAT (nuclear factor of activated T cells), Rel and NF-κB (nuclear factor-κB) proteins, CREB (cAMP response element-binding protein), ATF (activation transcription factor), and ZEB (zinc finger/E-box binding protein). Protein binding to each of the *cis*-acting response elements is induced in response to TcR ligation and CD28 costimulation with the exception of the Oct sites. AP-1, NFAT, Rel/NF-κB, CREB, and ATF transcription factors, in general, are associated with positive regulation of IL-2 expression (Jain *et al.* 1995). In contrast, ZEB binding to the NRE-A (negative response element A) and CREB/CREM binding to

Figure 6. Schematic of the 5' minimal essential regulatory region of the mouse IL-2 promoter. The region of the IL-2 promoter that is essential for gene transcription is located approximately 300 bp 5' of the transcription start site. Multiple *cis*-acting elements are localized within this 300 bp region as illustrated. In addition, numerous *trans*-acting factors, including AP-1, NFAT, NF-κB ZEB, CREB, and Oct bind to their respective response elements and function within the IL-2 promoter and function in a cooperative manner to regulate IL-2 gene transcription. The NRE-A represents the major negative regulatory cis-acting element in the IL-2 promoter.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the distal TRE (phorbol ester response element) negatively regulate IL-2 expression (Yasui *et al.* 1998).

While the precise integrative contribution of CD28 signaling to TcR-signaling remains elusive, evidence suggest that CD28 positively cooperates with the TcR at early stages of the signaling cascade to enhance TcR signaling through numerous mechanisms. For example, CD28 signaling induces tyrosine phosphorylation of several cellular substrates including TcRζ and ZAP-70 (Perez *et al.* 1997), enhances c-Fos expression (Tuosto and Acuto 1998), activates ERK (extracellular regulated kinase) and JNK (Jun N-terminal kinase) MAP (mitogen activated protein) kinases, (Jain *et al.* 1995; Kempiak *et al.* 1999) and potentiates NFAT, AP-1, and NF-κB transcriptional activity (Tuosto and Acuto 1998). Moreover, it has been demonstrated that full activation of the -164 bp CD28RE of the IL-2 promoter is dependent upon an adjacent AP-1 response element that is positioned 2 bp downstream of the CD28RE. Site-direction mutagenesis of either the CD28RE or the adjacent AP-1 binding motif disrupts function of the CD28RE (Iacobelli *et al.* 1999; Shapiro *et al.* 1997). Alternatively, CD28•B7 ligation also enhances T cell activation through post-transcriptional stabilization of IL-2 mRNA. The CD28RE also provides DNA binding sites for NF-AT, CREB/AFT, and NF-κB/Rel. The extensive integrative cross talk among multiple signaling pathways required for regulation of IL-2 expression is exemplified by the vast diversity of protein•DNA interactions that accompany T cell activation. A more complete description of the role of NF-κB, NFAT, AP-1, CREB, and ZEB t*rans*-acting factors on IL-2 gene transcription is provided.

1. NF-κB (nuclear factor–κB)

The mammalian NF-κB proteins include NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), c-Rel, and RelB. Inactive NF-κB proteins are retained in the cytoplasm of unstimulated T cells through an interaction with one or more of seven known IκB proteins (Baldwin 1996). TcR ligation results in rapid inactivation of IκBα, and subsequent translocation of active NF-κB dimers into the nucleus where they bind to κB motifs to regulate transcription. CD28 costimulation generates a rapid, sustained Rel/NF-κB induction. The duration of this expression is regulated by selective phosphorylation and degradation of IκBα and IκBβ, which leads to a persistent nuclear expression of Rel/NF-κB because unlike IκBα, IκBβ is not activated by NF-κB. c-Rel is the principal κB protein that binds to the CD28RE after CD28 ligation (Ghosh *et al.* 1993; Meyer *et al.* 1996). Overexpression of c-Rel or RelA activates κB-dependent transcription through the IL-2 CD28RE (Maggirwar *et al.* 1997). IL-2 expression is impaired in c-Rel knockout mice (Kontgen *et al.* 1995). Collectively, these results implicate a regulatory role of c-Rel in CD28-dependent IL-2 transcription. In addition to the CD28RE, a second κB binding DNA motif is positioned -208 bp upstream of the IL-2 gene transcriptional start. NF-κB proteins binding to each of these response elements is induced in response to TCR/CD28 costimulation.

2. NFAT (nuclear factor of activated T cells)

The NFAT family of transcription factors includes the cytosolic NFATc1, NFATc2, NFATc3, and NFATc4 and the nuclear NFATn. The DNA binding motifs of NFAT proteins (120 to 140 kD) resemble those of Rel-family proteins; c-Rel, p50, and p65 show some overlap with NFAT in their ability to bind to the CD28RE in the IL-2

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promoter. NFAT also cooperatively interacts with AP-1 proteins (Rao *et al.* 1997). As shown in **Figure 6**, five NFAT response elements are positioned at -280, -160, -135, -90, and -45 bp upsteam of the mouse IL-2 transcription start site (Rooney *et al.* 1995). Selective mutation of any one of these five sites attenuates TcR/CD28-induced IL-2 mRNA expression. Phosphorylated NFATc proteins reside in the cytoplasm, and translocate into the nucleus in an intracellular Ca^{2+}/calmodulin/calcineurin-dependent manner upon activation of the TcR. NFATc is dephosphorylated by calcineurin enabling its nuclear entry and subsequent transcriptional activity. However, induction of NFAT-dependent reporter gene expression and nuclear NFAT•DNA binding in CD28 plus PMA-activated T cells suggests that some NFAT proteins may be activated in a Ca^{2+}-independent manner (Rooney *et al.* 1995) and provides a putative mechanism for synergistic regulatory cross-signaling between TcR-mediated calcium/calcineurin-dependent and CD28-mediated calcium/calcineurin-independent pathways. The nuclear NFAT complex is comprised of an AP-1•NFATn heterodimer (Jain *et al.* 1992a). The AP-1 component confers stability of the protein•DNA interaction (Jain *et al.* 1993).

3. AP-1 (Activating protein-1)

AP-1 comprises homodimers of Jun (c-Jun, JunB, and JunD) or heterodimers of Jun and Fos (c-Fos, FosB, Fra-1, and Fra-2) that bind to the TGA(C/G)TCA TRE (Karin *et al.* 1997). Leucine zippers dictate the selectivity of AP-1 protein•protein dimerization, and basic amino acid sequences confer specificity of DNA•protein interactions. The AP-1 superfamily comprises the Jun family, the Fos family, and the maf family of transcription factors. Other families of *trans*-acting proteins are known to interact with AP-1, including additional bZIP (basic/leucine zipper

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interacting proteins), CREB/AFT, NFAT, NF-κB, Smads, the ETS proteins, and the Oct proteins of the POU family (Brodin *et al.* 2000; Liberati *et al.* 1999; Mulder 2000; Wisdom 1999; Wong *et al.* 1999; Xu *et al.* 2000b; Yingling *et al.* 1997; Zhang *et al.* 1998). AP-1 transcriptional activity depends on the activation of at least two independent, converging signaling cascades, namely the p56lck/PKC/p21/Ras/Raf-1/MEK1/MEK2/ERK1/ERK2 MAPK pathway activated through the TcR (Izquierdo *et al.* 1994a; Izquierdo *et al.* 1994b) and the JNK1/JNK2 MAPK pathways activated through CD28 ligation (Cheng *et al.* 2000; Su *et al.* 2001). JNK phosphorylation of c-Jun stabilizes the protein complex by suppressing ubiquitin-dependent degradation (Hermida-Matsumoto *et al.* 1996). Furthermore, JNK phosphorylation of the ser-63 and ser-73 residues of c-Jun augments c-Jun•CBP/p300 interactions to enhance AP-1 transcriptional activity presumably by enhancing binding affinity. Five AP-1 binding motifs have been identified in the mouse IL-2 promoter (**Figure 6**). Four of these DNA binding sites are located in conjunction with NFAT binding motifs (-280, -180 -160, -135, and -90) (Rooney *et al.* 1995). The AP-1 site at -180 bp upstream of the transcriptional start site is a consensus site in the reverse orientation, but varies from the consensus AP-1 binding site by one bp in the forward orientation.

Recent evidence suggest that a complex of CREB and cAMP elements bind to the -180 AP-1 site and functions to suppress IL-2 gene transcription in anergic $CD4^+$ T cells (Powell *et al.* 1999). The cooperative interaction of AP-1 and NFAT proteins increases DNA recognition motif selectivity as well as DNA•protein stabilization. NFAT transcriptional activity, as described above, is primarily Ca^{2+}-dependent; therefore, AP-1•NFAT interactions also provide a mechanism of transcriptional regulation through

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4. CREB (cAMP response element binding protein)

CREB, a 43 kD member of the ATF/CREB family transcription factor, contains a bZIP DNA binding motif that contains a cluster of basic amino acids and leucine zipper structures (Busch and Sassone-Corsi 1990; Maekawa *et al.* 1989). CREB family members form dimers through their leucine zipper regions and bind to the octanucleotide CRE (cAMP response element) element (TGANNTCA). CREB family members, including CREB, ATF-1, ATF-2, ATF-3, ATF-4, and CREM bind as homo- or heterodimers in combination with other CREB family members. Alternatively, CREB family members also heterodimerize with members of the Jun (i.e., c-Jun, JunB, and junD); Fos (i.e., c-Fos, FosB, Fra-1, and Fra-2); and maf (i.e., mafK, mafB, and nrl) families of *trans*-acting factors. This oligomerization is somewhat specific as c-Jun preferentially dimerizes with ATF-2, ATF-3, and ATF-4; whereas, c-Fos selectively heterodimerizes with ATF-4. TcR-induced CREB transcriptional activity is regulated exclusively by phosphorylation of ser-133 via a cAMP-independent p56lck/PKC/Ras/Raf-1/MEK/RASK2 (ribosome associated S6-kinase 2) pathway (Muthusamy and Leiden 1998). Dominant negative CREB mutant studies suggest that Fra-2 and FosB are the principal CREB *trans*-acting factors that are activated in response to TcR/CD28 signaling (Berkowitz and Gilman 1990; Berkowitz *et al.* 1989). The significance of Fra-2 and FosB selectivity in IL-2 expression has not yet been elucidated.

ATF-2 is phosphorylated and stimulated by JNK MAPK (MAP kinase) and p38 MAPK at Thr-69, Thr-71, and Ser-90 (Gupta and Terhorst 1994; Gupta *et al.* 1996; Gupta *et al.* 1995). Activated ATF-2 binds to CRE either as a homodimer or as a

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heterodimer with c-Jun (Hai and Curran 1991; Macgregor *et al.* 1990). ATF-2 oligomerizes with Smads and is directly phosphorylated by TAK-1 (TGF-β activating kinase-1) suggests that ATF-2 is a direct nuclear target of TGF-β-induced signaling (Hanafusa *et al.* 1999; Sano *et al.* 1999).

5. ZEB

ZEB (zinc finger/E-box binding protein) is a *trans*-acting factor that binds to the NRE-A (negative response element-A) located -108 bp upstream of the mouse IL-2 transcription start site (Yasui *et al.* 1998). ZEB•NRE-A binding confers negative regulation of IL-2 expression. ZEB contains two independent repressor regions that target distinct sets of transcription factors and regulate different tissues. Region I selectively represses hematopoietic-restricted transcription factors, including RelA, c-Fos, c-Jun, c-myb, E2F-1, and ETS family members (Postigo *et al.* 1999). The second region, Region II, specifically inhibits the activity of the myogenic transcription factor, MEF2C (myocyte enhancer factor 2C) to regulate muscle differentiation. ZEB binds to several different E box sequences, but has a higher affinity for the CACCTG sequence. In addition to repressing IL-2 expression, ZEB also silences the immunoglobulin heavy chain enhancer and GATA-3 transcriptional activity (Postigo *et al.* 1999). In contrast, ZEB•α4b1 confers co-stimulatory signaling in T cell activation.

V. TGF-β_1 signaling through Smads

The intracellular signaling mechanisms responsible for TGF-β_1-mediated immune modulation remain relatively unexplored. Therefore, this section will primarily, albeit not exclusively, describe the current understanding of Smad signaling in mammalian

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cells, as little information is yet available regarding Smad signaling in lymphoid tissue. As shown in **Figure 7**, eight TGF-β_1-dependent signaling cascades have been proposed: (1) PKC, (2) PLC, (3) protein phosphatase 1, (4) ras, (5) p38 MAPK, (6) ERK1/ERK2 MAPK, (7) JNK1/JNK2 MAPK, and (8) Smads [(Hartsough and Mulder 1997; Massague and Chen 2000; Mulder 2000; Piek *et al.* 1999; Roberts 1999; ten Dijke *et al.* 2000). It is likely that these pathways integrate at multiple levels to elicit an intricate regulatory control on the overall biological effects of TGF-β_1.

A. Structures and Functions of Smad proteins

Smad proteins are intracellular signaling mediators unique to members of TGF-β superfamily. At present, ten Smad proteins (i.e., Smad1-10) have been identified. Smad proteins range in molecular weight from 45 to 65 kD and, as illustrated in **Figure 8** contains three structurally distinct domains; i.e., two conserved domains, an N-terminal MH1 (Mad homology) domain and a C-terminal MH2 domain, and a variable proline rich linker region inserted between MH1 and MH2 domains (Chacko *et al.* 2001; Dennler *et al.* 1999; Shi *et al.* 1998; Wrana 2000). One of the principal functions of the MH1 domain of the receptor-activated Smads (i.e., Smad2 and Smad3) is to bind DNA sequences via its β-hairpin loop (Massague and Wotton 2000; Shi *et al.* 1998). A conserved lysine-rich motif in the N-terminus of Smad2 and Smad3 resemble the classic simian virus 40 large antigen NLS (nuclear localization signal) and may function as a nuclear localization signal for Smad3 (Shi *et al.* 1998; Xiao *et al.* 2000). In contrast to Smad3, Smad2 nuclear import appears to be NLS-independent (Xu *et al.* 2000a).

The precise regulatory mechanisms of Smad nuclear localization remains under investigation. The lysine rich MH1 domain functions predominantly in DNA•protein

Figure 7. Intracellular signaling pathways of TGF-β_1. TGF-β_1 mediates it biological effects by binding to a transmembrane TGF-β receptor complex with intrinsic serine/threonine kinase activity. Shown here are the eight known signaling pathways that are activated upon ligand binding to the TGF-β receptor complex.

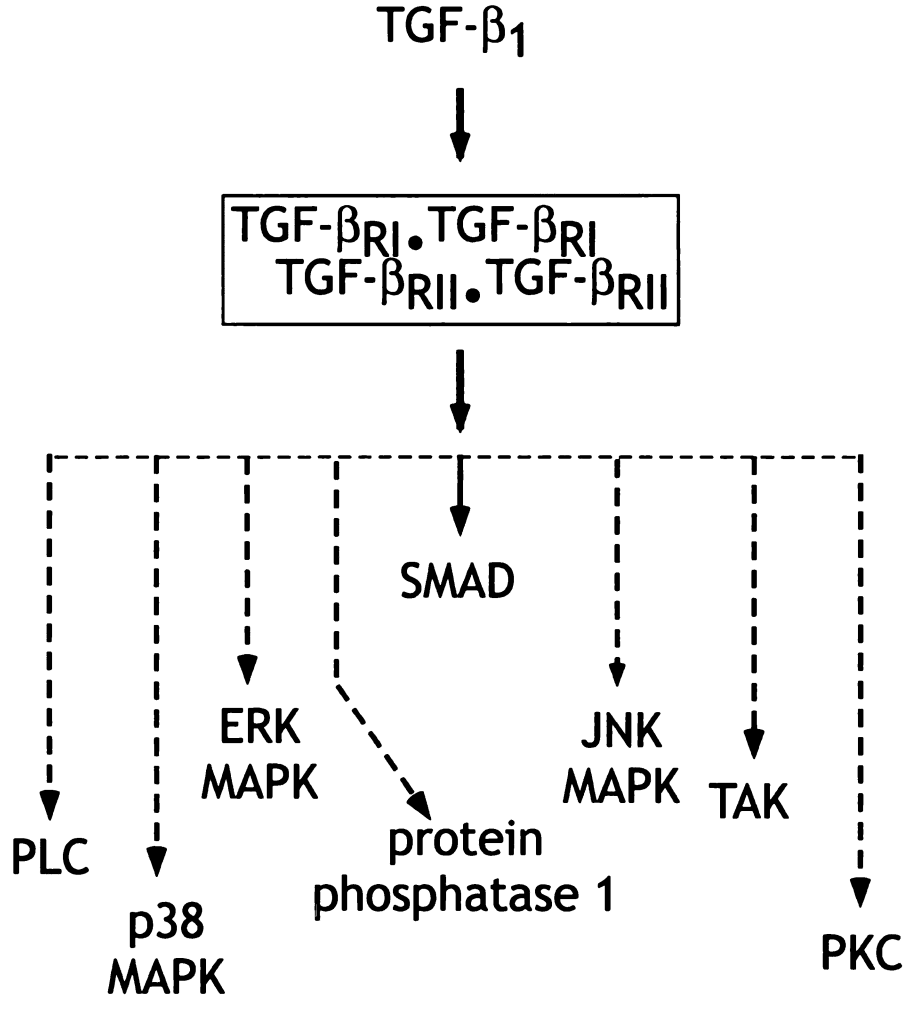
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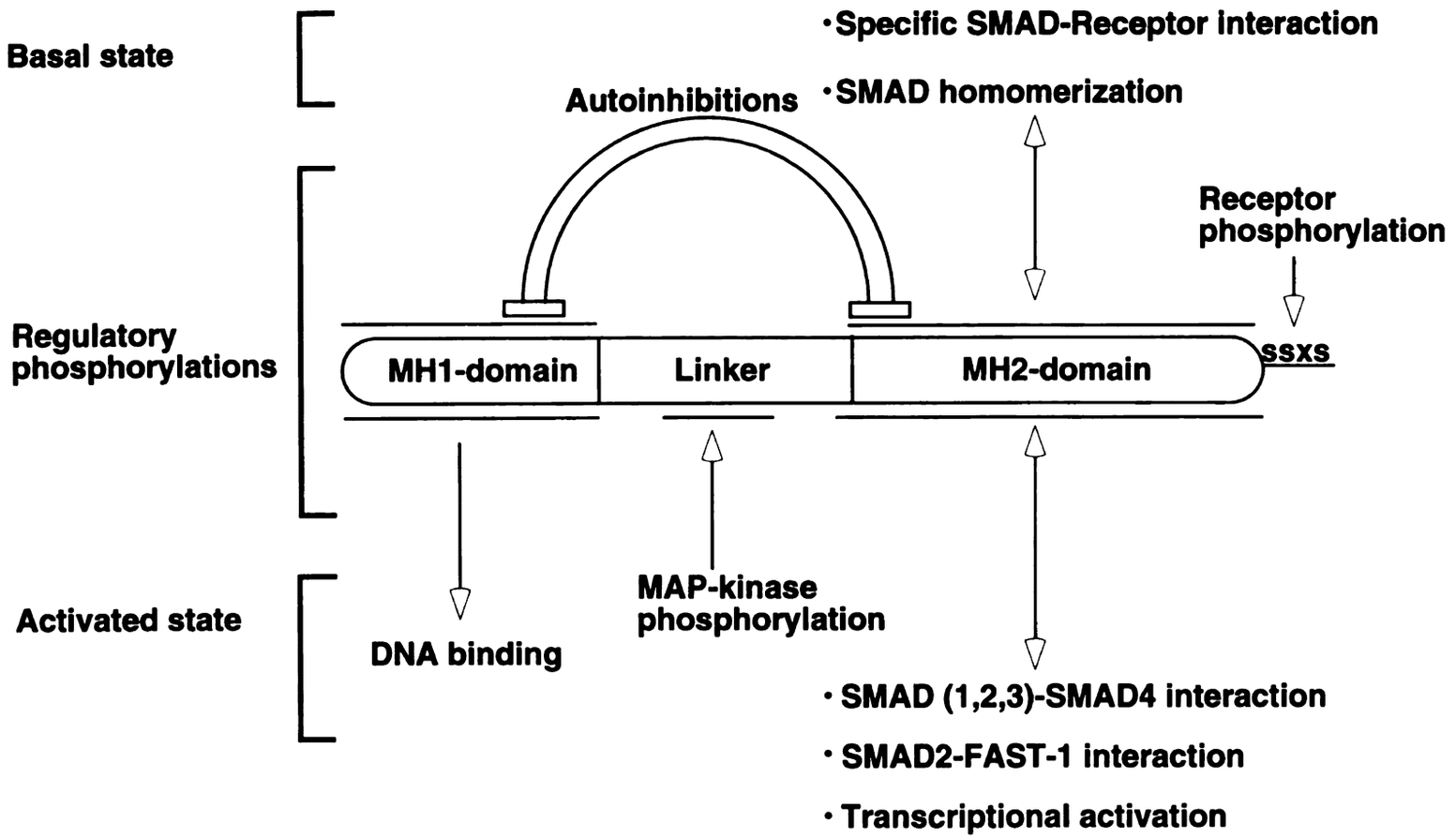
Figure 8. Schematic of the functional domains of the Smad proteins. The Smad proteins are intracellular signal transducers for TGF-β superfamily proteins. Shown here is a schematic structure of R-Smads and their functional domains. Smads are comprised of three distinct structural domains, including the MH1 domain, the MH2 domain, and the linker region. The MH1 domain is critical for DNA binding; whereas, the MH2 domain is essential for protein•protein interactions. The SSXS motif, located at the extreme carboxy terminus, represents the Ser phosphorylation sites for the TGF-β receptor. Co-Smad, Smad4 does not contain a SSXS domain. The PSSP site, located in the linker region, represents the phosphorylation site for ERK MAPK.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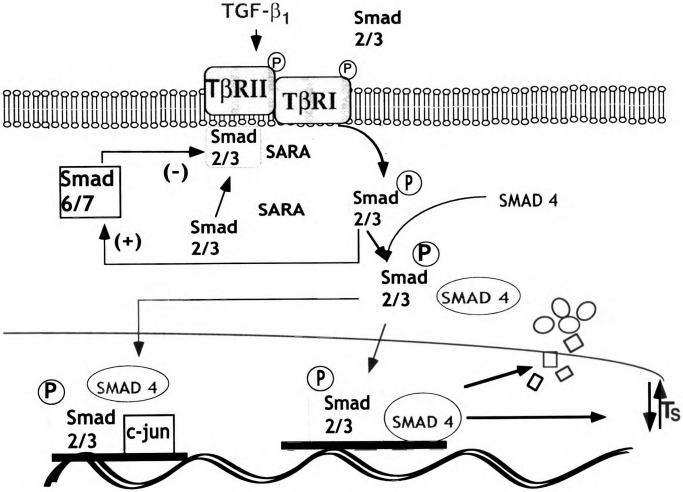
complex formation, whereas the MH2 domain, in general, confers protein•protein interactions. In addition, the MH2 domains contain the principal serine-threonine kinase phosporylation acceptor sites that confer specificity for ligand-induced activation (ten Dijke *et al.* 2000). In the inactive state, the Smad proteins reside in a folded conformation permitting an MH1•MH2 interaction which provides an autoinhibitory regulation of Smad protein activation. Functionally, the Smads are categorized into three classes: R-Smads (receptor-regulated Smads: Smad1, Smad2, Smad3, Smad5, and Smad10); co-Smads (common-mediator Smads: Smad4 and Smad9); and I-Smads (inhibitory Smads: Smad6 and Smad7). R-Smads interact with ligand-specific-activated TβRI. Smad2 and Smad3 are expressed in mammalian tissue and are TGF-β_1 responsive. The ligand specificity of the remaining R-Smads has been identified but will not be discussed here (Massague 1998).

Both Smad2 and Smad3 retain a SS(V/M)S motif at their extreme MH2 domain of which two serines are direct targets for phosphorylation by TβRI serine/threonine kinases. Substitution of the serine residues of the SSXS motif to negatively charged aspartate residues mimics TβRI-induced phosphorylation and results in constitutive Smad3 activation *in vitro* (Liu *et al.* 1997). SARA (Smad anchor for receptor activation) is a FYVE domain containing protein that complexes with inactive cytosolic Smad2 and Smad3 and functions to facilitate Smad•TβR1 interactions by trafficking Smads to the membrane (Tsukazaki *et al.* 1998; Wu *et al.* 2000). Tethering of Smads to the plasma membrane appears to be essential, as deletion of the FYVE domain of SARA abolishes TβR1 responsiveness (Tsukazaki *et al.* 1998). As illustrated in **Figure 9**, upon phosphorylation by TβRI, Smads dissociate from SARA and oligomerize with Smad4 for

Figure 9. TGF-β_1 signaling through Smad proteins. Upon ligand binding to the TGF-β receptor complex, R-Smads are recruited to the membrane in association with SARA, and are phosphorylated by the TGF-β receptor type I. The R-Smads then form homomeric complexes among themselves and then assembly into a heteromeric complex with Smad4. The heteromeric Smad complex then translocates into the nucleus where Smads interact with other transcription factors and accessory proteins in a cooperative manner to regulate transcription of TGF-β_1-responsive genes.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nuclear transport (Kawabata and Miyazono 1999; Raftery and Sutherland 1999). Smad4, previously identified as the tumor suppressor DPC4 (deleted in pancreatic cancer 4), is structurally and functionally distinct from the R-Smad family members. Smad4 complexes with Smad2 and Smad3 to induce Smad nuclear translocation and subsequent transcriptional activation (Massague 1996; Massague and Weis-Garcia 1996). SAD (Smad4 activation domain) is a 48-amino acid proline-rich regulatory element located within the middle linker domain of Smad4 and is essential for mediating signaling (de Caestecker *et al.* 2000; Qin *et al.* 1999). Interestingly, TGF-β_1-induces oligomerization of Smad2 and Smad3 as homo- and hetero-dimers and trimers in the absence of Smad4 *in vitro* (Kawabata and Miyazono 1999). This observation has not been confirmed *in vivo* and the biological relevance of this complex is unknown.

The TGF-β_1-responsive I-Smads (i.e., Smad6 and Smad7) lack the conserved SS(V/M)S motif and are not phosphorylated by TβRI. Smad6 and Smad7 antagonize TGF-β_1-mediated activation of Smad2 and Smad3 by preventing phoshorylation by TβR1. Smad6 prevents TGF-β_1-induced phosphorylation of Smad2 but not Smad3. Smad7, on-the-other-hand, prevents TGF-β_1-induced phosphorylation of Smad2 *and* Smad3. The mechanisms for differential inhibitory effects of Smad6 and Smad7 are not yet understood. However, these differential results exemplify an additional level of selectivity, specificity, and regulatory control of Smad signaling. The inhibitory Smads also function in a negative feedback loop, presumably to fine tune TGF-β_1 signaling, as they are transcriptionally up-regulated in a TGF-β_1-responsive manner (Stopa *et al.* 2000; von Gersdorff *et al.* 2000). Once in the nucleus, Smad complexes function as transcriptional regulators of TGF-β_1-responsive genes (Massague and Chen 2000;

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Massague and Wotton 2000; ten Dijke *et al.* 2000; Wrana 2000; Wrana and Attisano 2000).

Impairments in genes that encode for Smads have been associated with cancer and thus implicate a role for Smads as tumor suppressors. For example, Smad4 is deleted in about 50% of pancreatic cancers (Cook and Urrutia 2000; Hilgers *et al.* 2000). Germline mutation of Smad4 has been associated with familial juvenile polyposis, a disorder characterized by predisposition to gastrointestinal cancer (Howe *et al.* 1998; Roth *et al.* 2000; Takagi *et al.* 1998). Somatic mutations of Smad2 have been identified in sporadic cases of human colorectal cancer (Roth *et al.* 2000; Takagi *et al.* 1998; Takenoshita *et al.* 1998). Paradoxically, Smads also retain oncogenic properties. For instance, the oncogenes TGIF (TG-interacting factor), Ski (Slone Kettering Institute), and Sno (ski related novel) have all been associated with Smad-mediated signaling (Lo *et al.* 2001; Massague and Wotton 2000; Simeone *et al.* 2000; Wotton and Massague 2001)

B. Smad binding to sequence specific DNA motifs

Site directed mutagenesis studies employing recombinant mammalian Smad3 and Smad4 MH1 domains initially identified an 8 bp palindromic sequence (GTCTAGAC) as a SBE (Smad binding element); concatemers of this sequence conferred TGF-β_1 responsiveness to a minimal promoter (Piek *et al.* 1999; Zawel *et al.* 1998). Subsequent studies have demonstrated that a 4 bp CAGA DNA motif is sufficient for Smad3 and Smad4 binding (Dennler *et al.* 1998; Shi *et al.* 1998). Consistently, the MH1 domains of Smad3 and Smad4 bind directly to DNA motifs containing a common CAGA or GTCT 'Smad box' core sequence in the promoter of several TGF-β_1-responsive genes, including the human PAI-I (plasminogen activator inhibitor-I), human collagenase, junB, and

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TGF-β_1 itself. Targeted disruption of the CAGA sequences in the PAI-I promoter impairs TGF-β_1 responsiveness of reporter gene constructs, suggesting that CAGA is critical for TGF-β_1-induced gene expression (Stroschein *et al.* 1999a; Yingling *et al.* 1997; Zhang *et al.* 1998). It is becoming apparent that several similar, yet distinct, SREs containing the core CAGA sequence are critical for TGF-β_1-mediated Smad3 and Smad4 binding. For example, in the junB promoter an ACAGACA minimal sequence is essential for Smad3/Smad4 binding and TGF-β_1-responsiveness (Jonk *et al.* 1998). Noteworthy, unlike the CAGA sequence in the PAI-I promoter, which is activated by TGF-β_1, but not other members of the TGF-β superfamily, the CAGA sequence in the JunB promoter sequence is responsive to BMP *as well as* TGF-β_1 (Liberati *et al.* 1999). Moreover, an AGACA sequence has been identified as the minimal sequence to confer TGF-β_1 responsiveness for some genes (Chen *et al.* 2000; Chen *et al.* 1999; Ghosh *et al.* 2000; Jonk *et al.* 1998). High affinity binding of Smad2 to CAGA-containing TGF-β_1-responsive elements has not been demonstrated. Smad2/Smad4 complexes have been shown to bind to GC-rich DNA motifs like the p21 promoter to confer TGF-β_1-responsiveness (Moustakas and Kardassis 1998; Pardali *et al.* 2000b).

C. Smads Synergize with Transcription Factors

Evidence strongly suggests that collaboration of Smad proteins with other *trans*-acting factors and/or accessory proteins is critical for Smad-mediated TGF-β_1-responsive transcriptional activity. A well characterized example of this collaborative effort is demonstrated with the TGF-β_1-responsive human collagenase gene. In this model, a Smad3/Smad4 heterodimer and AP-1 bind as a complex to an overlapping AP-1/CAGA TGF-β_1-responsive site in the regulatory promoter region (Yingling *et al.* 1997). The

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Smad and AP-1 trans-acting factors are essential for transcriptional activity by TGF-β_1 at this site. Interestingly, both Smad3 and AP-1 directly bind to the DNA response element; however, Smad/CAGA binding is not essential for TGF-β_1-responsiveness. These results suggest that Smads function as co-activators via a Smad•AP-1 interaction to directly enhance AP-1 transcriptional activity. In support, Smad•AP-1 interaction does occur and is mediated through a strong Smad3 MH1/c-Jun interaction and a weak Smad3 MH2/ c-Fos interaction. Smad3 and Smad4 also bind to other AP-1 family members, including JunB, JunD, and Fra2 as well as to NF-κB, and appear to function in a similar co-activating manner to confer TGF-β_1 responsiveness (Kon *et al.* 1999; Liberati *et al.* 1999; Sano *et al.* 1999; Shen *et al.* 1998; Wong *et al.* 1999). Alternatively, Smad/CAGA interactions are necessary for transcriptional modulation of some TGF-β_1-responsive promoters. For example, direct DNA binding of both CBFA3 (Core Binding Factor subunit α3) and a Smad3/Smad4 complex to the TGF-β_1-inducible site in the immunoglobulin Cα promoter is essential for transcriptional activation of germline IgA by TGF-β_1 (Hanai *et al.* 1999).

In contrast to associating with *trans*-acting factors to induce transcription, Smads have also been demonstrated to heterodimerize with negative *trans*-acting factors to modulate transcription. For example, SIP-1 (Smad Interacting Protein) is a member of the ZEB family of negative transcription factors that binds to the NRE-A to inhibit transcriptional activity of TGF-β_1-responsive genes (Verschueren and Huylebroeck 1999). Smad2 and Smad3, but not Smad4 bind to SIP-1. SIP-1/Smad interactions have been demonstrated to abrogate SIP-1 binding to its response element in the promoter of

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D. Smads Interact with Transcriptional Co-Activators and Co-Repressors

Smad2, Smad3, and Smad4 have been demonstrated to interact via their MH2 domains with several transcriptional co-activators and co-repressors, e.g., CBP (CREB Binding Protein) and p300, that are known to modulate transcriptional activity of CREB, AP-1, NFAT, and NFκB. Smad•CBP/p300 interactions occur in a TGF-β_1-dependent manner, and have been shown to modulate the transcription of TGF-β_1-responsive genes (Janknecht *et al.* 1998; Waltzer and Bienz 1999).

VI. Implications for Smad Regulation of Immune Cell Function

TGF-β_1 regulates the expression of several genes associated with immune cell function, including cytokines, immunoglobulins, and cell cycle proteins. However, the mechanisms whereby TGF-β_1 modulates these genes at the transcriptional level are poorly understood. Recent data suggest Smad3 may play a critical role in immune cell responsiveness to TGF-β_1. Investigations involving disruption of the Smad3 signaling pathway in genetically engineered mice provide compelling evidence for a role by Smad3 in immune modulation (Yang *et al.* 1999). Phenotypic differences between TGF-β_1-null and TGF-β receptor-activated Smad3-null mice have been summarized (Letterio 2000) and are presented as **Table 1**. Smad3-null mice display defects in mucosal immunity characterized by the formation of severe bacterial abscesses along mucosal surfaces, including periorbital and periodontal surfaces and the gastrointestinal tract (Ashcroft *et*

Table 1. Phenotype comparison of Smad3-null and TGF-β_1-null mice.

TGF β1$^{-/-}$ mice	Smad3$^{-/-}$ mice
Strain-dependent defects in yolk sac vasculogenesis and embryonic lethality, 50% in mixed C57BL/6 x SV129 and 90% in pure C57BL/6	Normal embryonic development.

Table 1. Phenotype comparison of Smad3-null and TGF-β_1-null mice.

TGF-β1$^{-/-}$ mice	Smad3$^{-/-}$ mice
Strain-dependent defects in yolk sac vasculogenesis and embryonic lethality 50% in mixed C57B1/6 x SV129 and 99% in pure C57B1/6 backgrounds.	Normal embryonic development.
Normal thymocyte maturation during neonatal period. Decreased cellularity and reduced CD4$^+$ CD8$^+$ double positive progenitors with increased CD4$^+$ single positive cells in symptomatic mice.	Normal thymocyte maturation, with cortical atrophy and decreased thymic cellularity in symptomatic mice.
Peripheral lymphoid tissue with activated T cells, increased mitotic indices. Sensitive to growth inhibition by TGF-β_1.	Peripheral T cells show activated phenotype, increased proliferation, and lack growth arrest response to TGF-β.
Autoimmune syndrome with pathogenic IgG autoantibodies, immune complex deposition. Reversed by *in vivo* depletion of CD4$^+$ or CD8$^+$ T cells, and in immune deficient backgrounds.	No evidence for autoimmunity. Severe mucosal abscess formation, containing Providencia bacterial strains.
Decreased B220$^+$ B cells in peripheral lymphoid organs and bone marrow. Accumulation of plasma cells in lymph nodes and bone marrow. Reduced IgA producing cells in mucosa and increased serum IgE. Cultured B lymphocytes retain responsiveness to TGF-β.	Normal B cell development. Increased plasma cells in lymph nodes of symptomatic mice. Normal numbers of IgA positive B cells in intestinal mucosa. LPS activated B cells retain sensitivity to growth inhibition by TGF-β.
Myeloid hyperplasia; increased circulating monocytes, neutrophils, and platelets, and marrow megakaryocytes. Platelet aggregation defect.	Increased circulating granulocytes and monocytes in mice with mucosal infection. Platelet function not evaluated.
Normal chemotaxis to TGF-β.	Defective chemotaxis to TGF-β in monocytes, keratinocytes and neutrophils.
Death by 4 weeks of age.	Survival varies according to model, strain, and severity of mucosal disease.

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al. 1999; Yang *et al.* 1999). Neutrophil and macrophoge chemotactic responsiveness to TGF-β_1 are also significantly impaired (Ashcroft *et al.* 1999; Yang *et al.* 1999). Smad3-null T cells display an activated *in vivo* phenotype and are unresponsive to TGF-β_1-induced growth inhibition *in vitro* (Datto *et al.* 1999; Yang *et al.* 1999). In contrast, growth inhibition by TGF-β_1 *in vitro* is unaffected in LPS (lipopolysaccharide)-activated Smad3-null B cells (Datto *et al.* 1999; Yang *et al.* 1999). Normal hematopoiesis and an apparent undisrupted marrow response to ongoing infections in these mice suggest that the breakdown of mucosal immune response is not due to defective myloid or lymphoid cell development (Yang *et al.* 1999). In contrast to TGF-β_1 deficiency, Smad3-null mice present no evidence for autoimmunity (Yang *et al.* 1999). In further contrast to the TGF-β_1-null mice, B cell development and IgA production *in vivo* are seemingly unaffected with deletion of the Smad3 gene (Yang *et al.* 1999). Smad2 and Smad4 null mice die during embryogenesis (Dunker and Krieglstein 2000; Waldrip *et al.* 1998).

A second line of evidence for a role of Smad3 in immune cells stems from functional cooperation between Smad3 and the transcription factor, AML (adult myeloid leukemia) in regulating TGF-β_1 responsiveness (Kurokawa *et al.* 1998a; Kurokawa *et al.* 1998b). AML1/Evi-1 transcriptional activity regulates TGF-β_1-mediated myeloid cell growth inhibition and plays a profound functional role in leukemogenesis (Imai *et al.* 2001; Izutsu *et al.* 2001).

Additionally, Smad3 has been implicated in the transcriptional regulation of germline Igα by TGF-β_1. Transcriptional regulation of Igα by TGF-β_1 is mediated through a proximal promoter/enhancer region located approximately -130 bp upstream of the human and mouse intron (I) α transcription start site. This sequence contains an array

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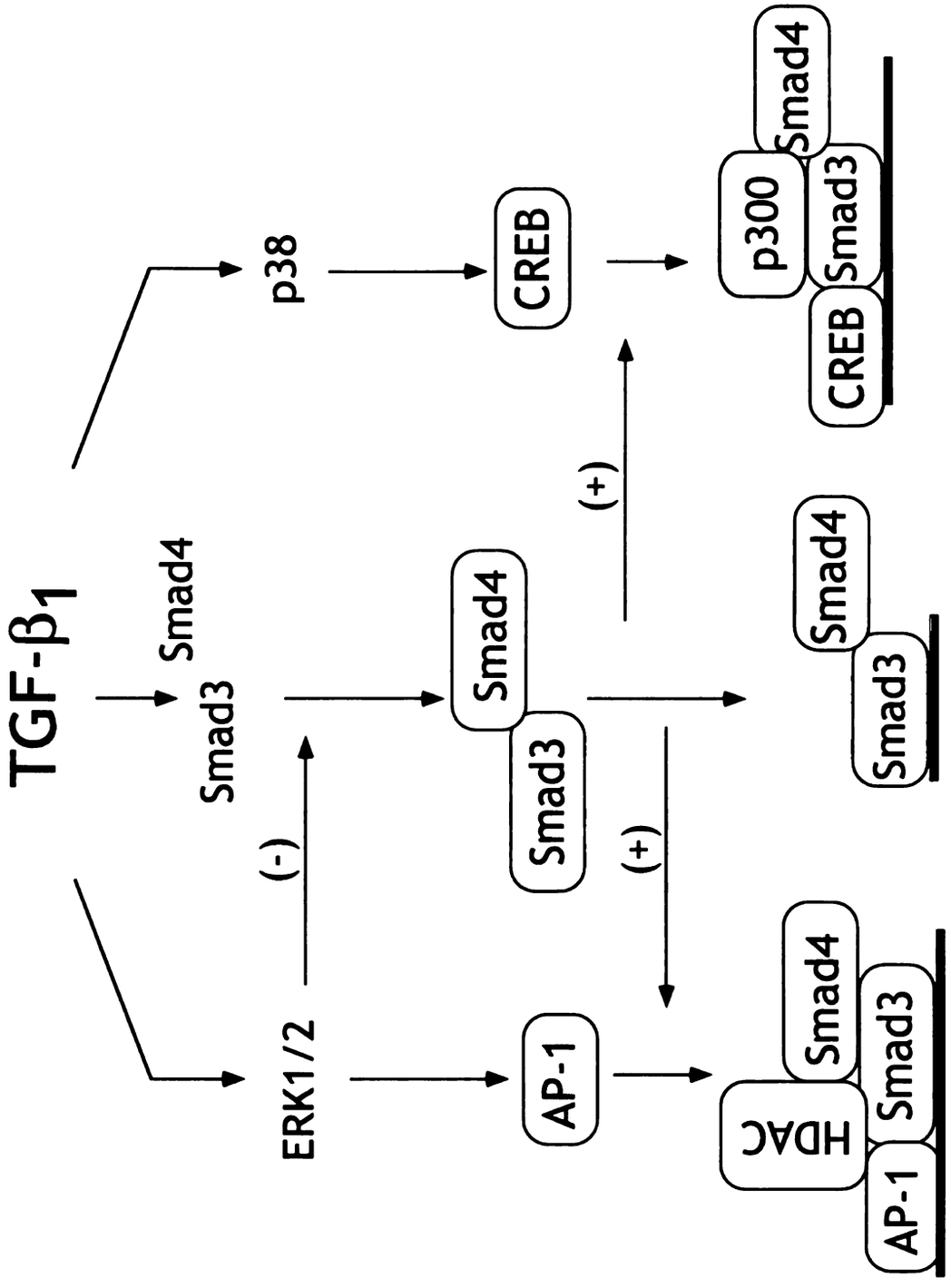
of interspersed Smad, acute myloid leukemia (AML), and cAMP-response element-binding protein (CREB) response elements that cooperatively, via protein•protein and protein•DNA interactions, mediate *in vitro* TGF-β_1 transcriptional responsiveness (Pardali *et al.* 2000a; Zhang and Derynck 2000). *In vitro* EMSA analyses using GST (glutathione-*s*-transferase)-fusion proteins have revealed direct binding of both Smad3 and Smad4 to the CAGA elements within this region (Zhang and Derynck 2000). Furthermore, in the presence of TGF-β_1, over-expression of Smad3 and Smad4 selectively increases both surface IgA expression and IgA production by murine B lymphoma cells *in vitro* (Park *et al.* 2001). Collectively, these results provide direct evidence that Smad3 plays a role in immune cell responsiveness to TGF-β_1 *in vitro* and *in vivo*.

VII. Regulatory cross talk between Smad and MAP kinase Signaling Cascades

Significant progress has been made in elucidating intracellular signaling pathways and establishing the biochemical moieties that are activated by individual ligand•receptor interactions. More recently, it has become apparent that synergistic as well as antagonistic cross talk at multiple hierarchical levels among individual pathways influences the overall physiological significance of ligand binding. In support of this, TGF-β_1-induced activation of the TβR complex initiates signaling through multiple non-Smad-mediated signaling cascades (**Figure 10**). For example, TGF-β_1 upregulates p38 MAPK and ERK MAPK signaling in multiple epithelial and fibroblast cell models (Fanger 1999; Mulder 2000; Terada *et al.* 1999; Yue and Mulder 2000). The putative regulatory crosstalk between TGF-β_1-activated Smad and TGF-β_1-activated MAPK

Figure 10. Putative regulatory cross talk between Smad signaling and MAPK signaling cascades. TGF-β_1-induced biological activity is likely to be regulated through cross talk between multiple signaling pathways that are upregulated with ligation of the TGF-β receptor complex. Illustrated here are examples of positive and negative regulation between Smad and MAPK signaling.

TGF-β_1


TGF-β1
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pathways is an intense focus of current research. It has been demonstrated that ATF-2 oligomerizes with Smad3 and Smad4 in a TGF-β_1-dependent manner (Hanafusa *et al.* 1999; Hocevar *et al.* 1999; Sano *et al.* 1999). ATF-2, a member of the CREB family that binds to the cAMP response element, is activated by TcR-induced p38 MAPK-mediated phosphorylation (Zhang *et al.* 1999a). Moreover, Smad3 and Smad4 also complex with CBP and p300, two co-activator accessory molecules that also hetero-oligomerize with ATF-2 to augment the stability of ATF-2 binding to the CRE (Nishihara *et al.* 1999; Nishihara *et al.* 1998). Synergism of ATF-2 transcriptional activity has been confirmed using the TGF-β_1-responsive p3TP-Lux reporter plasmid (Hanafusa *et al.* 1999). Collectively, these results demonstrate that Smad and p38 MAPK signaling act in a synergistic manner to enhance TGF-β_1-responsive transcriptional activity. These results are particularly interesting in light of the fact that TcR ligation also induces p38 MAPK activity and ATF-2 binding to DNA response elements in the IL-2 promoter.

A second line of evidence suggesting Smad and MAPK signaling crosstalk is evident from oncogenic Ras-mediated inhibition of Smad nuclear translocation. Specifically, prolonged Ras-induced ERK MAPK activation has been demonstrated to result in phosphorylation of Smad2 and Smad3 at four Ser/Thr-Pro sites located within linker region, resulting in the inability of Smad2 and Smad3 to dimerize with Smad4 (**Figure 8**) (Kretzschmar *et al.* 1999; Ulloa *et al.* 1999). Mutation of these Ser/Thr-Pro ERK MAPK phoshorylation sites confers responsiveness to TGF-β_1 in the presence of oncogenic Ras (Kretzschmar *et al.* 1999). Transcriptional modulation of CREB and AP-1 *trans*-acting factors by Smads through direct protein•protein interactions provides a third distinct line of evidence implicating crosstalk between Smad and MAPK pathways

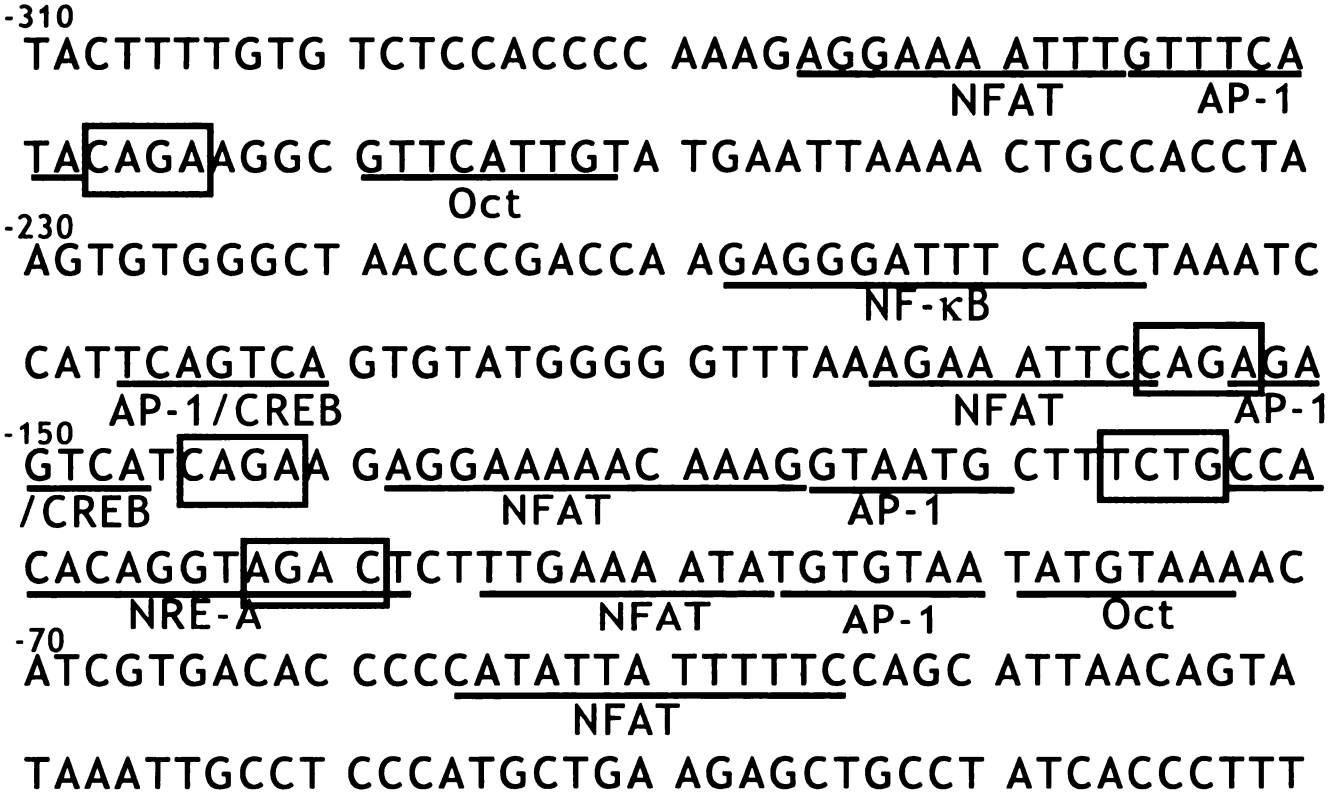
(Dennler *et al.* 1998; Dennler *et al.* 2000; Sano *et al.* 1999; Wong *et al.* 1999). Collectively, these data suggest that Smad regulation by MAPKs may be a general phenomenon for controlling TGF-β_1-mediated signaling.

VIII. Objective and specific aims

TGF-β_1 is a multifunctional cytokine that plays a profound role in maintaining a physiological balance between positive and negative regulatory signals essential for immune homeostasis. Experimental and clinical data suggest that disruption of TGF-β_1 contributes to aberrant pathogenic immune and immunological responses. One of the cell populations that is strongly regulated by TGF-β_1 is the T lymphocyte. Direct modulation of T cell activation and clonal expansion is one mechanism by which TGF-β_1 elicits profound regulatory control of T cell-dependent humoral immunity. The mechanisms underlying the effects of TGF-β_1 on T cells remains elusive. The overall goal of this research is to elucidate the mechanisms underlying the ability of TGF-β_1 modulate T cell effector function differently. Towards this end, the first objective was to establish an *in vitro* model to characterize the effects of TGF-β_1 on T cell activation and proliferation.

Smads, a novel family of transcription factors, have recently been identified as TGF-β_1-inducible intracellular signaling mediators. A role for Smads in lymphocytes has not been well characterized. CAGA DNA sequences have been identified as Smad3 response elements. We have identified five CAGA sites in the 5' minimal essential regulatory region of the mouse IL-2 promoter (**Figure 11**). Thus, the second objective was to determine whether Smads play a role in regulation of IL-2 by TGF-β_1.

Figure 11. Identification of CAGA sequences in the 5' minimal essential regulatory region of the mouse IL-2 promoter. Smad3 acts as a transcription factor by binding to sequence specific DNA motifs located in the promoter of TGF-β_1-responsive genes. Five CAGA sequences have been identified within the minimal essential regulatory region of the mouse IL-2 promoter. As illustrated, each CAGA sequences is located adjacent to or overlaps a DNA binding element for another transcription factor(s) that regulate IL-2 transcription including NFAT, NF-κB, AP-1, CREB and ZEB. It is hypothesized that the CAGA "Smad boxes" are sequence specific DNA binding motifs for Smad3 in the IL-2 promoter.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NF-κB
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In addition to Smad signaling, MAPKs are also activated downstream of ligand binding to the TGF-β receptor. Crosstalk between these two signaling pathways has been established in several cell types. Our preliminary results implicate activation of MAPKs by TGF-β_1 in T cells. Thus the final objective of this research was to determine whether signaling cross talk between Smad and MAPK pathways plays a regulatory role in IL-2 expression by TGF-β_1 in T cells. The overall working hypothesis for this is as follows:

HYPOTHESIS:

TGF-β_1 acts directly on T cells to modulate IL-2 expression positively and negatively in a concentration-dependent manner. TGF-β receptor serine/threonine kinases activate Smads, which translocate into the nucleus to modify AP-1, CREB, NFAT, NF-κB, and ZEB transcription factor activity at their respective response elements located within the regulatory region of the IL-2 promoter. The concentration-dependent bifunctional effect of TGF-β_1 on IL-2 expression in α-CD3 + α-CD28-activated splenic T cells is, in part, resultant of regulatory cross talk between Smad and MAPK signaling.

VIV. Relevance

TGF-β_1 is a growth factor that plays a profound role in establishing normal tissue and organ development. TGF-β_1 also plays a critical role in defending the body against toxicity by regulating immune responses, wound repair, and tissue regeneration following xenobiotic-induced insult. The mechanisms underlying these TGF-β_1-mediated effects remain an enigma. The studies herein provide critical insight regarding the putative

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MATERIALS AND METHODS

I. Animals

Pathogen-free female B6C3F1 mice, 6 weeks of age, were purchased from Charles River Breeding (Portage, MI). On arrival, mice were randomized, transferred to plastic cages containing sawdust bedding (five animals/cage) and quarantined for a week. Mice homozygous for a null mutation in the gene encoding the TGF-β receptor-activated Smad3 ($Smad3^{-/-}$) and age-matched wild type littermates ($Smad3^{+/+}$) were a generous gift from Dr. John Letterio (National Cancer Institute, Bethesda, MD). $Smad3^{-/-}$ mice were produced by disrupting exon 8 of the SMAD3 gene in embryonic stem cells using homologous recombination (Yang *et al.* 1999). $SMAD3^{+/+}$ mice were generated by mating $Smad3^{+/-}$ mice; genotypes were analyzed by PCR using tail DNA. Mice were provided Purina Certified Laboratory Chow and water *ad lib* and housed at 21-24°C and 40-60% relative humidity with a 12-h light/dark cycle.

II. Cell lines

The C57BL/6 mouse T cell lymphoma EL-4 cells were a gift from Dr. Atsuo Ochi (Ontario, Canada). The human T cell leukemic Jurkat E6-1 cell line was purchased from the American Type Tissue Culture Collection (Rockville, MD). EL-4 and Jurkat cells were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 5% BCS (Hyclone, Logan, UT), 100 U penicillin/mL, 100 U streptomycin/mL, 50 μM 2-mercaptoethanol, and 2 mM L-glutamine (RBI/Sigma, St. Louis, MO) and incubated at 37°C in a humidified atmosphere of 5%-CO_2-95% air. Cells

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were passaged as needed to maintain log phase growth. The EL-4 cells were stimulated with 10 μg/mL plate-bound anti-mouse CD3ε (hamster monoclonal IgG, clone 145-2C11) + 10 μg/mL plate-bound anti-mouse CD28 (hamster monoclonal IgG, clone 37.51). The Jurkat cells were stimulated with 10 μg/mL plate-bound anti-human CD3ε (mouse monoclonal IgG, clone UCHT1) + 10 μg/mL plate-bound anti-human CD28 (mouse monoclonal IgG, clone CD28.2). CD3 and CD28 antibodies were purchased from PharMingen (San Diego, CA). Alternatively, in some experiments, EL-4 and Jurkat cells were stimulated with PMA/Io (80 nM/1 μM).

III. TGF-β_1 (Transforming growth factor-beta$_1$)

Biologically active recombinant human TGF-β_1, purchased from R & D Systems (Minneapolis, MN), was reconstituted in 1X PBS supplemented with 4 mM HCl and 0.1% BSA to prepare a 2 μg/mL stock solution. Aliquots of the reconstituted TGF-β_1 were stored at 80°C until use.

IV. Chemicals

PMA (phorbol-12-myristate-13 acetate), Io (ionomycin), PD98059, and UO126 were purchased from RBI/Sigma. PMA and Io were prepared as 10 mM stock solutions and stored at -80°C. A 1000X PMA/Io working solution (80 μM PMA/1 mM Io) was prepared by combining PMA and Io stock solutions in DMSO (dimethylsulfoxide). PD98059 and UO126 were prepared as 1000X stock solutions in DMSO. Aliquots of all stock concentrations of reagents were stored at -80°C until use. Working solutions were prepared fresh just prior to each experiment.

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V. Antibodies

The antibodies used in Western immunoblot analyses were as follows: The mouse monoclonal anti-Smad3 (H-2), recognizing amino acids 1-465, the goat polyclonal anti-Smad2/Smad3 (N-19), recognizing the amino terminal domain, the mouse monoclonal anti-Smad4 (B-8), recognizing amino acids 1-552, and the goat polyclonal anti-Smad2 (H-20) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse antisera specifically recognizing Smad2, Smad3, or Smad4 were gifts from Dr. Peter ten Dijke (Amsterdam, The Netherlands). Rabbit polyclonal anti-Smad2 was purchased from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal anti-phospho-ERK1/ERK2 MAPK, rabbit polyclonal anti-phospho JNK MAPK, and rabbit polyclonal anti-phospho or total MEK1/MEK2 were purchased from Promega (Madison, WI).

VI. Oligonucleotides

A total of eight oligonucleotides corresponding to four α-CD3-inducible DNA response elements in the IL-2 promoter were synthesized at Michigan State University (Macromolecular Structure, Biochemistry Department). Olignucleotides representing the naïve and CAGA-mutated proximal AP-1, CD28RE, NRE-A, and distal NFAT response elements were annealed to their complementary strand and 5'-end-labeled with [γ-^{32}P]-dATP (NEN, Boston, MA) using T4 kinase (Pharmacia Biotech, Piscataway, NJ). The sequence for each probe is provided in **Table 2**. The naïve CAGA sequences are indicated by a single underline, and the mutated CAGA sequences are indicated by a double underline.

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Table 2. Oligonucleotides used for EMSA analyses*

DNA Binding Site	DNA Sequence
Proximal AP-1	AATTCCAGAGAGTCATCAGAAGA
Mutated proximal AP-1	AATTCTTATGAGTCATTTATAGA
CD28RE	TTAAAGAAATTCCAGAGAGTCATCAGAAGA
Mutated CD28RE	TTAAAGA AATTCTTATGAGTCATTTATAGA
NRE	ATAGCTTTCTGCCACAGGTAGACTCTTTG
Mutated NRE	ATAGCTTTTTACCACAGGTTATTTCTTTG
Distal NFAT	AGAGGAAAATTTGTTTCATACAGAAGGCGT
Mutated NFAT	AGAGGAAAATTTGTTTCATATTATAGGCGT

*The oligonucleotides used for EMSA experiments. The naïve CAGA sequences are indicated by a single underline, and the mutated CAGA sequences are indicated by a double underline.

VII. Reporter Gene Plasmids

Plasmids containing SEAP (secreted alkaline phosphatase) vectors driven by either NFAT or NF-κB reporter genes were purchased from Clontech (Palo Alto, CA). These reporter plasmids were used in transient transfection studies to assess regulation of NFAT and NF-κB transcription in response to TGF-β_1. The NFAT reporter plasmid is under the regulatory control of three copies of NFAT, and the NF-κB reporter plasmid is under the regulatory control of four copies of NF-κB. The pSVB-β-galactosidase (Promega) control vector was used as a control for transfection efficiency.

VIII. Isolation and *In Vitro* Activation of Primary Lymphocytes

Mice were sacrificed via cervical dislocation and spleens or thymi were aseptically removed. Single cell suspensions were prepared by isolating the lymphocytes from the spleen or thymus. The cells were then washed in RPMI 1640 media followed by centrifugation at 200 x g for 5 minutes. Cell counts were obtained using a Coulter Particle Counter Z1 (lower threshold = 4 μm) and cell density was adjusted to $5x10^6$ cells/mL unless otherwise specified. Cells were cultured in RPMI 1640 (Gibco, Gaithersburg, MD) supplemented with 100 U penicillin/mL, 100 U streptomycin/mL, 50 μM 2-mercaptoethanol, and 1% BCS (HyClone, Logan, UT) unless otherwise specified. Splenocytes and thymocytes were cultured in tissue culture plates that were pre-coated (overnight) with 2 μg/mL α-CD3ε (PharMingen). Co-stimulation was provided with soluble or plate-bound α-CD28 (1 μg/mL) as indicated. Alternatively, cells were activated with PMA/Io (80 nM/1 μM). All cell cultures were maintained at 37°C in a humidified atmosphere.

$CD90^+$ (Thy1.2) T cells were isolated using murine CD90-specific magnetic Microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Flow cytometric analysis of the positively selected purified cells subsequently stained with CD90-FITC (Miltenyi Biotec) indicated >98% purity. Briefly, a pooled single cell suspension was isolated from the spleens of four animals, washed in labeling buffer, centrifuged at 300 x g for 10 min and resuspended in 90 µL per 10^7 total cells. A 10 µL aliquot of MACs CD90 Microbeads (Miltenyi Biotec) were added to the cell suspension and incubated for 15 min at 12°C. The cell suspension was applied to a LS^+/VS^+ positive selection column (Miltenyi Biotec). After the negative cells were allowed to pass through the column, the column was rinsed, removed from the separator, and placed into a collection tube. The positive cells were removed by adding 5 mL of LS^+/VS^+ buffer and gently forcing the contents of the column with a plunger.

IX. *In vitro* Proliferation Assays

Single cell splenocyte or thymocyte preparations were cultured in 96 well culture plates at 2.5 x 10^6 cells/mL in the presence or absence of TGF-β_1. T cells were activated with plate-bound α-CD3 (2 µg/mL) + α-CD28 (1 µg/mL) or LPS (10 µg/mL). Cells were cultured at 37°C and 5% CO_2 for 48 hours (LPS) or 72 hours (α-CD3/α-CD28) and pulsed with 1.0 µCi/well of [^{3}H]-thymidine (NEN, Boston, MA) for the last 16 hours of culture. Cells were harvested onto glass fiber filters with a PHD Cell Harvester (Cambridge Technology, Inc., Cambridge, MA), and tritium incorporation was quantified using a Packard 460C liquid scintillation counter.

X. ELISA (Enzyme-Linked Immunosorbent Assay)

A. Mouse IL-2 ELISA

Splenocytes or thymocytes were cultured in triplicate (5 x 10^6 cells/mL) in 48 well culture plates or in quadruplicate (5 x 10^6 cells/mL) in 96 well plates for 24 hours. Supernatants were collected and quantified for secreted mouse IL-2 by sandwich ELISA. Immulon IV Removawell microtiter strip wells (Dynex Technologies, Inc., Chantilly, VA) were coated overnight at 4°C with 50 µl of purified rat α-mouse IL-2 antibody (1.0 µg/mL) in 0.1 M sodium bicarbonate buffer (pH 8.2). Blocking buffer, 300 µl of BSA (bovine serum albumin) (3% v/v) in 0.01 M PBS (phosphate buffered saline) containing 0.1% (v/v) Tween 20 (BSA-PBST), was added to each well and incubated at 37°C for 30 minutes. Wells were washed four times with PBST followed by addition of IL-2 standard or sample (50 µl) and incubated at 37°C for one hour. After incubation, the plate was washed four times with PBST and once with distilled water. The biotinylated anti-mouse IL-2 (1.5 µl/mL), diluted in 3% BSA-PBST, was added to each well (50 µl) and incubated at room temperature for one hour. The plate was washed six times using the PBST solution and once with distilled water followed by addition of 50 µl streptavidin-horseradish peroxidase (1.5 µg/mL) for one hour at room temperature. Samples were then washed eight times and the bound peroxidase conjugate was detected by addition of a substrate solution (100 µl/well) containing 0.1 M citric-phosphate buffer (pH 5.5), 0.1 mg/mL TMB (tetramethylbenzidine) (Fluka Chemical Corp., Ronkonkoma, NY) and 1% H_2O_2. The reaction was terminated with an equal volume of 6N H_2SO_4, and absorbance was quantified at 450 nm using an EL808 automated microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). A standard curve for each assay was

generated using recombinant mouse IL-2 (PharMingen). The DeltaSoft 3-computer analysis program (BioMetallics, Princeton, NJ) was used to quantify secreted IL-2.

B. Mouse IL-4 ELISA

Secreted mouse IL-4 was quantified as described for mouse IL-2 with the following modifications. Immulon IV Removawell microtiter strip wells were coated overnight at 4°C with 50 µl of purified rat anti-mouse IL-4 antibody (1.0 µg/mL) in 0.1 M sodium bicarbonate buffer (pH 8.2). Wells were washed four times with PBST prior to the addition of IL-4 standard or sample (100 µl) and incubated at 37°C for one hour. Biotinylated anti-mouse IL-4 (1.5 µl/mL), diluted in 3% BSA-PBST, was added to each well (50 µl) and incubated at room temperature for one hour. The reaction was terminated with an equal volume of 6N H_2SO_4, and absorbance was quantified at 450 nm using an EL808 automated microplate reader (Bio-Tek Instruments, Inc). A standard curve for each assay was generated using recombinant mouse IL-4 (PharMingen). The DeltaSoft 3-computer analysis program (BioMetallics) was used to quantify secreted IL-4.

C. Mouse IFN-γ (interferon gamma) ELISA

Secreted mouse IFN-γ was quantified as described for mouse IL-2 with the following modifications. Immulon IV Removawell microtiter strip wells were coated overnight at 4°C with 50 µl of purified rat anti-mouse IFN-γ antibody (1.0 µg/mL) in 0.1 M sodium bicarbonate buffer (pH 8.2). Wells were washed four times with PBST prior to the addition of IFN-γ standard or sample (50 µl) and incubated at 37°C for one hour. Biotinylated anti-mouse IFN-γ (1.5 µl/mL), diluted in 3% BSA-PBST, was added to each well (50 µl) and incubated at room temperature for one hour. The reaction was

terminated with an equal volume of 6N H_2SO_4, and absorbance was quantified at 450 nm using an EL808 automated microplate reader (Bio-Tek Instruments, Inc.). A standard curve for each assay was generated using recombinant mouse IFN-γ (PharMingen). The DeltaSoft 3-computer analysis program (BioMetallics) was used to quantify secreted IFN-γ.

D. Human IL-2 ELISA

Secreted human IL-2 was quantified as described for mouse IL-2 with the following modifications. Immulon IV Removawell microtiter strip wells were coated overnight at 4°C with 50 µl of purified rat α-human IL-2 antibody (1.0 µg/mL) in 0.1 M sodium bicarbonate buffer (pH 8.2). Wells were washed four times with PBST prior to the addition of human IL-2 standard or sample (50 µl) and incubated at 37°C for two hours. Biotinylated anti-human IL-2 (1.5 µl/mL), diluted in 3% BSA-PBST, was added to each well (50 µl) and incubated at room temperature for two hours. A standard curve for each assay was generated using recombinant human IL-2 (PharMingen). The DeltaSoft 3-computer analysis program (BioMetallics) was used to quantify secreted IL-2.

E. Mouse IgM ELISA

Immulon IV Removawell microtiter strip wells (Dynex Technologies, Inc., Chantilly, VA) were coated overnight at 4°C with 50 µl with anti-mouse immunoglobulin (Ig) capture antibody (Boehringer Mannheim, Indianapolis, IN) in 0.1 M sodium bicarbonate buffer (pH 8.2). Blocking buffer, 300 µl of BSA (3% v/v) in 0.01 M PBS containing 0.1% (v/v) Tween 20 (BSA-PBST), was added to each well and incubated at

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37°C for 30 minutes. Wells were washed 4 times with PBST followed by addition of sample (100 µl) of supernatant and incubated at 37°C for 90 minutes. After incubation, the plate was washed four times with PBST and once with distilled water. Horseradish peroxidase conjugated-anti-mouse IgM detection antibody (Sigma, St. Louis, MO) was added to each well and incubated at 37°C for 90 minutes. The plate was washed 6 times using the PBST solution and once with distilled water to remove unbound antibody. ABTS (2,2'-azinobis (3-ethybenzthiazoline-sulfonic acid) substrate (Boehringer Mannheim, Indianapolis, IN) was added and the rate of colorimetric change was monitored at 405 nm for 60 minutes using an EL808 automated microplate reader (Bio-Tek Instruments, Inc.) Secreted IgM concentrations were generated from the standard curve generated from known IgM concentrations using the DeltaSoft 3-computer analysis program (BioMetallics). The pronase viability assay was used to determine the number of viable splenocytes/well recovered following incubation. Results from quadruplicate cultures are expressed as the mean µg IgM/10^6 recovered viable splenocytes ± SEM.

F. Mouse IgA ELISA

Immulon II Removawell microtiter strip wells (Dynex Technologies, Inc., Chantilly, VA) were coated overnight at 4°C with 50 µl purified rat anti-mouse IgA antibody (1.0 µg/mL) in 0.1 M sodium bicarbonate buffer (pH 8.2). Blocking buffer, 300 µl of BSA (3% v/v) in 0.01 M PBS containing 0.1% (v/v) Tween 20 (BSA-PBST), was added to each well and incubated at 37°C for 30 minutes. Wells were washed four times with PBST followed by addition of sample (100 µl) of supernatant and incubated at 37°C for 90 minutes. The standard curve was generated using mouse recombinant IgA, (Sigma, St. Louis, MO). After the incubation period, the wells were washed with PBST,

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and 50 µl biotinylated anti-mouse IgA in 3% BSA-PBST (1.5 µl/mL) was added to each well for 60 minutes at 37°C. The wells were washed with PBST solution, and 50 µl streptavidin-peroxidase (1.5 µg/mL) was added for one hour at room temperature. Following a final PBST wash, bound peroxidase conjugate was detected by addition of a substrate solution (100 µl/well) containing 0.1 M citric-phosphate buffer (pH 5.5), 0.1 mg/mL TMB, and 1% H_2O_2. The reaction was terminated with an equal volume of 6N H_2SO_4, and absorbance was measured at 450 nm using a microplate reader (Bio-Tek Instruments, Inc.). IgA concentrations were generated from the standard curve generated from known IgA concentrations using the DeltaSoft 3-computer analysis program (BioMetallics). The pronase viability assay was used to determine the number of viable splenocytes/well recovered following incubation. Results from quadruplicate cultures are expressed as the mean ng IgA/10^6 recovered viable splenocytes $\pm$ SEM.

XI. Quantitative RT-PCR (Reverse Transcriptase-Polymerase Chain Reaction)

A. Preparation of mouse IL-2 Internal Standard for RT-PCR

A recombinant IL-2 IS (internal standard) was generated using a rat β-globin sequence as the spacer gene as described (Condie *et al.* 1996). The design of the IS primers used was (5' to 3'): IS forward primer = T7 promoter (TAATACGACTCACTATAGG), IL-2 forward primer (TGCTCCTTGTCAACAGCG), and rat β-globin forward primer (GGTGCTTGGAGACAGAGGTC); and IS reverse primer = $(dT)_{18}$, IL-2 reverse primer (TCATCATCGAATTGGCACTC), and rat β-globin reverse primer (TCCTGTCAACAATCCACAGG). PCR conditions for making the internal standard were performed using 100 ng of rat genomic DNA as described

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(Vanden Heuvel *et al.* 1993). Purification of PCR products was performed using the Wizard PCR Prep DNA purification system (Promega, Madison, WI), and was followed by transcription of the products into RNA using Promega's Gemini II *In Vitro* Transcription System. The IS was treated with RNase-free DNase to remove the DNA template.

B. Quantitative Competitive RT-PCR for mouse IL-2

Total RNA was isolated using TriReagent (Sigma, St. Louis, MO) for competitive RT-PCR. One hundred ng total RNA and known amounts of IS rcRNA were reverse-transcribed into cDNA using oligo(dT)$_{15}$ as primers. A PCR reaction buffer, (4 mM MgCl2, 6 pmol of IL-2 forward and reverse primers, and 2.5 units of Taq DNA polymerase) was added to the cDNA samples. Samples were heated to 94°C for 15 seconds, 55°C for 30 seconds, and 72°C for 30 seconds followed by a single extension step at 72°C for 5 minutes. Thirty-five cycles were used to amplify the IL-2 PCR product, which were then electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining; the IL-2 product generated from the sample RNA was 391 bp and the IS product was 474 bp. A Gel Doc 1000 video imaging system (BioRad, Hercules, CA) was used to quantify the 391 bp and 474 bp bands. The number of transcripts was calculated from a standard curve generated from the density ratio between the gene of interest (IL-2) and varying known amounts of IS.

XII. Protein Extractions

A. Whole Cell

Cells were cultured in 60 mm^2 tissue culture plates at a density of $5x10^6$ cells/mL (5 mL/plate). For whole cell protein extraction, cells were lysed in 500 µl of RIPA buffer (1x PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) supplemented with 1mM DTT, 1 mM PMSF, and 1 µg/mL of aprotinin and leupeptin, homogenized with a dounce homogenizer (30 strokes), and incubated on ice for 30 minutes. Following lysis, samples were centrifuged at 17,500 x g for 20 minutes and the supernatant retained as whole cell protein and stored at -80°C until use.

B. Nuclear

Cells were cultured in 60 mm^2 tissue culture plates at a density of $5x10^6$ cells/mL (5 mL/plate). Cells were lysed with a hypotonic buffer (10 mM HEPES and 1.5 mM $MgCl_2$, 1mM DTT, 1mM PMSF, and 1 µg/mL of aprotinin and leupeptin) and the nuclei were pelleted by centrifugation at 6,700 x g for 5 minutes. Nuclei were lysed in hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM NaCl, 0.3 mM EDTA, and 10% glycerol) supplemented with 1mM DTT, 1 mM PMSF, and 1 µg/mL of aprotinin and leupeptin for 30 minutes on ice. Following nuclear lysis, the samples were centrifuged at 17,500 x g for 15 minutes, and the supernatant was retained and stored at -80°C until use.

C. Cytosolic

During isolation of nuclear proteins, the supernatants from the hypertonic lysis were retained for cytosolic protein extraction, whereas the pellet was used to extract nuclear proteins. Briefly, the supernatant was centrifuged for one hour at 100,000 x g at

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4°C. Glycerol was added to the resultant supernatant to 10% final concentration. Samples were stored at -80°C until use.

XIII. Protein Determination

Protein determination was performed using a BCA protein assay kit (Sigma, St. Louis, MO). A standard curve was prepared using increasing concentrations of BSA (0, 2.5, 5, 10, 15, and 20 μg) in a total volume of 100 μl. Unknown protein samples (5 μl) in a final volume of 100 μl were added to a 2 mL volume of bicinchoninic acid plus copper (II) sulfate, and incubated for 30 minutes at 37°C. Absorbance at 562 nm as measured using a Beckman DU-600 spectrophotometer (Fullerton, CA).

XIV. Western Immunoblotting

Proteins (25 μg) were incubated with 4x loading dye (62.5 mM Tris, 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 1% 2-mercaptoethanol) and heated for 10 minutes at 95°C. Samples were then resolved on a 10% SDS-PAGE gel and transferred overnight at 4°C to nitrocellulose in transfer buffer (24 mM Tris, 191 mM glycine, 20% methanol). The nitrocellulose was blocked for one hour with BSA-TBST (Tris buffered saline plus Tween 20) prior to incubation with the primary antibody. A corresponding Ig horseradish peroxidase-linked secondary antibody was used for protein detection using the ECL chemiluminescent system (Amersham, Arlington Heights, IL). Band intensity was quantified using a Gel Doc 1000 video imaging system (BioRad, Hercules, CA).

XV. EMSA (Electrophoretic Mobility Shift Assay)

Binding experiments were performed with 5 μg of nuclear extract and 20,000 cpm of [γ-^{32}P]-dATP-labelled oligonucleotide. The binding reactions were resolved by electrophoresis in 4% nondenaturing polyacrylamide gels in 0.5x TBE buffer (1X TBE: 89 mM Tris, 89 mM boric acid, and 2 mM EDTA). Nuclear extract were incubated in binding buffer (70-100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 0.05% Nonidet P-40, 1mM DTT, 1 mM PMSF, and 1 μg/mL of aprotinin and leupeptin) with 0.5-1.0 μg of poly (dI-dC). Following electrophoresis, the gel was dried and autoradiographed for analysis. Supershift experiments were performed by adding 1 μg of an anti-Smad3, Fos, or Jun antibody to the reaction mixture 20 minutes before electrophoresis.

XVI. Transient Transfections

Transient cell transfections of mouse T cell lymphoma EL-4 and human T cell leukemic Jurkat cells were performed using the Cytofectene Transfection Reagent (Bio-Rad, Melville, NY). Cells were resuspended (EL-4, 2 X 10^5 cells/mL; Jurkat, 5 X 10^6 cells/mL) in fresh medium (1X RPMI) containing 5% BCS and left overnight at 37°C. Cells were harvested, washed, resuspended (2 X 10^5 cells/mL) in transfection buffer (Bio-Rad) and incubated for one hour at 37°C. The pSV-β-galactosidase control vector was co-transfected in every experiment to monitor transfection efficiencies. The transfected cells were transferred to α-CD3 + α-CD28-pre-coated plates, treated with TGF-β_1, and incubated for an additional 23 hours at 37°C. Following incubation, supernatants were collected and stored at -80°C prior to use. Cells from each well were

lyzed for β-galactosidase activity. Briefly, cells were washed twice with PBS, and 50 µl of 1X Reporter Lysis Buffer (Promega) was added to each well of a 96 well plate. Samples were mixed by pipetting and incubated at room temperature for 15 minutes while rocking. Plates were centrifuged at 200 x g for 5 minutes to remove cellular debri and cleared lysates were transferred to clean eppendorf tubes and stored at -80°C prior to being assayed.

SEAP activity was quantified using a chemiluminescent detection kit (Clontech). Briefly, 60 µl supernatant from each well of a 96-well plate was pipetted into a clean eppendorf tube and incubated for 30 minutes at 65°C in a water bath. Samples were cooled by placing them on ice for 5 minutes, and then equilibrated to room temperature. A 60 µl aliquot of SEAP assay buffer (Clontech) was added to each sample and incubated at room temperature. Following 5 minutes, 60 µl of 62.5 µM CSPD (Clontech SEAP detection) substrate was added for 60 minutes at room temperature. Luminescence was quantified for 15 seconds using a Turner TD-20e luminometer.

The luciferase activity was normalized using the β-galactosidase activity. Briefly, 50 µl of 2X β-galactosidase assay buffer (Promega) was added to 50 µl cell lysate. Samples were mixed by pipetting, covered, and incubated overnight at room temperature on a rocker. Absorbance of the samples was read at 450 nm for 15 seconds using a Turner TD-20e luminometer.

XVII. *In vitro* AFC (Antibody Forming Cell Response)

Spleens from untreated mice were isolated aseptically and made into a single cell suspension. The splenocyte suspension was adjusted to 1 X 10^7 cells/mL (sRBC and DNP-Ficoll) or 5 X 10^6 cells/mL (LPS) in RPMI supplemented with 10% BCS

(Hyclone), 50 μM 2-ME, 100-units/mL penicillin, and 100 μg/mL streptomycin. Cell aliquots (500 μL) were transferred to a 48 well Costar culture plate (Cambridge, MA). Quadruplicate cultures were assayed for each treatment group. Five μl of vehicle (0.02 % PBS, pH 3.5, containing 0.1% BSA) or TGF-β_1 was added directly to the respective wells of the 48 well plates just prior to antigen sensitization. Respective wells were sensitized with 6.5 x 10^6/well sRBC, 50 μg/well LPS (Sigma), or 50 ng/mL DNP-Ficoll. Cells were subsequently cultured in a Bellco stainless steel tissue culture chamber pressurized to 6.0 psi with a gas mixture consisting of 10% O_2, 7% CO_2, and 83% N_2 for 5 days (sRBC and DNP-Ficoll) or 3 days (LPS). The culture chamber was placed on a rocking platform for the duration of the culture period.

Antibody producing cells were enumerated by their ability to hemolyze intact sRBC, when sRBC were used as the sensitizing antigen, or TNP-haptenated sRBC, when the sensitizing antigen was DNP-FICOLL or LPS as previously described (Kaminski and Stevens 1992). Sheep erythrocytes were haptenated with trinitrophenol (TNP) using picryl sulfonic acid (Sigma) as the source of TNP as previously described (Kaminski and Stevens 1992). Briefly, 5 mL of sRBC were centrifuged at 400 x g for 10 minutes and supernatant was removed. The sRBC were resuspended in 20 mL cacodylate buffer containing 4.0% picryl sulfonic acid and incubated at 37°C with gentle rocking for 10 minutes. The sRBC were centrifuged at 400 x g for 10 minutes, supernatant was removed, and the erythrocytes were resuspended in 50 mL cacodylate buffer containing 0.8% glycylglycine. Following three washes with EBSS (Earles' balanced salt solution), the TNP-haptenated sRBC were stored under sterile conditions at 4°C for up to one week.

Results from quadruplicate cultures were expressed as the mean AFC/10^6 recovered viable splenocytes ± SE. The pronase viability assay (as described below) was used to determine the number of viable splenocytes/well recovered following incubation.

XVIII. Pronase Viability Determination

Resuspended cells (100 µl) were added to an equal volume of pronase (225 proteolytic units/mL) (Calbiochem-Behring Corp., San Diego, CA) and incubated for 10 minutes at 37°C. Following incubation, the cell suspension was diluted with 10 mL of Isoton (Coulter, Addison, NJ) and counted on a Coulter counter. The percent viability was calculated by the following equation: (cell counts with pronase/cell counts without pronase) x 100 = viable cells.

XIX. Densitometry

The optical density of each treatment group was obtained using the Multi-Analyst program and a GS-700 imaging densitometer (BioRad, Hercules, CA). Using the density values, the ratio between the control and treated samples was calculated. The control group was designated with the value of 1.0 in order to assess qualitative changes between treatments. Alternatively, density was reported as optical value/mm^2.

XX. Statistical Analysis

The mean ± SE (standard error) was determined for each treatment group in the individual experiments. Homogeneous data were evaluated by a parametric analysis of variance, and Dunnett's two-tailed *t-test* was used to compare treatment groups to the vehicle control when significant differences were observed (Dunnett 1955).

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For the temporal relationship studies, IL-2 ELISA experiments were replicated three times using triplicate wells for each individual experiment. The cell proliferation experiments were replicated five times using quadruplicate wells for each individual experiment. The values for both control and experimental groups were pooled across experiments to yield an n=3 and n=5 for the IL-2 protein secretion and cell proliferation experiments, respectively. Specifically, (1) the data were transformed to a percent of the control, (2) the mean $\pm$ SE of the replicates from each experimental group with each experiment was calculated, and (3) the overall mean $\pm$ SE across experiments was calculated from the individual means. The data are expressed as a percent of the control. The results are reported as the overall mean $\pm$ SE. Statistical significance was determined at the $p < 0.05$ level using the Dunnett's two-tailed *t-test* to compare each experimental group with the untreated control.

EXPERIMENTAL RESULTS

I. Concentration- and time-dependent effects of TGF-β_1 on T cell proliferation and IL-2 expression

TGF-β_1 has been reported to attenuate as well as stimulate T cell growth (Cerwenka *et al.* 1994; Kehrl *et al.* 1986c; Rich *et al.* 1996; Swain *et al.* 1991b). IL-2 is a cytokine that is principally recognized for its ability to function as an autocrine growth factor in promoting T cell activation, differentiation, and clonal expansion (Gomez *et al.* 1998; Waldmann *et al.* 1998). Attenuated IL-2 production has been associated with impaired T cell growth (Gomez *et al.* 1998; Waldmann *et al.* 1998). TGF-β_1 reportedly augments as well as attenuates IL-2 expression (Cerwenka *et al.* 1994; D'Angeac *et al.* 1991). The mechanisms for these seemingly disparate effects by TGF-β_1 are unknown. The objective of this series of experiments was to test the hypothesis that the mode of T cell activation, the concentration of TGF-β_1, and the time of addition of TGF-β_1 relative to T cell activation influence the effects of TGF-β_1 on T cell growth and IL-2 expression.

A. Effects of TGF-β_1 on [^{3}H]-thymidine incorporation in mouse splenic T cells and thymocytes

As diagrammed in **Figure 12**, splenocytes and thymocytes were isolated from naïve B6C3F1 mice and activated in culture with α-CD3 + α-CD28 or α-CD3 alone. TGF-β_1 was added directly to the cell cultures concurrently with T cell activation or at various intervals either prior to or after T cell activation. [^{3}H]-thymidine incorporation was quantified following 72 hours of T cell activation. Interestingly, CD28 co-stimulation augmented α-CD3-induced splenic T cell growth (**Figure 13a**), but

Figure 12. Experimental design for characterizing the effects of TGF-β_1 on T cell activation and growth. The following variables were investigated: (1) target cell population, (2) mode of T cell activation, (3) TGF-β_1 concentration, and (4) temporal relationship between T cell activation and addition of TGF-β_1 to the cell cultures.

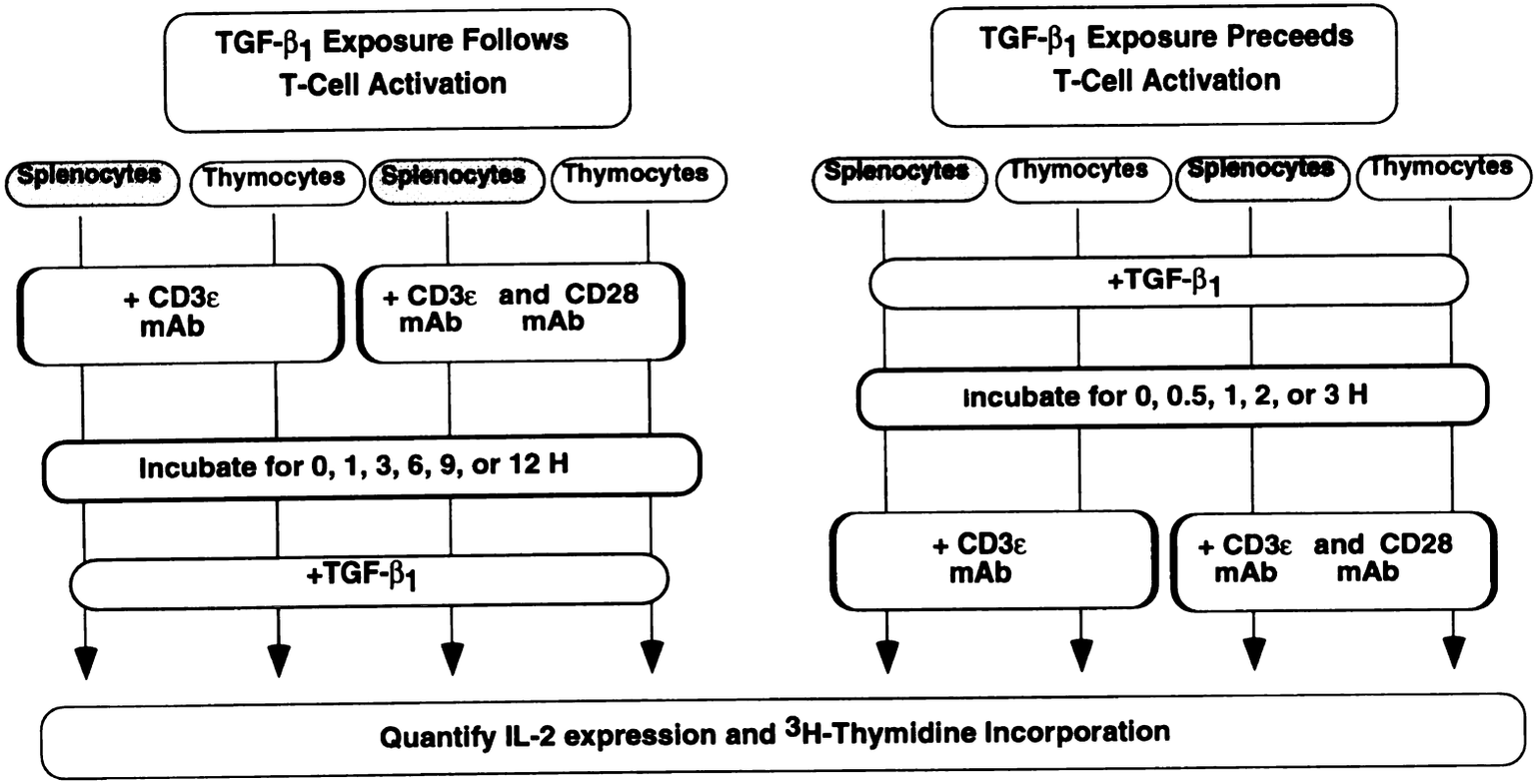
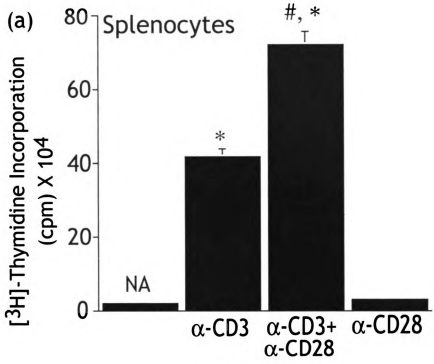
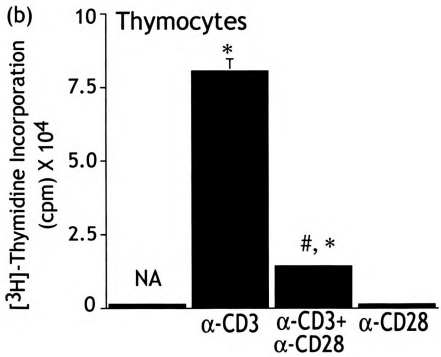

TGF-β1 Exposure Follows T-Cell Activation
Splenocytes
Thymocytes
Splenocytes
Thymocytes
+ CD3ε mAb
+ CD3ε mAb and CD28 mAb
Incubate for 0, 1, 3, 6, 9, or 12 H
+TGF-β1
TGF-β1 Exposure Preceeds T-Cell Activation
Splenocytes
Thymocytes
Splenocytes
Thymocytes
+TGF-β1
Incubate for 0, 0.5, 1, 2, or 3 H
+ CD3ε mAb
+ CD3ε mAb and CD28 mAb
Quantify IL-2 expression and 3H-Thymidine Incorporation

Figure 13. Proliferation of activated mouse splenic T cells and thymocytes. Naïve B6C3F1 splenocytes (a) or thymocytes (b) were cultured (2.5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation (Naïve) or in the presence of immobilized α-CD3 (2 μg/mL), immobilized α-CD3 (2 μg/mL) + soluble α-CD28 (1 μg/mL), or soluble α-CD28 (1 μg/mL), as indicated. Following a 56 hour incubation at 37°C, [^{3}H]-thymidine (1 μCi/mL) was added for a final 16 hour incubation. Cells were harvested and [^{3}H]-thymidine incorporation was quantified using a beta scintillation counter. The data are expressed as mean [^{3}H]-thymidine incorporation (cpm) ± SE for five experiments in quadruplicate. #,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the Naïve group. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the immobilized α-CD3 group.


(a)
Splenocytes
[3H]-Thymidine Incorporation (cpm) X 10^4
80
60
40
20
0
NA
*
#, *
α-CD3
α-CD3+ α-CD28
α-CD28


(b)
Thymocytes
[3H]-Thymidine Incorporation (cpm) X 10^4
10
7.5
5.0
2.5
0
NA
*
#, *
α-CD3
α-CD3+ α-CD28
α-CD28

attenuated α-CD3-activated thymocyte growth (**Figure 13b**). TGF-β_1, when added concurrently with T cell activation, inhibited α-CD3 + α-CD28-induced splenic T cell and thymocyte growth in a concentration-dependent manner (**Figure 14**). TGF-β_1 also inhibited α-CD3-induced splenic T cell and thymocyte growth in a concentration-dependent manner (**Figure 14**).

The observed concentration-dependent inhibition of T cell growth by TGF-β_1 was further demonstrated to be dependent upon the temporal relationship between T cell activation and addition of TGF-β_1 to the cell cultures. Specifically, increasing the interval between T cell activation and addition of TGF-β_1 to splenocyte cultures attenuated the inhibitory effect of TGF-β_1 on α-CD3 + α-CD28-induced (**Figures 15a and 15b**) and α-CD3-induced (**Figures 16a and 16b**) T cell growth. Interestingly, a bimodal time-dependent growth inhibitory effect by TGF-β_1 was observed when TGF-β_1 was added to cultures of naive splenocytes at varying intervals prior to T cell activation (**Figure 16a**). A similar bimodal time-dependent growth inhibitory effect by TGF-β_1 was also observed when TGF-β_1 was added to α-CD3-activated splenic T cells (**Figure 16b**). These bimodal temporal responses were concentration-dependent with 1 ng/mL and 10 ng/mL TGF-β_1 eliciting maximal responses (**Figures 16b**).

Increasing the interval between T cell activation and addition of TGF-β_1 to thymocyte cultures also attenuated the inhibitory effects of TGF-β_1 on α-CD3 + α-CD28-induced T cell growth (**Figures 17a and 17b**). A modest concentration-dependent-bimodal temporal response was observed when TGF-β_1 was added to thymocytes at various intervals after α-CD3 + α-CD28-induced activation (**Figure 17b**). Inhibition of α-CD3-induced thymocyte growth by TGF-β_1 was not markedly influenced by altering

Figure 14. Concentration-dependent effect of TGF-β_1 on proliferation of activated splenic T-cells and thymocytes. Naïve B6C3F1 splenocytes or thymocytes were cultured (2.5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the presence of immobilized α-CD3 (2 μg/mL) or immobilized α-CD3 (2 μg/mL) + soluble α-CD28 (1 μg/mL), as indicated. TGF-β_1 (0, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 ng/mL) was immediately added directly to the cultures. Following a 56 hour incubation at 37°C, [^{3}H]-thymidine (1 μCi/mL) was added for the final 16 hour incubation. Cells were harvested and [^{3}H]-thymidine incorporation was quantified using a beta scintillation counter. The data are expressed as a percentage of vehicle control [α-CD3 + 0 ng/mL TGF-β_1 (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture or α-CD3 + α-CD28 + 0 ng/mL] ± SE for three experiments in triplicate. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the appropriate vehicle control group. Vehicle control values (mean ± SE): α-CD3 + α-CD28 activated splenic T-cells, 7.2 X 10^4 ± 4.5 X 10^3 cpm; α-CD3-activated splenic T-cells, 4.1 X 10^4 ± 0.9 X 10^3 cpm; α-CD3 + α-CD28-activated thymocytes, 1.4 X 10^4 ± 1.6 X 10^3 cpm; α-CD3-activated thymocytes, 8.0 X 10^4 ± 6 X 10^3 cpm. The hatched box represents the vehicle values (mean ± SE).

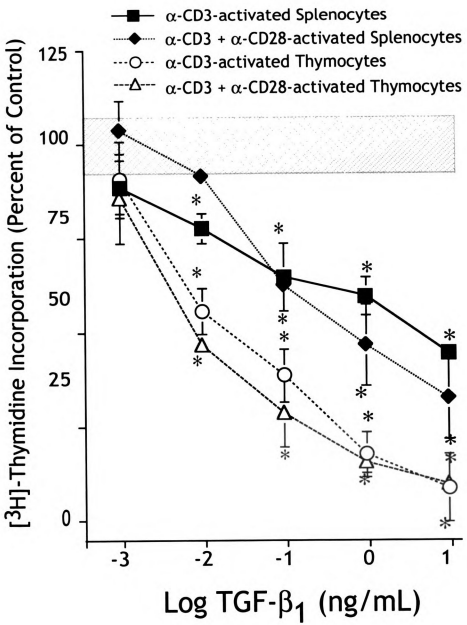

α-CD3-activated Splenocytes
α-CD3 + α-CD28-activated Splenocytes
α-CD3-activated Thymocytes
α-CD3 + α-CD28-activated Thymocytes
[3H]-Thymidine Incorporation (Percent of Control)
125
100
75
50
25
0
-3
-2
-1
0
1
Log TGF-β1 (ng/mL)

Figure 15. Time of addition effect of TGF-β_1 on α-CD3 + α-CD28-induced splenic T cell proliferation. Naïve B6C3F1 splenocytes were cultured (2.5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation (Naïve) or in the presence of immobilized α-CD3 (2 μg/mL) + α-CD28 (1 μg/mL) as indicated. TGF-β_1 (0, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 ng/mL) was added directly to the cultures. TGF-β_1 was added 3, 2, 1, 0.5, or 0 hours prior to T cell activation (a), or 0, 1, 3, 6, 9, or 12 hours after T cell activation (b) as indicated. Following a 56 hour incubation at 37°C, [^{3}H]-thymidine (1 μCi/mL) was added for the final 16 hour incubation. Cells were harvested and [^{3}H]-thymidine incorporation was quantified using a beta scintillation counter. The data are expressed as a percentage of vehicle control [α-CD3 + α-CD28 + 0 ng/mL TGF-β_1 (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture] $\pm$ SE for five experiments in quadruplicate. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the appropriate vehicle control group. Vehicle control value (mean $\pm$ SE): α-CD3 + α-CD28 activated splenic T-cells, 7.2 X $10^4 \pm 4.5$ X 10^3 cpm. The hatched box represents the vehicle values (mean $\pm$ SE).

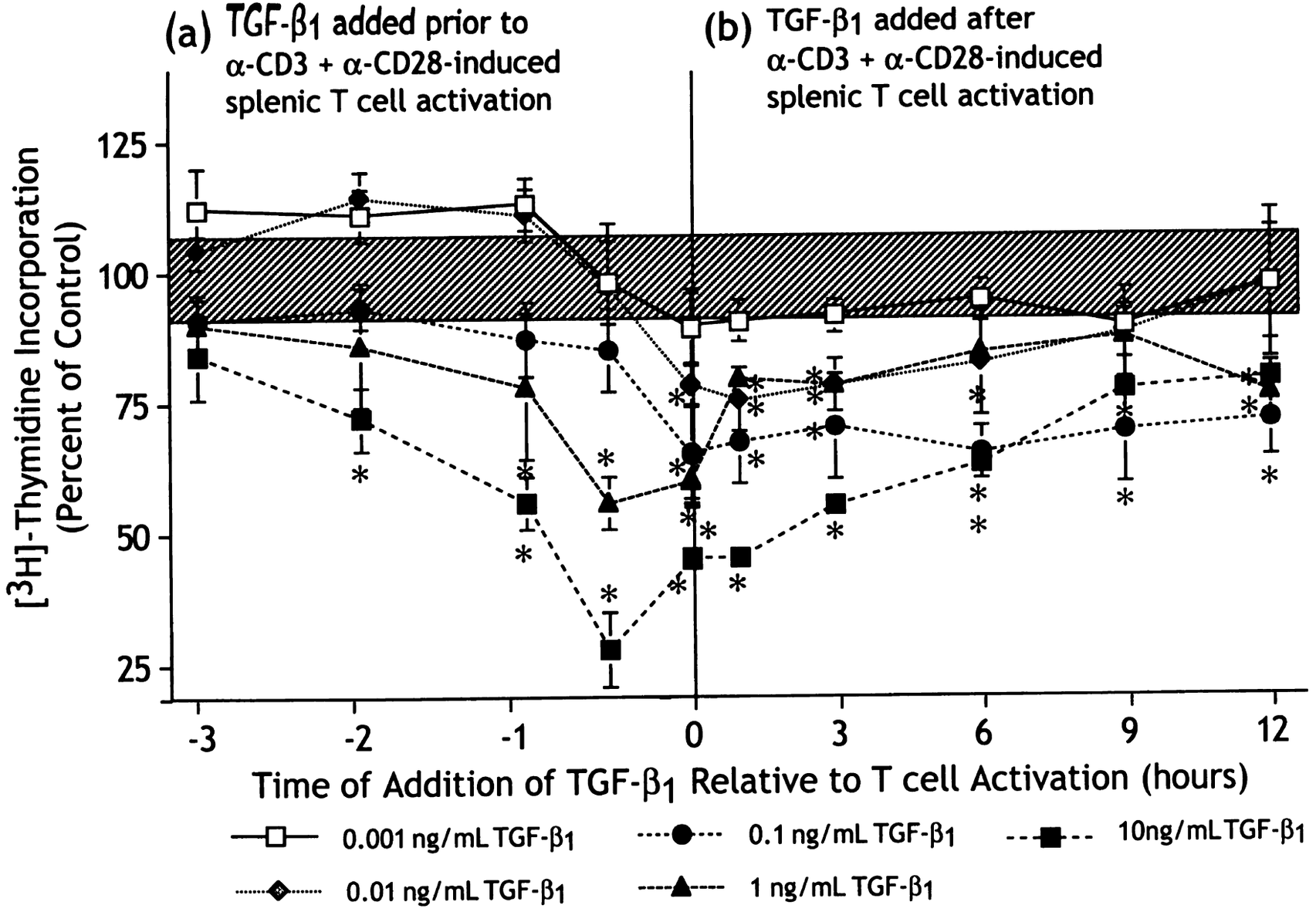

(a) TGF-β1 added prior to α-CD3 + α-CD28-induced splenic T cell activation
(b) TGF-β1 added after α-CD3 + α-CD28-induced splenic T cell activation
[3H]-Thymidine Incorporation (Percent of Control)
125
100
75
50
25
-3
-2
-1
0
3
6
9
12
Time of Addition of TGF-β1 Relative to T cell Activation (hours)
0.001 ng/mL TGF-β1
0.1 ng/mL TGF-β1
10ng/mL TGF-β1
0.01 ng/mL TGF-β1
1 ng/mL TGF-β1

Figure 16. Time of addition effect of TGF-β_1 on α-CD3-induced splenic T cell proliferation. Naïve B6C3F1 splenocytes (2.5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation (Naïve) or in the presence of immobilized α-CD3 (2 µg/mL) as indicated. TGF-β_1 (0, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 ng/mL) was added directly to the cultures. TGF-β_1 was added 3, 2, 1 0.5 or 0 hours prior to T cell activation (a), or 0, 1, 3, 6, 9, or 12 hours after T cell activation (b). Following a 56 hour incubation at 37°C, [^{3}H]-thymidine (1 µCi/mL) was added for the final 16 hour incubation. Cells were harvested and [^{3}H]-thymidine incorporation was quantified using a beta scintillation counter. The data are expressed as a percentage of vehicle control [α-CD3 + 0 ng/mL TGF-β_1 (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture] $\pm$ SE for five experiments in quadruplicate. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the appropriate vehicle control group. Vehicle control value (mean $\pm$ SE): α-CD3-activated splenic T-cells, 4.1 X $10^4 \pm$ 0.9 X 10^3 cpm. The hatched box represents the vehicle values (mean $\pm$ SE).

(b) TGF-β1 added after

(a) TGF-β1 added prior to

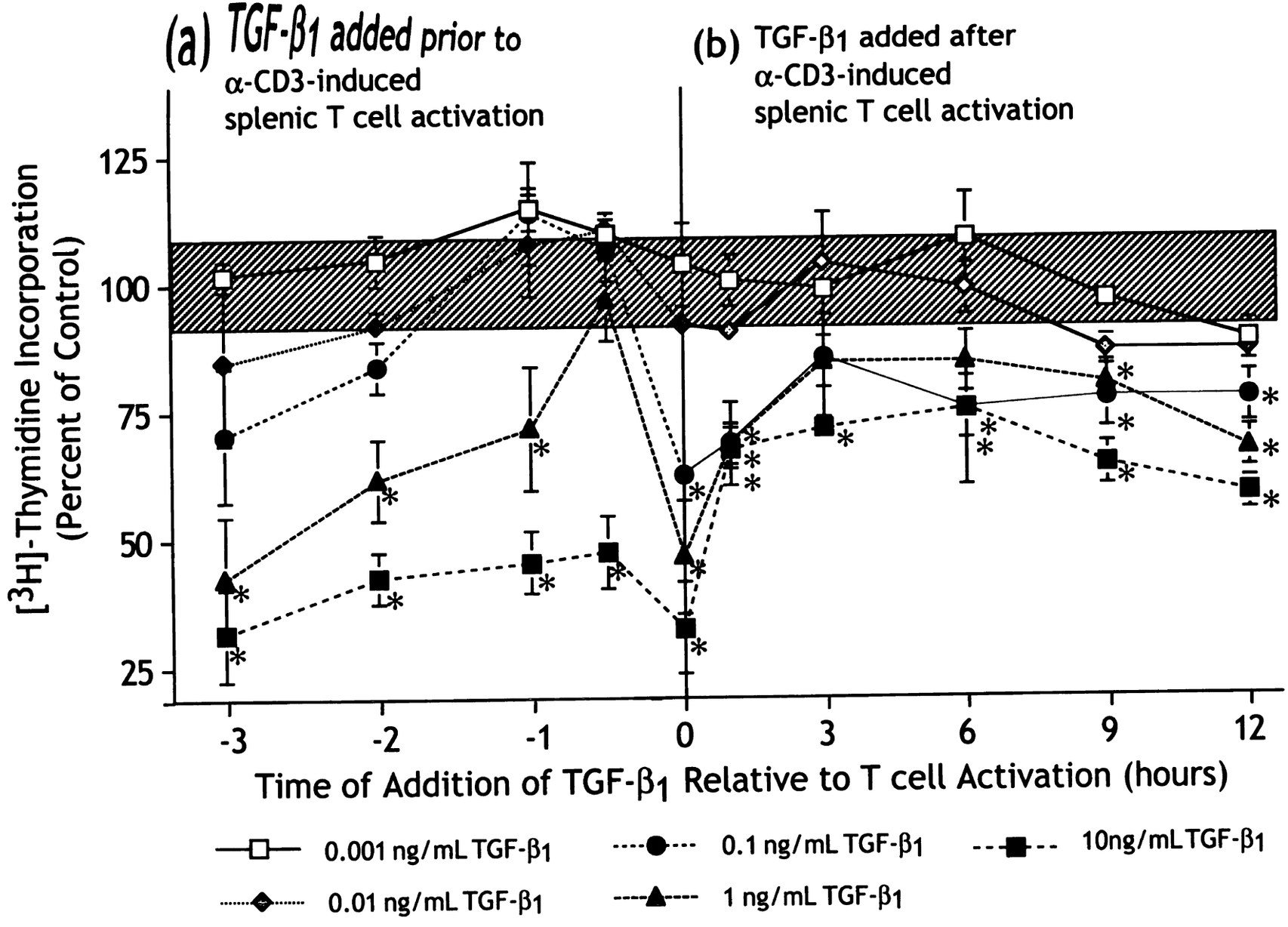
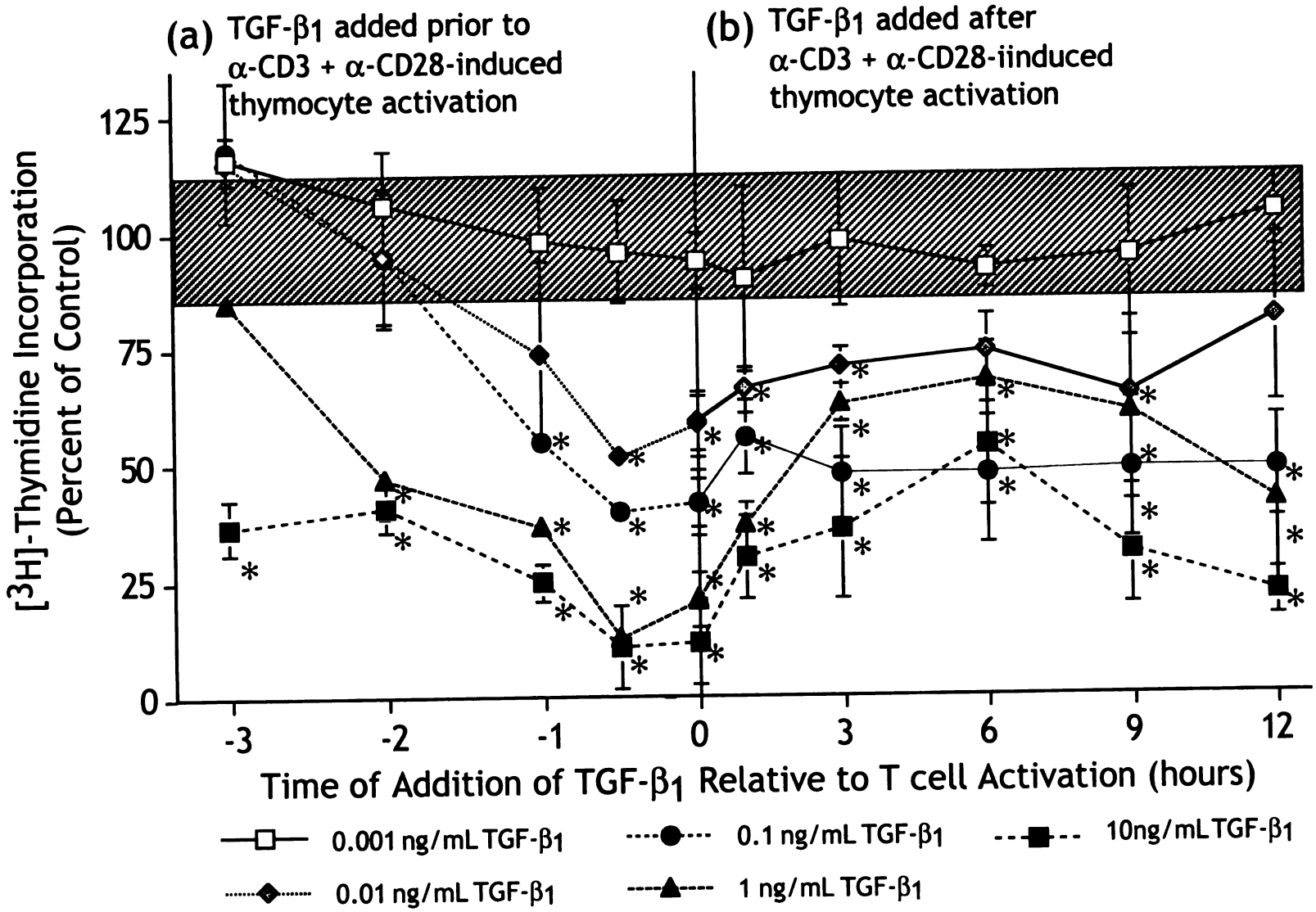

(a) TGF-β1 added prior to
α-CD3-induced
splenic T cell activation
(b) TGF-β1 added after
α-CD3-induced
splenic T cell activation
[3H]-Thymidine Incorporation
(Percent of Control)
125
100
75
50
25
-3
-2
-1
0
3
6
9
12
Time of Addition of TGF-β1 Relative to T cell Activation (hours)
0.001 ng/mL TGF-β1
0.01 ng/mL TGF-β1
0.1 ng/mL TGF-β1
1 ng/mL TGF-β1
10ng/mL TGF-β1

Figure 17. Time of addition effect of TGF-β_1 on α-CD3 + α-CD28-induced thymocyte proliferation. Naïve B6C3F1 thymocytes were cultured (2.5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation (Naïve) or in the presence of immobilized α-CD3 (2 μg/mL) + α-CD28 (1 μg/mL) as indicated. TGF-β_1 (0, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 ng/mL) was added directly to the cultures. TGF-β_1 was added 3, 2, 1, 0.5 or 0 hours prior to T cell activation (a), or 0, 1, 3, 6, 9, or 12 hours after T cell activation (b). Following a 56 hour incubation at 37°C, [^{3}H]-thymidine (1 μCi/mL) was added for a final 16 hour incubation. Cells were harvested and [^{3}H]-thymidine incorporation was quantified using a beta scintillation counter. The data are expressed as a percentage of vehicle control [α-CD3 + α-CD28 + 0 ng/mL TGF-β_1 (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture] $\pm$ SE for five experiments in quadruplicate. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the appropriate vehicle control group. Vehicle control value (mean $\pm$ SE): α-CD3 + α-CD28-activated thymocytes, 1.4 X 10^4 $\pm$ 1.6 X 10^3 cpm. The hatched box represents the vehicle values (mean $\pm$ SE).


(a) TGF-β1 added prior to α-CD3 + α-CD28-induced thymocyte activation
(b) TGF-β1 added after α-CD3 + α-CD28-iinduced thymocyte activation
[3H]-Thymidine Incorporation (Percent of Control)
125
100
75
50
25
0
-3
-2
-1
0
3
6
9
12
Time of Addition of TGF-β1 Relative to T cell Activation (hours)
0.001 ng/mL TGF-β1
0.01 ng/mL TGF-β1
0.1 ng/mL TGF-β1
1 ng/mL TGF-β1
10ng/mL TGF-β1

the temporal relationship between T cell activation and addition TGF-β_1 (**Figures 18a and 18b**). Noteworthy, TGF-β_1 did not augment T cell growth under any T cell activation condition tested (**Figures 15, 16, 17 and 18**). In summary, these results establish that inhibition of α-CD3-induced splenic T cell and thymocyte growth by TGF-β_1 *in vitro* is dependent on the concentration of TGF-β_1, CD28 co-stimulation, and the temporal relationship between addition of TGF-β_1 and T cell activation.

B. Effects of TGF-β_1 on IL-2 protein secretion and steady state IL-2 mRNA expression

Splenocytes and thymocytes were isolated from naïve B6C3F1 mice and activated in culture with α-CD3 + α-CD28 or α-CD3 alone. TGF-β_1 was added directly to the cell cultures concurrently with T cell activation or at various intervals either prior to or after T cell activation. Supernatants were collected 24 hours after T cell activation and analyzed for IL-2 protein secretion by ELISA. Total RNA was isolated from splenocytes activated in culture for 60 minutes. Steady state IL-2 mRNA was quantified using RT-PCR.

Anti-CD3-induced IL-2 protein secretion was augmented with α-CD28 co-stimulation in splenic T cells and thymocytes (**Figures 19a** and **19b,** respectively). When added concurrently with T cell activation, TGF-β_1 either augmented or attenuated α-CD3 + α-CD28-induced IL-2 secretion from splenic T cells at fM (femtomolar) and pM (picomolar) concentrations, respectively (**Figure 20**). TGF-β_1 (10^{-2} ng/mL) augmented α-CD3-induced IL-2 protein secretion in splenic T cells (**Figure 20**). In contrast, TGF-β_1 did not augment, but rather, attenuated IL-2 protein secretion in activated thymocytes in a

Figure 18. Time of addition effect of TGF-β_1 on α-CD3-induced thymocyte proliferation. Naïve B6C3F1 splenocytes thymocytes were cultured (2.5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation (Naïve) or in the presence of immobilized α-CD3 (2 μg/mL) as indicated. TGF-β_1 (0, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 ng/mL) was added directly to the cultures. TGF-β_1 was added 3, 2, 1, 0.5 or 0 hours prior to T cell activation (a), or 0, 1, 3, 6, 9, or 12 hours after T cell activation (b). Following a 56 hour incubation at 37°C, [^{3}H]-thymidine (1 μCi/mL) was added for a final 16 hour incubation. Cells were harvested and [^{3}H]-thymidine incorporation was quantified using a beta scintillation counter. The data are expressed as a percentage of vehicle control [α-CD3 + 0 ng/mL TGF-β_1 (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture] $\pm$ SE for five experiments in quadruplicate. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the appropriate vehicle control group. Vehicle control value (mean $\pm$ SE): α-CD3-activated thymocytes, 8.0 X $10^4 \pm 6$ X 10^3 cpm. The hatched box represents the vehicle values (mean $\pm$ SE).

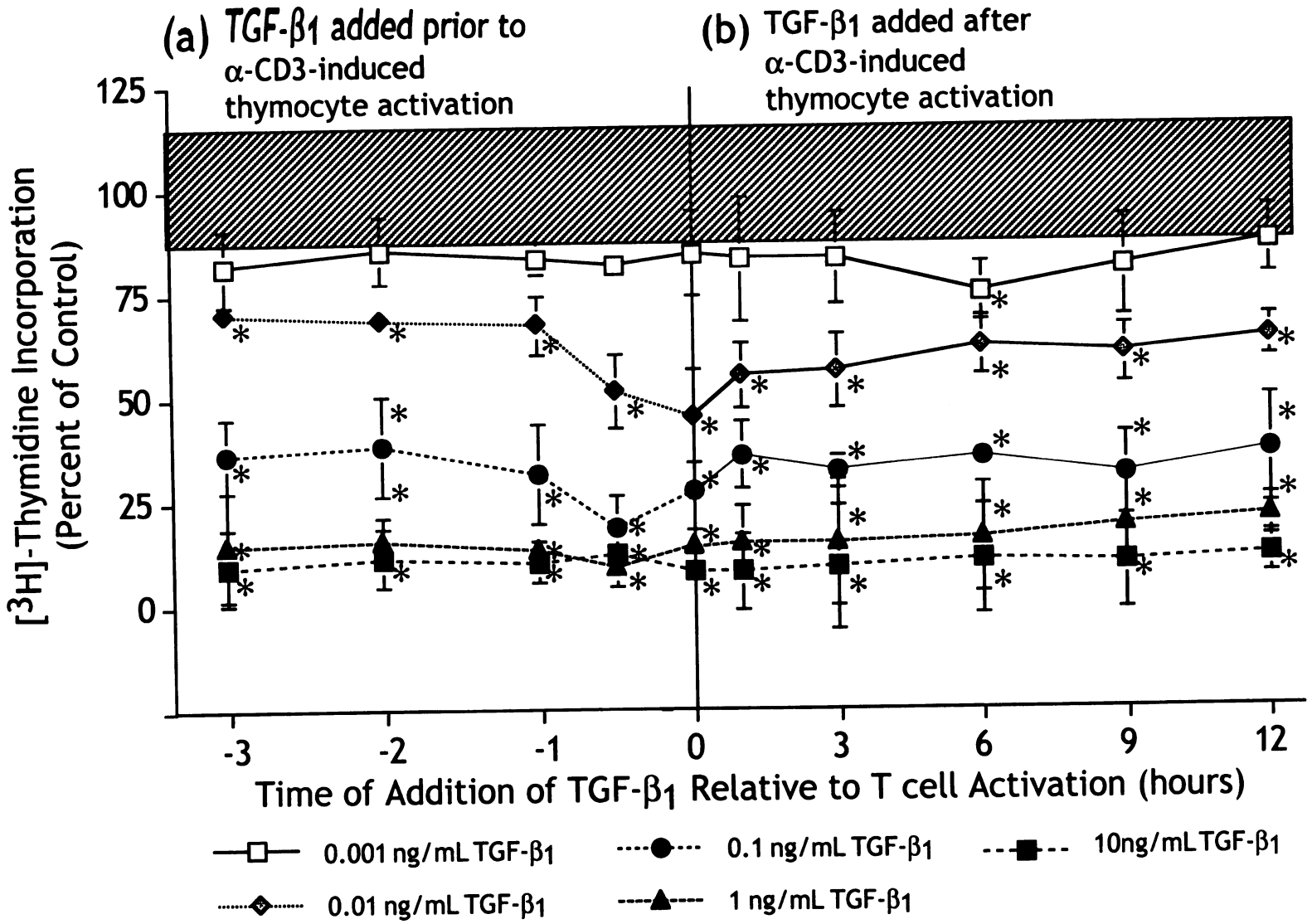
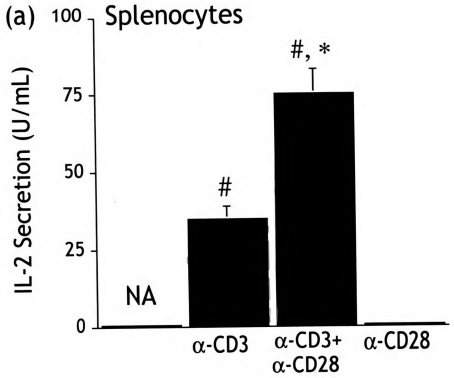

(a) TGF-β1 added prior to α-CD3-induced thymocyte activation
(b) TGF-β1 added after α-CD3-induced thymocyte activation
[3H]-Thymidine Incorporation (Percent of Control)
125
100
75
50
25
0
-3
-2
-1
0
3
6
9
12
Time of Addition of TGF-β1 Relative to T cell Activation (hours)
0.001 ng/mL TGF-β1
0.1 ng/mL TGF-β1
10ng/mL TGF-β1
0.01 ng/mL TGF-β1
1 ng/mL TGF-β1

Figure 19. IL-2 protein secretion in activated mouse splenic T cells and thymocytes. Naïve B6C3F1 splenocytes (a) or thymocytes (b) were cultured (5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation (Naïve) or in the presence of immobilized α-CD3 (2 μg/mL), immobilized α-CD3 (2 μg/mL) + soluble α-CD28 (1 μg/mL), or soluble α-CD28 (1 μg/mL), as indicated. Following a 24 hour incubation at 37°C, supernatants were collected and stored at -80°C until analyzed. IL-2 protein secretion was quantified using standard sandwich ELISA. The data are expressed as mean IL-2 protein secretion (units/mL) ± SE for three experiments in triplicate. #,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the Naïve group. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the immobilized α-CD3 group.


(a)
Splenocytes
IL-2 Secretion (U/mL)
100
75
50
25
0
#, *
#
NA
α-CD3
α-CD3+
α-CD28
α-CD28

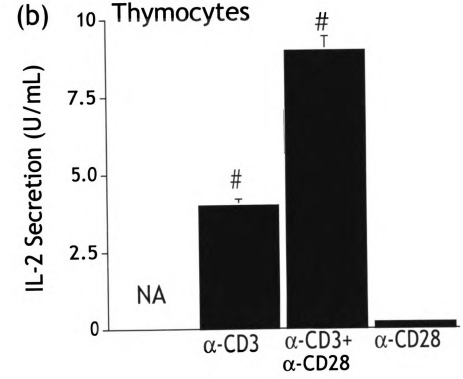
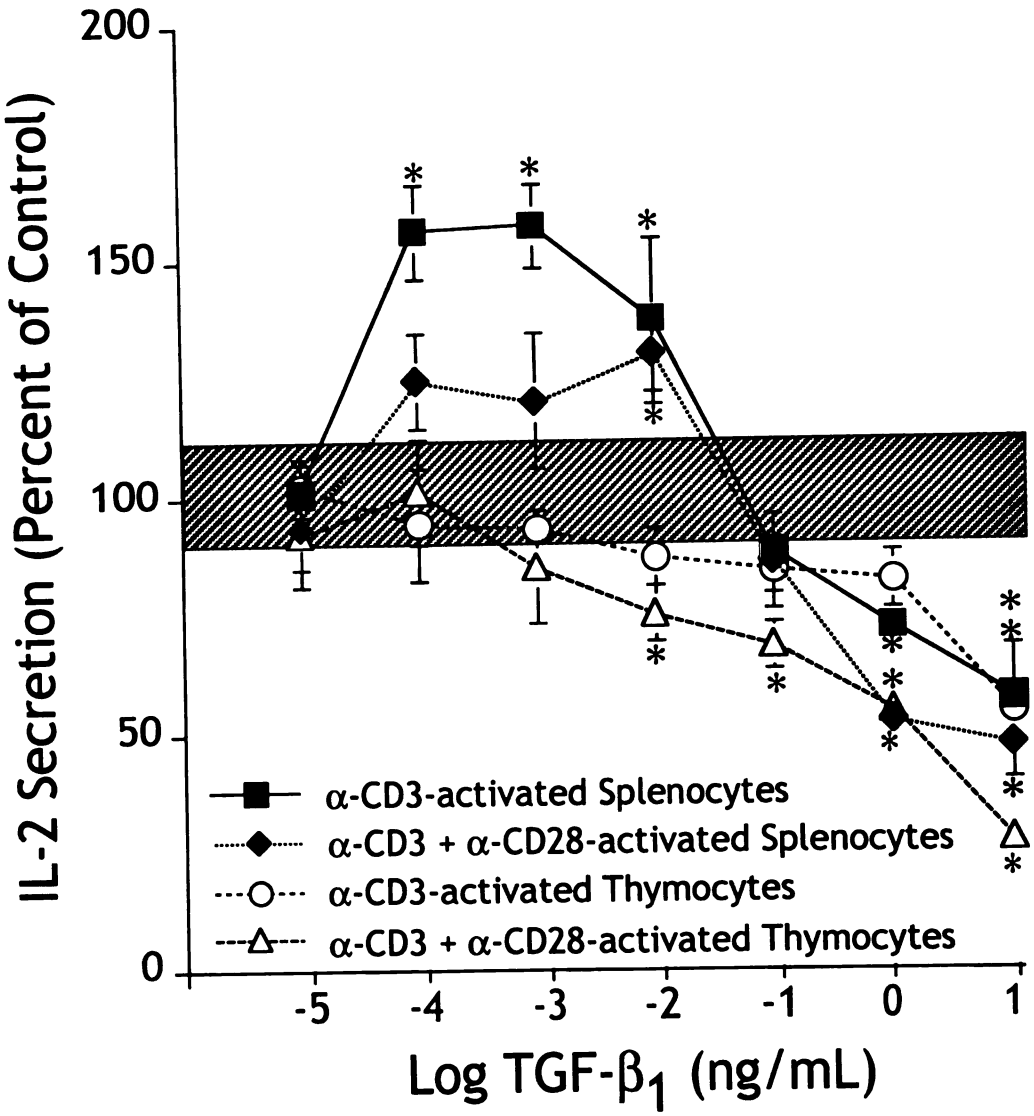

(b)
Thymocytes
IL-2 Secretion (U/mL)
10
7.5
5.0
2.5
0
#
#
NA
α-CD3
α-CD3+
α-CD28
α-CD28

Figure 20. Concentration-dependent effect of TGF-β_1 on IL-2 protein secretion in activated splenic T cells and thymocytes. Naïve B6C3F1 splenocytes or thymocytes were cultured (5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation (Naïve) or in the presence of immobilized α-CD3 (2 μg/mL) or immobilized α-CD3 (2 μg/mL) + soluble α-CD28 (1 μg/mL), as indicated. TGF-β_1 (0, 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 ng/mL) was immediately added directly to the cultures, as indicated. Following a 24 hour incubation at 37°C, supernatants were collected and stored at -80°C until analyzed. IL-2 protein secretion was quantified using standard sandwich ELISA. The data are expressed as a percentage of vehicle control [α-CD3 + 0 ng/mL TGF-β_1 (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture or α-CD3 + α-CD28 + 0 ng/mL TGF-β_1] ± SE for three experiments in triplicate. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the appropriate vehicle control group. Vehicle control values (mean ± SE): α-CD3 + α-CD28 activated splenic T-cells, 74.5 ± 4.2 U/mL; α-CD3-activated splenic T-cells, 37.5 ± 3.5 U/mL; α-CD3 + α-CD28-activated thymocytes, 9.0 ± 4.2 U/mL; α-CD3-activated thymocytes, 4.2 ± 2.1 U/mL. The hatched box represents the vehicle values (mean ± SE).


200
150
100
50
0
IL-2 Secretion (Percent of Control)
α-CD3-activated Splenocytes
α-CD3 + α-CD28-activated Splenocytes
α-CD3-activated Thymocytes
α-CD3 + α-CD28-activated Thymocytes
-5
-4
-3
-2
-1
0
1
Log TGF-β1 (ng/mL)

concentration-dependent manner (**Figure 20**). TGF-β_1 did not induce IL-2 secretion in the absence of T cell activation (**Figure 21**). Simultaneous addition of TGF-β_1 and T cell activation also resulted in either augmentation or attenuation of α-CD3 + α-CD28-induced steady state IL-2 mRNA expression by TGF-β_1 in splenic T cells at fM and pM concentrations, respectively (**Figure 22**).

The bimodal concentration-dependent stimulatory and inhibitory effects of TGF-β_1 on α-CD3 + α-CD28-induced IL-2 secretion was further dependent on the temporal relationship between T cell activation and addition of TGF-β_1 to cell cultures. Augmentation of IL-2 protein secretion by TGF-β_1 was abrogated when TGF-β_1 was added either 30 minutes prior to or 30 minutes after T cell activation (**Figures 23a, 23b 24a, and 24b**). Notably TGF-β_1 (1 ng/mL and/or 10 ng/mL) stimulated splenic T cell IL-2 secretion when TGF-β_1 was added at 2 or 3 hours prior to T cell activation (**Figure 23a** and **Figure 24a**). A similar response by TGF-β_1 was not observed when added at various intervals *after* the T cells were activated (**Figure 23b** and **24b**).

In contrast to splenic T cells, the effect of TGF-β_1 on IL-2 secretion in α-CD3 + α-CD28-activated thymocytes was relatively time-independent (**Figure 25**). However, as illustrated in **Figure 26**, a marked temporal response was observed in α-CD3-activated thymocytes. In summary, these data demonstrate TGF-β_1 differentially regulates IL-2 secretion *in vitro* in a manner that is dependent on concentration, CD28 co-stimulation, and the activation status of the T cells. In addition, TGF-β_1 differentially regulates IL-2 secretion in splenocytes and thymocytes, suggesting that T cell maturation is also a factor; however, secondary indirect B cell and/or macrophage-mediated effects in splenocyte cultures may contribute to this differential response by TGF-β_1. Collectively,

Figure 21. TGF-β_1 does not induce IL-2 protein secretion in naive splenocytes or thymocytes *in vitro*. Naïve B6C3F1 splenocytes (a) or thymocytes (b) were cultured (5 X 10^6 cells/mL) in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation. Following a 24 hour incubation at 37°C, supernatants were collected and stored at -80°C until analyzed. IL-2 protein secretion was quantified using standard sandwich ELISA. The data are expressed as mean IL-2 protein secretion (units/mL) ± SE for three experiments in triplicate.

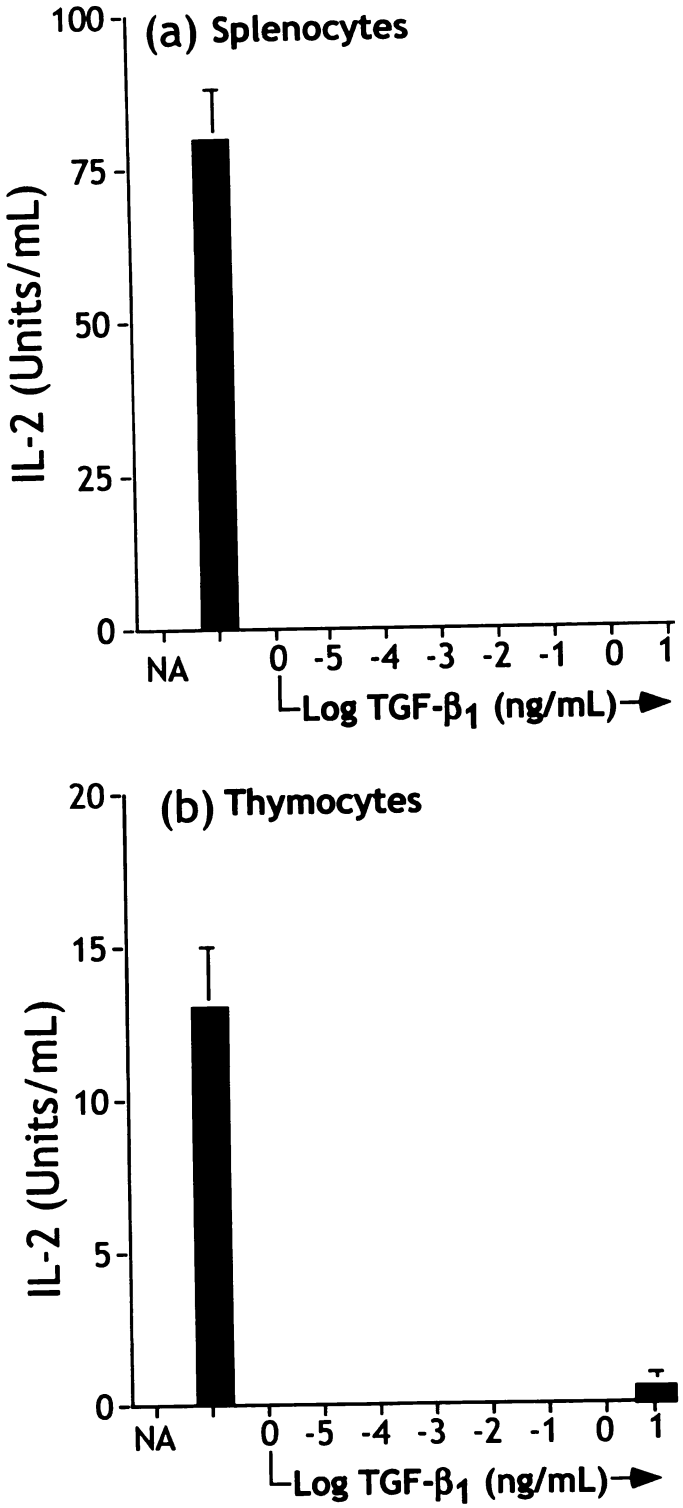

(a) Splenocytes
100
75
50
25
0
IL-2 (Units/mL)
NA
0 -5 -4 -3 -2 -1 0 1
Log TGF-β1 (ng/mL)
(b) Thymocytes
20
15
10
5
0
IL-2 (Units/mL)
NA
0 -5 -4 -3 -2 -1 0 1
Log TGF-β1 (ng/mL)

Figure 22. Concentration-dependent effect of TGF-β_1 on IL-2 mRNA. Splenocytes (5 X 10^6 c/mL) were added to culture plates pre-coated with α-CD3 + α-CD28. TGF-β_1 was immediately added thereafter for one hour. Total RNA was extracted for RT-PCR analysis for IL-2 mRNA expression as described in "Materials and Methods". IL-2 expression without treatment is shown (NA). The data are reported as the mean $\pm$ SE (n=3). *$p < 0.05$ with comparison to the α-CD3 + α-CD28 + 0 ng/mL TGF-β_1 group (VH) .

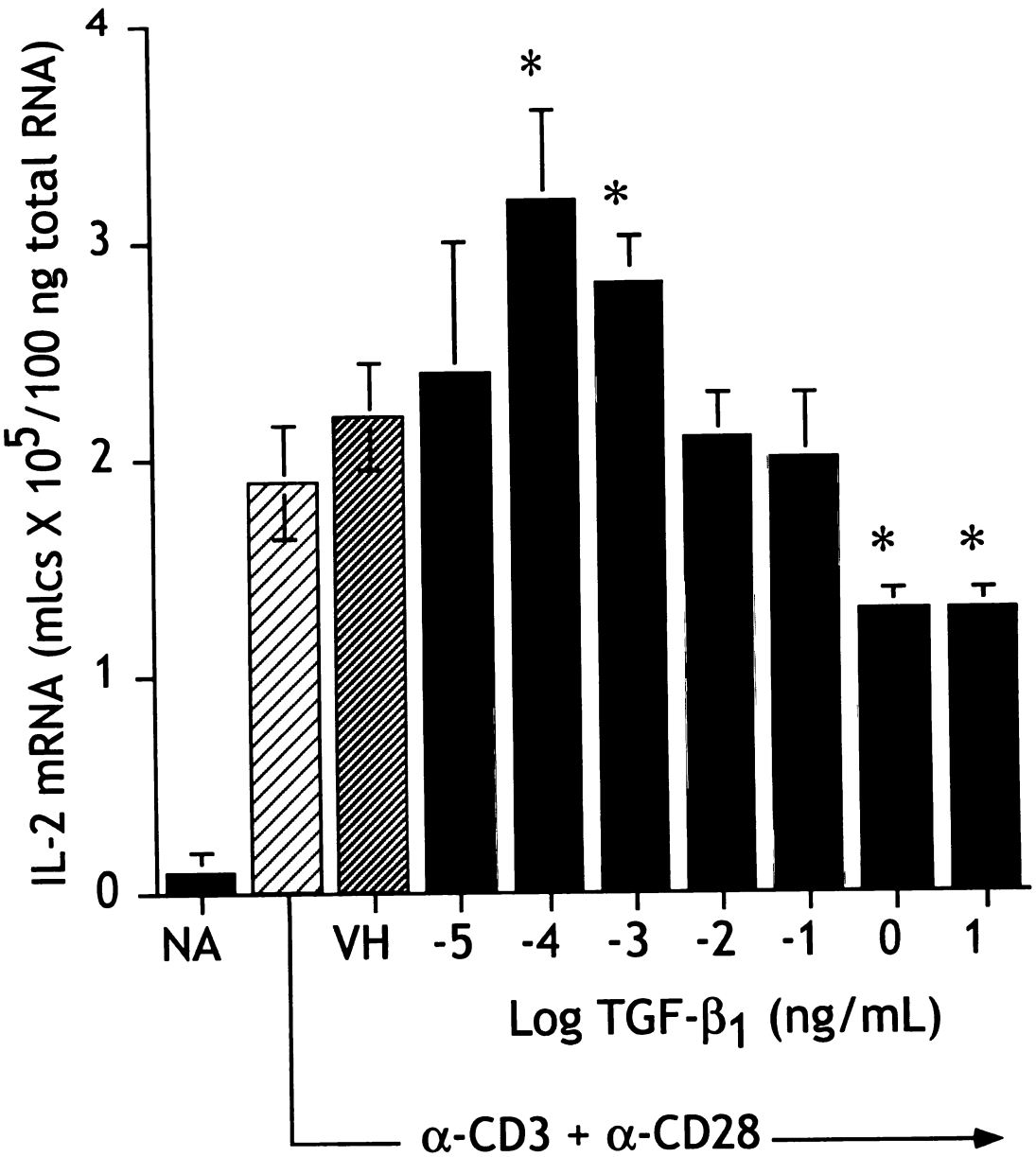

IL-2 mRNA (mlcs X 10^5/100 ng total RNA)
4
3
2
1
0
*
*
*
*
NA
VH
-5
-4
-3
-2
-1
0
1
Log TGF-β_1 (ng/mL)
α-CD3 + α-CD28

Figure 23. Time of addition effect of TGF-β_1 on α-CD3 + α-CD28-induced IL-2 protein secretion in splenic T cells. Naïve B6C3F1 splenocytes were cultured (5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation (Naïve) or in the presence of immobilized α-CD3 (2 μg/mL) + soluble α-CD28 (1 μg/mL), as indicated. TGF-β_1 (0, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 ng/mL) was added directly to the cultures. TGF-β_1 was added 3, 2, 1, 0.5, or 0 hours prior to T cell activation (a), or 0, 1, 3, 6, 9, or 12 hours after T cell activation (b) as indicated. Following a 24 hour incubation at 37°C, supernatants were collected and stored at -80°C until analyzed. IL-2 protein secretion was quantified using standard sandwich ELISA. The data are expressed as a percentage of vehicle control [α-CD3 + α-CD28+ 0 ng/mL TGF-β_1 (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture] ± SE for three experiments in triplicate. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the appropriate vehicle control group. Vehicle control values (mean ± SE): α-CD3 + α-CD28 activated splenic T-cells, 74.5 ± 4.2 U/mL. The hatched box represents the vehicle values (mean ± SE).

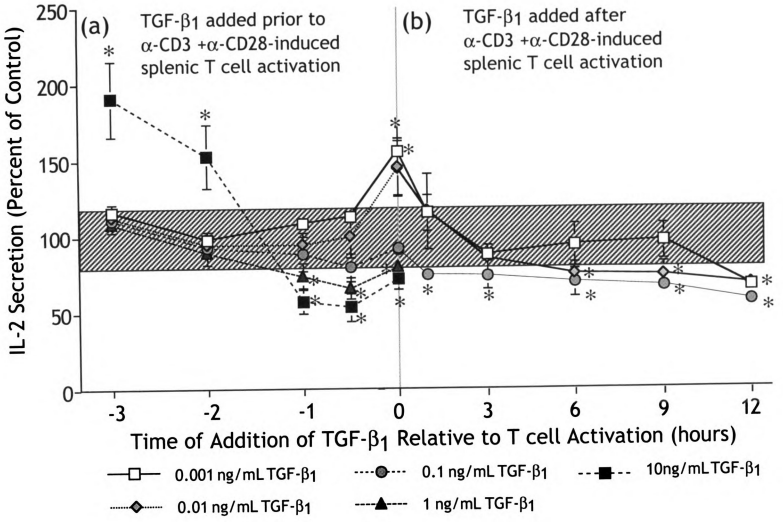

(a)
TGF-β1 added prior to
α-CD3 +α-CD28-induced
splenic T cell activation
(b)
TGF-β1 added after
α-CD3 +α-CD28-induced
splenic T cell activation
IL-2 Secretion (Percent of Control)
250
200
150
100
50
0
-3
-2
-1
0
3
6
9
12
Time of Addition of TGF-β1 Relative to T cell Activation (hours)
0.001 ng/mL TGF-β1
0.1 ng/mL TGF-β1
10ng/mL TGF-β1
0.01 ng/mL TGF-β1
1 ng/mL TGF-β1

Figure 24. Time of addition effect of TGF-β_1 on αCD3-induced IL-2 protein secretion in splenic T cells. Naïve B6C3F1 splenocytes thymocytes were cultured (5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation (Naïve) or in the presence of immobilized α-CD3 (2 μg/mL) + soluble α-CD28 (1 μg/mL), as indicated. TGF-β_1 (0, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 ng/mL) was added directly to the cultures. TGF-β_1 was added 3, 2, 1, 0.5, or 0 hours prior to T cell activation (a), or 0, 1, 3, 6, 9, or 12 hours after T cell activation (b) as indicated. Following a 24 hour incubation at 37°C, supernatants were collected and stored at -80°C until analyzed. IL-2 protein secretion was quantified using standard sandwich ELISA. The data are expressed as a percentage of vehicle control [α-CD3 + α-CD28+ 0 ng/mL TGF-β_1 (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture] ± SE for three experiments in triplicate. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the appropriate vehicle control group. Vehicle control values (mean ± SE): α-CD3 + α-CD28-activated thymocytes, 9.0 ± 4.2 U/mL. The hatched box represents the vehicle values (mean ± SE).

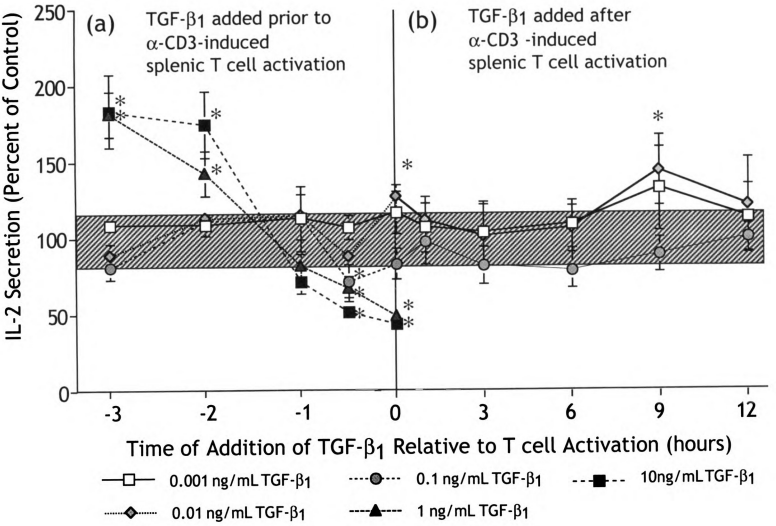

(a)
TGF-β1 added prior to
α-CD3-induced
splenic T cell activation
(b)
TGF-β1 added after
α-CD3 -induced
splenic T cell activation
IL-2 Secretion (Percent of Control)
250
200
150
100
50
0
-3
-2
-1
0
3
6
9
12
Time of Addition of TGF-β1 Relative to T cell Activation (hours)
0.001 ng/mL TGF-β1
0.1 ng/mL TGF-β1
10ng/mL TGF-β1
0.01 ng/mL TGF-β1
1 ng/mL TGF-β1

Figure 25. Time of addition effect of TGF-β_1 on α-CD3 + α-CD28-induced IL-2 protein secretion in thymocytes. Naïve B6C3F1 splenocytes were cultured (5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation (Naïve) or in the presence of immobilized α-CD3 (2 μg/mL) as indicated. TGF-β_1 (0, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 ng/mL) was added directly to the cultures. TGF-β_1 was added 3, 2, 1, 0.5, or 0 hour prior to T cell activation (a), or 0, 1, 3, 6, 9, or 12 hours after T cell activation (b) as indicated. Following a 24 hour incubation at 37°C, supernatants were collected and stored at -80°C until analyzed. IL-2 protein secretion was quantified using standard sandwich ELISA. The data are expressed as a percentage of vehicle control [α-CD3 + 0 ng/mL TGF-β_1 (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture] $\pm$ SE for three experiments in triplicate. *$p<0.05$ (determined by Dunnett's *t*-test) as compared to the appropriate vehicle control group. Vehicle control values (mean $\pm$ SE): α-CD3 + α-CD28-activated thymocytes, 9.0 $\pm$ 4.2 U/mL The hatched box represents the vehicle values (mean $\pm$ SE).

(a)
TGF-β1 added prior to
α-CD3 + α-CD28-induced
thymocyte activation
(b)
TGF-β1 added after
α-CD3 + α-CD28-induced
thymocyte activation
IL-2 Secretion (Percent of Control)
150
125
100
75
50
-3
-2
-1
0
3
6
9
12
Time of Addition of TGF-β1 Relative to T cell Activation (hours)
0.001 ng/mL TGF-β1
0.01 ng/mL TGF-β1
0.1 ng/mL TGF-β1
1 ng/mL TGF-β1
10ng/mL TGF-β1

Figure 26. Time of addition effect of TGF-β_1 on α-CD3-induced IL-2 secretion in thymocytes. Naïve B6C3F1 thymocytes were cultured (5 X 10^6 cells/mL in RPMI 1640 supplemented with 5% BCS) in the absence of exogenous stimulation (Naïve) or in the presence of immobilized α-CD3 (2 μg/mL) as indicated. TGF-β_1 (0, 10^{-3}, 10^{-2}, 10^{-1}, 1, or 10 ng/mL) was added directly to the cultures. TGF-β_1 was added 3, 2, 1, 0.5 or 0 hours prior to T cell activation (a), or 0, 1, 3, 6, 9, or 12 hours after T cell activation (b) as indicated. Following a 24 hour incubation at 37°C, supernatants were collected and stored at -80°C until analyzed. IL-2 protein secretion was quantified using standard sandwich ELISA. The data are expressed as a percentage of vehicle control [α-CD3 + 0 ng/mL TGF-β_1 (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture] $\pm$ SE for three experiments in triplicate. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the appropriate vehicle control group. Vehicle control values (mean $\pm$ SE): α-CD3-activated thymocytes, 4.2 $\pm$ 2.1 U/mL. The hatched box represents the vehicle values (mean $\pm$ SE).

(a) TGF-β1 added prior to α-CD3-induced thymocyte activation
(b) TGF-β1 added after α-CD3-induced thymocyte activation
IL-2 Secretion (Percent of Control)
150
125
100
75
50
25
-3
-2
-1
0
3
6
9
12
Time of Addition of TGF-β1 Relative to T cell Activation (hours)
0.001 ng/mL TGF-β1
0.01 ng/mL TGF-β1
0.1 ng/mL TGF-β1
1 ng/mL TGF-β1
10ng/mL TGF-β1

these results support the hypothesis of cooperative signaling interactions among the TcR, the TβR, and CD28 to regulate IL-2 expression by TGF-β_1. The influence of TGF-β_1 on IL-2 secretion does not parallel its modulatory effects on T cell growth under similar T cell activation conditions (**Figure 27**). These results reflect direct effects by TGF-β_1 on other parameters associated with cell growth, for example cell cycle proteins and apoptosis.

II. TGF-β_1 bifunctionally modulates *in vitro* IgM AFC responses in a concentration-dependent manner

Picomolar concentrations of TGF-β_1 inhibit *in vitro* IgM AFC responses (Delaney *et al.* 1994; Jeon *et al.* 1997). In light of the observation that fM concentrations of TGF-β_1 augment IL-2 secretion (**Figure 20**), steady state IL-2 mRNA (**Figure 22**) and chemotaxis (Wahl *et al.* 1987), the objective of these studies was to determine whether fM concentrations of TGF-β_1 also enhance *in vitro* DNP-Ficoll, sRBC, and LPS-induced AFC responses. Splenocytes were isolated from naïve B6C3F1 mice and sensitized in culture with sRBC, DNP-Ficoll, or LPS in the presence of TGF-β_1. The effects of TGF-β_1 on T cell-dependent and T cell-independent immunoglobulin production were investigated using the hemolytic plaque assay.

TGF-β_1 augmented and attenuated the *in vitro* T cell-dependent anti-sRBC AFC response at fM (0.4 – 2.4 pg/mL) and pM (0.001 – 0.005 ng/mL) concentrations, respectively (**Figure 28**). Concentration-dependent augmentation as well as attenuation by TGF-β_1 was also observed in the *in vitro* T cell-independent DNP-Ficoll AFC response (**Figure 29**). In contrast, TGF-β_1 attenuated the *in vitro* T cell-independent B

Figure 27. TGF-β_1 differentially regulates IL-2 secretion and [^{3}H]-thymidine incorporation in α-CD3 + α-CD28-activated splenic T cells in a concentration- and time-dependent manner. Data are abstracted and summarized from previously described results to illustrate the differential response by TGF-β_1 on T cell activation, as determined by IL-2 protein secretion, and T cell proliferation, as determined by [^{3}H]-thymidine incorporation. The data are expressed as a percentage of vehicle control [α-CD3 + 0 ng/mL TGF-β_1 (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture] $\pm$ SE for five experiments in quadruplicate. *,$p<0.05$ (determined by Dunnett's *t*-test) as compared to the appropriate vehicle control group. The hatched box represents the control values expressed as a percentage (mean $\pm$ SE).

-thymidine
ration- and
previously
activation, a
ned by [³H]-
e control (fo
concentration
etermined by
hatched bar

Figure 28. Effect of TGF-β_1 on the *in vitro* AFC response to sRBC. Spleens from naïve (NA) female B6C3F1 mice were isolated aseptically and made into single cell suspensions. The splenocytes were washed and adjusted to 1 X 10^7 c/mL and transferred in 500-µL to the wells of a 48-well culture plate. Quadruplicate cultures were prepared with NA, vehicle (VH; 0.02 % PBS final concentration in culture, pH 3.5, containing 0.1% BSA) or TGF-β_1 (0.0003, 0.0006, 0.001, 0.002, 0.005, 0.01, 0.02, 0.04, 0.08, 0.2, 0.3, 0.6, 1.25, or 2.5 ng/mL final concentration in culture), and sensitized with sRBC. Cultures were subsequently analyzed for a day 5 antibody response by enumerating the number of antibody forming cells (AFC); spleen cell viability and total recovered cells/culture were also determined. The results from quadruplicate determinations are expressed as the mean AFC per 10^6 recovered viable cells $\pm$ SE (n=4). $*p < 0.05$ as determined by Dunnett's t test as compared to the vehicle control. The results are representative of three independent experiments.

AFC/10⁶ Recovered Splenocytes
1000
750
500
250
0
No Stimulation
0
0.0003
0.0006
0.0012
0.0024
0.0048
0.0098
0.0195
0.0391
0.0781
0.1563
0.3125
0.625
1.25
2.5
TGF-β1 (ng/mL)
sRBC
Recovered Viable Splenocytes X 10⁶
6
5
4
3
2
1
0
0
0.0003
0.0006
0.0012
0.0024
0.0048
0.0098
0.0195
0.0391
0.0781
0.1563
0.3125
0.625
1.25
2.5
TGF-β1 (ng/mL)
sRBC

Figure 29. Effect of TGF-β_1 on the *in vitro* AFC response to DNP-Ficoll. Spleens from naïve (NA) female B6C3F1 mice were isolated aseptically and made into single cell suspensions. The splenocytes were washed and adjusted to 1 X 10^7 c/mL and transferred in 500-µL to the wells of a 48-well culture plate. Quadruplicate cultures were prepared with NA, vehicle (VH; 0.02 % PBS final concentration in culture, pH 3.5, containing 0.1% BSA) or TGF-β_1 (0.0003, 0.0006, 0.001, 0.002, 0.005, 0.01, 0.02, 0.04, 0.08, 0.2, 0.3, 0.6, 1.25, or 2.5 ng/mL final concentration in culture), and sensitized with DNP-Ficoll (50 ng/mL). Cultures were subsequently analyzed for a day 3 antibody response by enumerating the number of AFC; spleen cell viability and total recovered cells/culture were also determined. The results from quadruplicate determinations are expressed as the mean AFC per 10^6 recovered viable cells $\pm$ SE (n=4). $*p < 0.05$ as determined by Dunnett's t test as compared to the vehicle control. The results are representative of three independent experiments.

oll. Spleens
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transferred
re prepared
containing
3, 0.08, 0.2
with DNP-
esponse by
ells/culture
xpressed as
ermined by
ive of three

cell polyclonal mitogen AFC response in a concentration- dependent manner (**Figure 30**). Augmentation of AFC responses by TGF-β_1, as described above, was not due to an increase the number of viable cells recovered (see inserts for **Figures 28, 29,** and **30**). In summary, these results demonstrate that TGF-β_1 modulates T cell-dependent and T cell-independent AFC responses in a bifunctional concentration manner. The observed profile of impaired immunoglobulin production is highly suggestive of a cell-type specific response.

III. Characterization of Smad proteins in mouse lymphoid tissue

A. Smad2, Smad3, and Smad4 protein expression in mouse splenocytes and thymocytes

Whole cell lysates from splenocytes and thymocytes isolated from naïve B6C3F1 mice were used to investigate Smad protein expression in lymphoid tissue by Western immunoblot analyses. As illustrated in **Figure 31**, Smad2 Smad3, and Smad4 are expressed in mouse splenocytes and thymocytes. Importantly, the splenocyte preparation represents a mixed population of B cells, T cell, macrophages, and dendritic cells. Thymocytes are devoid of B cells, macrophages, and dendritic cells, and represent a relatively pure preparation of immature T cells. Thymocytes were employed in this set of experiments specifically to determine the expression pattern of Smad proteins in T cells. Polyclonal antibodies recognizing proline-rich linker regions revealed a 62 kD band corresponding to Smad2 (**Figure 31a**), 56 kD band corresponding to Smad3 (**Figure 31b**), and a 68 kD band corresponding to Smad4 (**Figure 31c**). A second antibody against Smad4 demonstrated less cross-reactivity with the other Smads (**Figure 31d**).

Figure 30. Effect of TGF-β_1 on the *in vitro* AFC response to LPS. Spleens from naïve (NA) female B6C3F1 mice were isolated aseptically and made into single cell suspensions. The splenocytes were washed and adjusted to 5 X 10^6 c/mL and transferred in 500-µL to the wells of a 48-well culture plate. Quadruplicate cultures were prepared with NA, vehicle (VH; 0.02 % PBS, final concentration in culture, pH 3.5, containing 0.1% BSA) or TGF-β_1 (0.0003, 0.0006, 0.001, 0.002, 0.005, 0.01, 0.02, 0.04, 0.08, 0.2, 0.3, 0.6, 1.25, or 2.5 ng/mL final concentration in culture), and activated with LPS (10 µg/mL LPS). Cultures were subsequently analyzed for a day 3 antibody response by enumerating the number of AFC; spleen cell viability and total recovered cells/culture were also determined. The results from quadruplicate determinations are expressed as the mean AFC per 10^6 recovered viable cells $\pm$ SE (n=4). *$p < 0.05$ as determined by Dunnett's t test as compared to the vehicle control. The results are representative of three independent experiments.

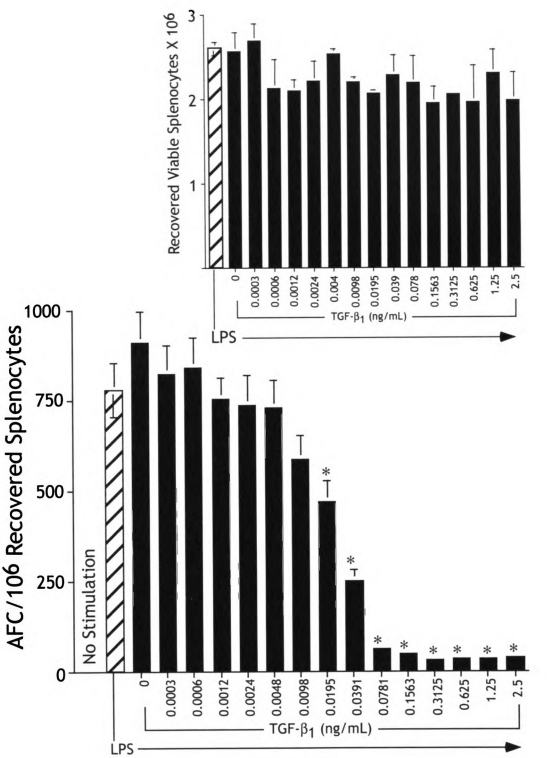
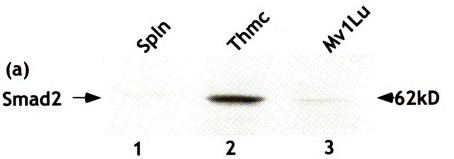
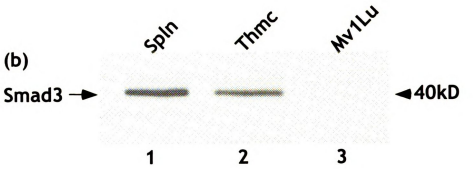
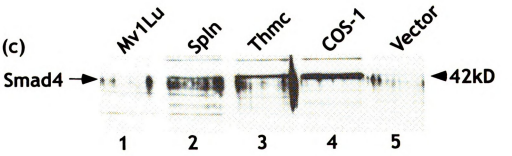
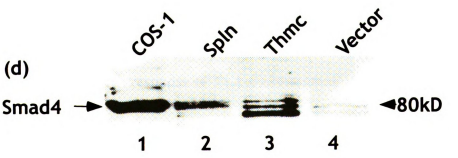

AFC/10^6 Recovered Splenocytes
1000
750
500
250
0
No Stimulation
LPS
0
0.0003
0.0006
0.0012
0.0024
0.0048
0.0098
0.0195
0.0391
0.0781
0.1563
0.3125
0.625
1.25
2.5
TGF-β1 (ng/mL)
*
Recovered Viable Splenocytes X 10^6
3
2
1
LPS
0
0.0003
0.0006
0.0012
0.0024
0.004
0.0098
0.0195
0.039
0.078
0.1563
0.3125
0.625
1.25
2.5
TGF-β1 (ng/mL)

Figure 31. Smad2, Smad3, and Smad4 protein expression in mouse splenocytes and thymocytes. Whole cell protein lysates obtained from splenocytes, Mv1Lu cells, COS-1 cells transfected to overexpress Smad4, or COS-1 transfected with the vector control for the Smad4 gene. Protein (25 μg) was loaded in each lane, resolved on a 10% non-denaturing SDS-PAGE gel, transferred to nitrocellulose, and incubated with 2 μg/mL anti-Smad2 (a), anti-Smad3, H-2 (b), anti-Smad4, B-8 (c), or anti-Smad4, S-20 (d). Ig horseradish peroxidase-linked secondary antibodies were used for protein detection using the ECL system


(a)
Spln
Thmc
Mv1Lu
Smad2
62kD
1
2
3


(b)
Spln
Thmc
Mv1Lu
Smad3
40kD
1
2
3


(c)
Mv1Lu
Spln
Thmc
COS-1
Vector
Smad4
42kD
1
2
3
4
5


(d)
COS-1
Spln
Thmc
Vector
Smad4
80kD
1
2
3
4

Smad4 specificity was further characterized by comparing whole cell splenic and thymic extracts with whole cell extracts from Smad4 transfected COS-1 cells (Lane 4 in **Figure 31c** and Lane 1 of **Figure 31d**). The Smad3 antibody modestly cross-reacts with Smad2, as suggested by a second slower migrating band (**Figure 31b**, lanes 1, 2, and 3). Cell lysates from Mv1Lu mink epithelial cells, commonly used as a positive control in the *in vitro* TGF-β_1 bioassay, were incorporated as a comparative TGF-β_1-responsive control in this set of experiments.

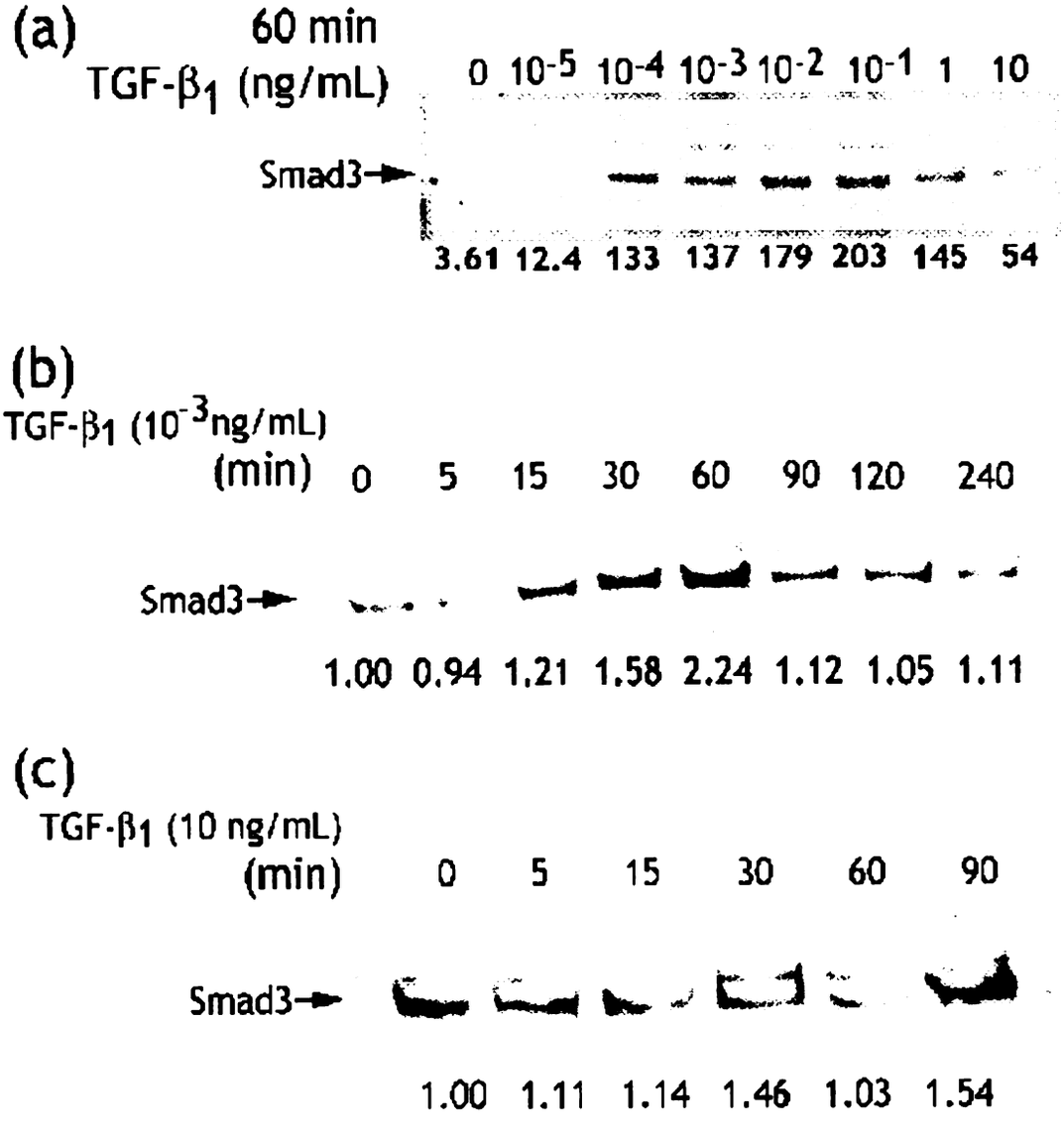
B. Concentration-dependent effects of TGF-β_1 on nuclear expression of Smad3 in mouse splenocytes

Having confirmed constitutive Smad protein expression in splenocytes, the next series of experiments investigated the responsiveness of splenic Smad3 to TGF-β_1. Splenocytes were isolated from naïve B6C3F1 mice, cultured in the presence of TGF-β_1 for 60 minutes, and nuclear lysates were isolated for Western blot analyses. TGF-β_1 enhanced Smad3 nuclear protein expression in a bimodal, concentration-dependent manner (**Figure 32a**). Maximal nuclear expression of Smad3 was demonstrated in response to a 10^{-2} - 10^{-3} ng/mL TGF-β_1 treatment. A mechanistic understanding of how Smads are regulated once they enter the nucleus is required to determine whether these results reflect increased Smad3 nuclear translocation or enhanced nuclear stabilization.

C. Temporal response of TGF-β_1-induced activation of Smad3 in mouse splenocytes

The temporal response of Smad3 activation by TGF-β_1 was investigated. Splenocytes were isolated from naïve B6C3F1 mice, treated with TGF-β_1 (10^{-3} ng/mL or

Figure 32. Concentration- and time-dependent effect of TGF-β_1 on nuclear expression of Smad3 in mouse splenocytes. Splenocytes (5 X10^6 c/mL) were treated with (a) increasing concentrations of TGF-β_1 for 60 minutes, (b) 10^{-3} ng/mL TGF-β_1 for 0, 5, 15, 30, 60, 90, 120, and 240 minutes, or (c) 10 ng/mL TGF-β_1 for 0, 5, 15, 30, 60, and 90 minutes. Nuclear proteins (25 μg) were isolated and resolved on a 10% denaturing polyacrylamide gel. Proteins were transferred to nitrocellulose and incubated with a mouse polyclonal Smad3. An anti-mouse Ig horseradish peroxidase-linked secondary antibody were used for protein detection using the ECL system. The fold induction of Smad expression is shown as intensity relative to vehicle control (0 ng/mL TGF-β_1; 0.02 % PBS, pH 3.5, containing 0.1% BSA).


(a)
60 min
TGF-β1 (ng/mL)
0 10-5 10-4 10-3 10-2 10-1 1 10
Smad3
3.61 12.4 133 137 179 203 145 54
(b)
TGF-β1 (10-3ng/mL)
(min)
0 5 15 30 60 90 120 240
Smad3
1.00 0.94 1.21 1.58 2.24 1.12 1.05 1.11
(c)
TGF-β1 (10 ng/mL)
(min)
0 5 15 30 60 90
Smad3
1.00 1.11 1.14 1.46 1.03 1.54

10 ng/mL) for 0, 5, 15, 30, 60, 90, 120, or 240 minutes, and nuclear lysates were isolated for Western blot analyses. Increased nuclear expression of Smad3 protein by TGF-β_1 (10^{-3} ng/mL) was detected at 15 minutes (**Figure 32b**). Peak nuclear Smad3 expression was observed at 60 minutes and near basal expression levels were detected 240 minutes after treatment. Ten ng/mL TGF-β_1 only modestly increased Smad3 nuclear expression (**Figure 32c**).

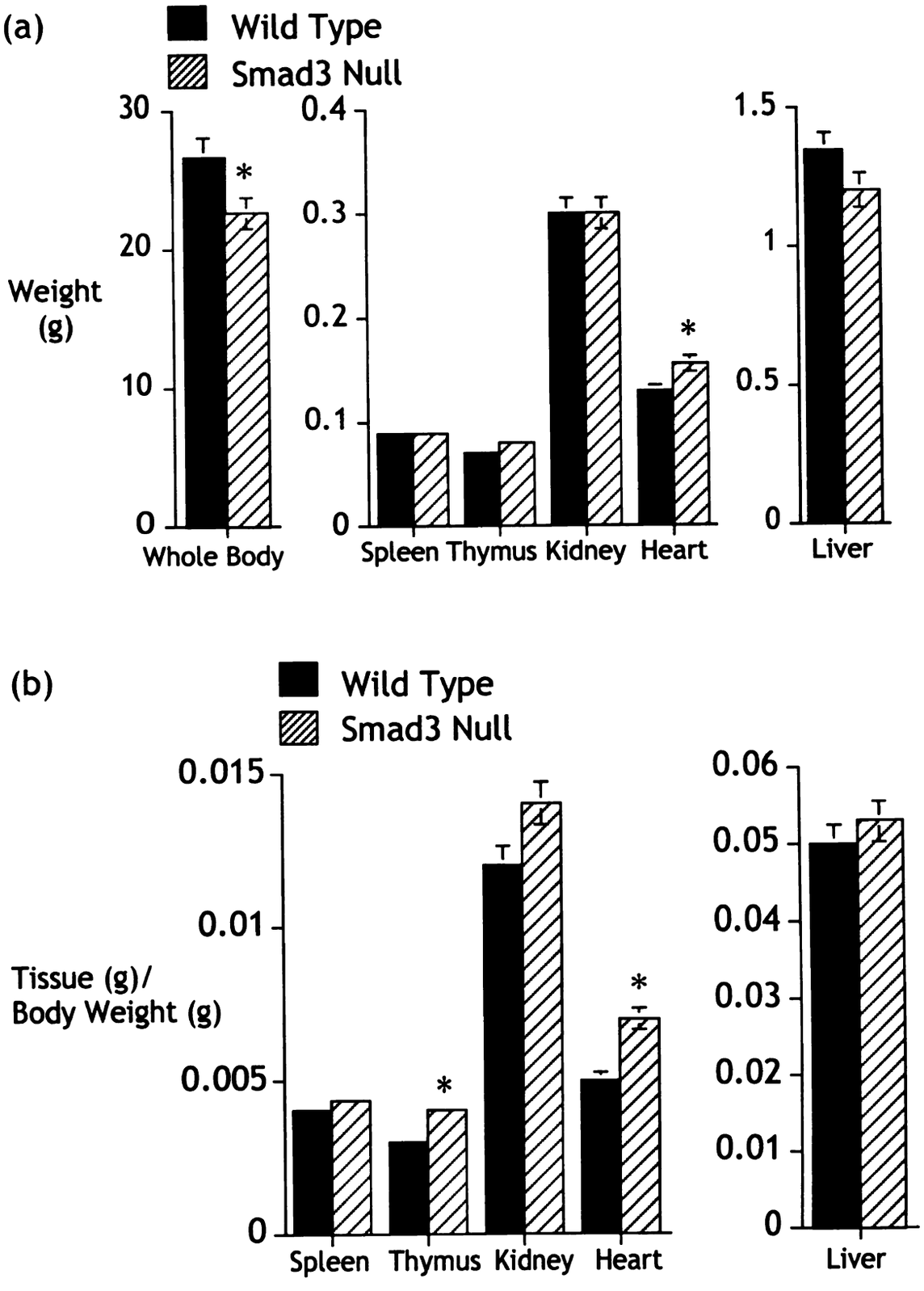
IV. Pathology of Smad3-null mice

Smad3-null mice were used to investigate a role for Smad3 in modulation of IL-2 expression by TGF-β_1. Mice homozygous for a null mutation in the gene encoding Smad3 ($Smad3^{-/-}$) are phenotypically indistinguishable from their littermate controls at birth, except 70% of $Smad3^{-/-}$ are smaller prior to weaning (Yang *et al.* 1999). Usually within 3 months of age, these $Smad3^{-/-}$ mice develop a wasting syndrome that is characteristically associated with multifocal formation of pyogenic abscesses, kyphotic posture, enlarged lymph nodes, reduced spleen weight, and an involuted thymus (Yang *et al.* 1999).

A. Body and organ weights

Grossly asymptomatic $Smad3^{-/-}$ mice were used in these studies as demonstrated by an absence of periorbital and peridontal pyogenic abscesses, absence of kyphotic posture, and similar body weight relative to littermate controls. Collectively, the $Smad3^{-/-}$ mice used in these studies were of slightly lower body weight than the $Smad3^{+/+}$ littermates (**Figure 33**). The $Smad3^{-/-}$ mice were further examined to ascertain any differences in gross organ weights. Organ weights are reported as tissue weight (**Figure**

Figure 33. Effects of targeted deletion of Smad3 on body and organ weights. Animal body, spleen, thymus, kidney, heart, and liver weights were recorded at necropsy from the Smad3-null mice and age-matched wild type littermates used in these studies. All mice were aged 6-9 wks. The data are reported as (a) tissue weight (g) or (b) tissue weight (g)/body weight (g) and are expressed as the mean $\pm$ SE (n = 12/group).


(a)
Wild Type
Smad3 Null
Weight (g)
30
20
10
0
0.4
0.3
0.2
0.1
0
1.5
1
0.5
0
*
Whole Body
Spleen
Thymus
Kidney
Heart
Liver
(b)
Wild Type
Smad3 Null
Tissue (g)/ Body Weight (g)
0.015
0.01
0.005
0
0.06
0.05
0.04
0.03
0.02
0.01
0
*
Spleen
Thymus
Kidney
Heart
Liver

33a) or as tissue weight per total body weight (**Figure 33b**) to account for the difference in body weights between the Smad3$^{-/-}$ and Smad3$^{+/+}$ mice. When compared on a tissue per body weight basis, spleen, kidney, and liver weights were similar; however, thymus and heart weights were elevated in the Smad3$^{-/-}$ mice (**Figure 33b**).

B. Thymic and splenic cellularity

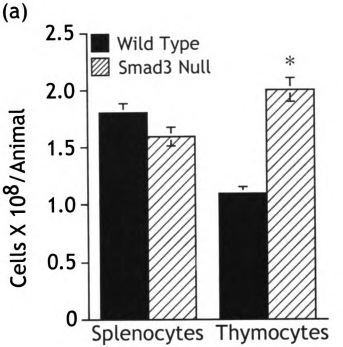
A more extensive analysis was conducted on the immune target organs of interest by assessing spleen and thymic cellularity. Single cell suspensions of splenocytes and thymocytes were obtained and counted on a per individual animal basis, and reported as either cells per mouse or cells per total body weight in order to compensate for the smaller size of the Smad3$^{-/-}$ mice (**Figure 34**). Interestingly, thymic cellularity was increased in the Smad3$^{-/-}$ mice relative to littermates suggesting a putative role for Smad3 in thymic T cell development.

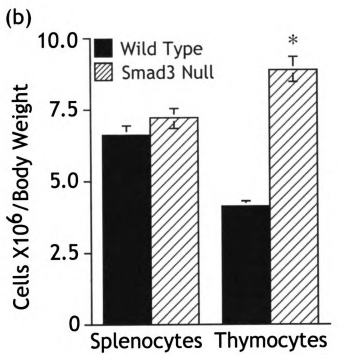
V. A role for Smad3 in the inhibition of IL-2 expression by TGF-β_1

A. Inhibition of IL-2 protein secretion by TGF-β_1 is attenuated in α-CD3 + α-CD28-activated Smad3-null splenic T cells and thymocytes

The objective of these studies was to investigate a role for Smad3 in the inhibition of IL-2 protein secretion by TGF-β_1 in activated T cells. Splenocytes and thymocytes were isolated from Smad3$^{-/-}$ mice and activated *in vitro* with α-CD3 + α-CD28. Following 24 hours in culture, supernatants were collected and IL-2 protein secretion was quantified by ELISA. TGF-β_1 attenuated IL-2 protein secretion in α-CD3 + α-CD28-activated splenic T cells and thymocytes from wild type littermate control mice

Figure 34. Splenic and thymic cellularity in Smad3-null mice. Cell counts were obtained from single cell isolations of splenocytes and thymocytes from asymptomatic Smad3-null mice (aged 6-9 wks of age) and age-matched wild type littermates. The data are reported as (a) cells X 10^8/animal or (b) cells X 10^6/ body weight (g) and are expressed as the mean $\pm$ SE (n = 12/group).


(a)
Wild Type
Smad3 Null
*
Cells X 10^8/Animal
2.5
2.0
1.5
1.0
0.5
0
Splenocytes
Thymocytes

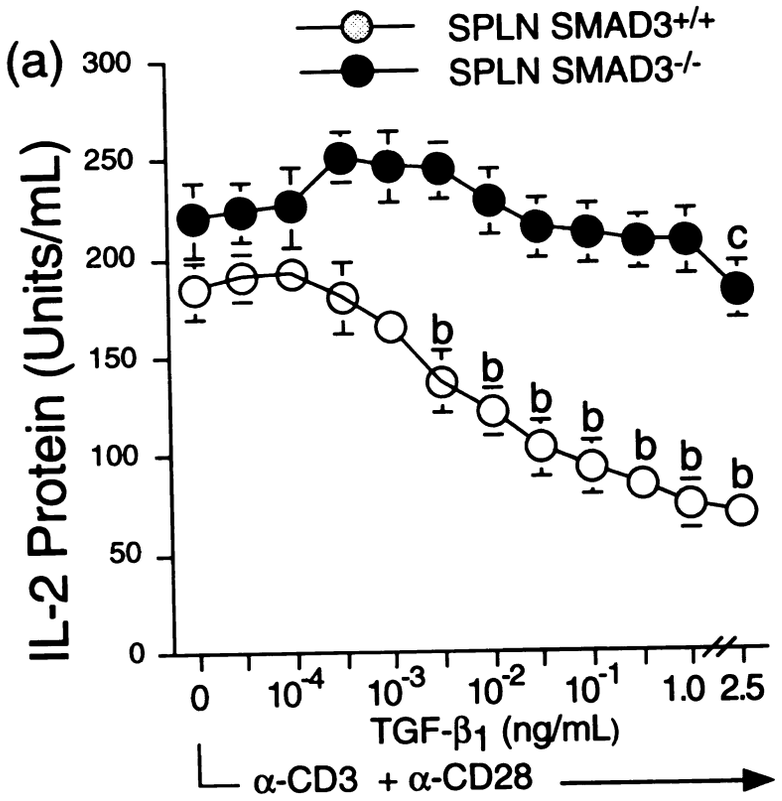

(b)
Wild Type
Smad3 Null
*
Cells X10^6/Body Weight
10.0
7.5
5.0
2.5
0
Splenocytes
Thymocytes

(Smad3$^{+/+}$) in a concentration-dependent manner (**Figure 35a** and **36a**, respectively). Inhibition of IL-2 protein secretion by TGF-β_1 was markedly attenuated in α-CD3 + α-CD28-activated Smad3$^{-/-}$ splenic T cells and thymocytes (**Figure 35a** and **36a,** respectively). Notably, the magnitude of α-CD3 + α-CD28-induced IL-2 secretion was markedly reduced in SMAD3$^{-/-}$ thymocytes relative to SMAD3$^{+/+}$ thymocytes (**Figure 36b**). IL-2 protein secretion was detected in supernatants from untreated SMAD3$^{-/-}$ splenocytes (**Figure 35b**). The magnitude of α-CD3 + α-CD28-induced IL-2 secretion was also elevated in splenocytes from SMAD3$^{-/-}$ relative to SMAD3$^{+/+}$ mice (**Figure 35b**). In contrast, IL-2 protein secretion was not elevated in untreated SMAD3$^{-/-}$ thymocytes. Collectively these results suggest a role for Smad3 in regulating IL-2 protein secretion by TGF-β_1 *in vitro*. These results also suggest that Smad3 may play a role in regulating IL-2 protein expression *in vivo*.

A. Inhibition of steady state IL-2 mRNA by TGF-β_1 is attenuated in activated Smad3-null splenic T cells and thymocytes

Having established a role for Smad3 in IL-2 protein secretion, the next objective was to investigate a putative role for Smad3 in regulating IL-2 transcription. Splenocytes and thymocytes were isolated from Smad3$^{-/-}$ mice and activated *in vitro* with α-CD3 + α-CD28. Following one hour in culture, total RNA was isolated for quantitative RT-PCR. TGF-β_1 inhibited steady state IL-2 mRNA expression in α-CD3 + α-CD28-activated splenocytes and thymocytes from Smad3$^{+/+}$ mice in a concentration-dependent manner (**Figure 37a** and **38a**, respectively). Inhibition of α-CD3 + α-CD28-induced steady state IL-2 mRNA by TGF-β_1 was abrogated in Smad3$^{-/-}$ splenic T cells and thymocytes (**Figures 37a** and **38a**, respectively). Steady state IL-2 mRNA expression was detected

Figure 35. Inhibition of IL-2 protein secretion by TGF-β_1 is attenuated in α-CD3 + α-CD28-activated Smad3-null splenic T cells. Splenocytes were activated with immobilized α-CD3 (2 μg/mL) + α-CD28 (1 μg/mL) in the presence (a) or absence (b) of TGF-β_1, as indicated for 24 hours at 37^{0}C. IL-2 was determined by ELISA. The data are expressed as the mean $\pm$ SE of three separate experiments of triplicate cultures. "a" denotes $p < 0.05$, $SMAD3^{-/-}$ significantly different from the $SMAD3^{+/+}$. "b" denotes $p < 0.05$, $SMAD3^{+/+}$ significantly different from vehicle. "c" denotes $p < 0.05$, $SMAD3^{-/-}$ significantly different from vehicle. N.D., IL-2 protein below the level of detection.


(a)
SPLN SMAD3+/+
SPLN SMAD3-/-
IL-2 Protein (Units/mL)
300
250
200
150
100
50
0
a
b
b
b
b
b
b
c
0
10-4
10-3
10-2
10-1
1.0
2.5
TGF-β1 (ng/mL)
α-CD3 + α-CD28

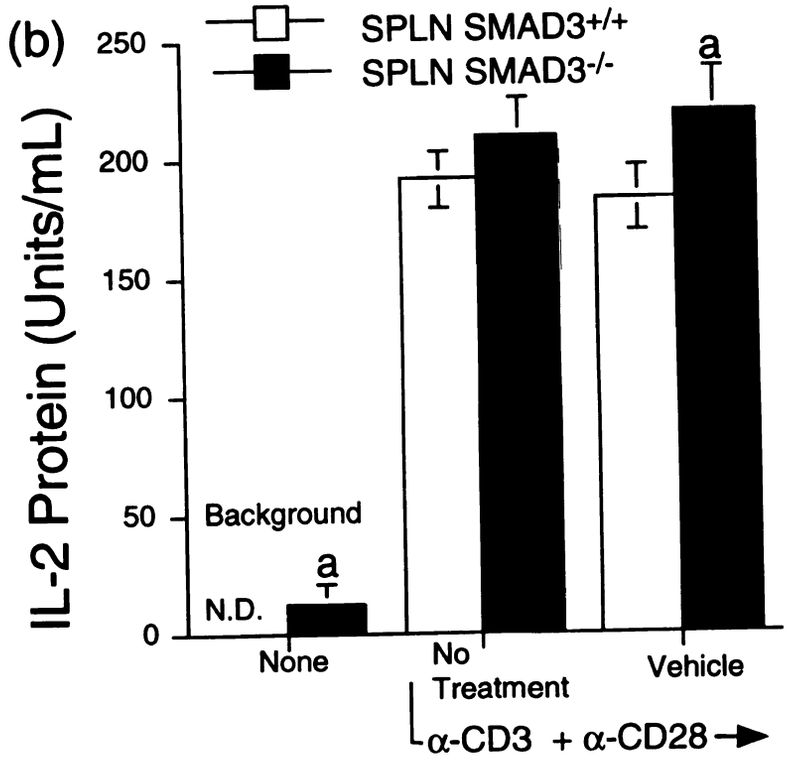
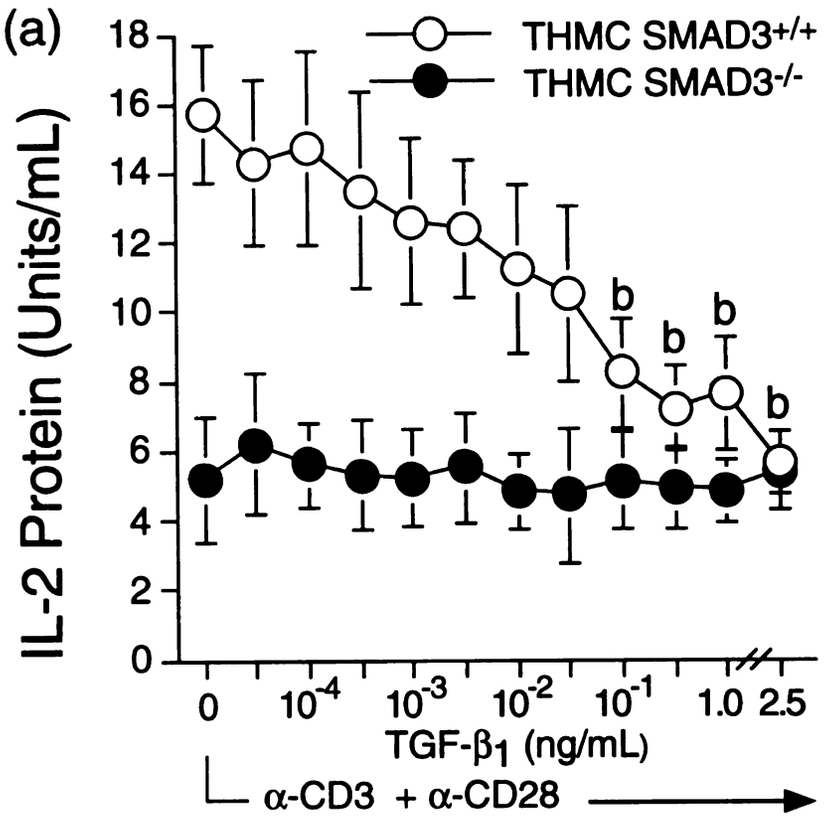

(b)
SPLN SMAD3+/+
SPLN SMAD3-/-
IL-2 Protein (Units/mL)
250
200
150
100
50
0
Background
N.D.
a
a
None
No Treatment
Vehicle
α-CD3 + α-CD28

Figure 36. Inhibition of IL-2 protein secretion by TGF-β_1 is attenuated in α-CD3 + α-CD28-activated Smad3-null thymocytes. Thymocytes were activated with immobilized α-CD3 (2 μg/mL) + α-CD28 (1 μg/mL) in the presence (a) or absence (b) of TGF-β_1, as indicated for 24 hours at 37^0C. IL-2 was determined by ELISA. The data are expressed as the mean $\pm$ SE of three separate experiments of triplicate cultures. "a" denotes $p < 0.05$, $SMAD3^{-/-}$ significantly different from the $SMAD3^{+/+}$. "b" denotes $p < 0.05$, $SMAD3^{+/+}$ significantly different from vehicle. "c" denotes $p < 0.05$, $SMAD3^{-/-}$ significantly different from vehicle. N.D., IL-2 protein below the level of detection.


(a)
THMC SMAD3+/+
THMC SMAD3-/-
IL-2 Protein (Units/mL)
18
16
14
12
10
8
6
4
2
0
b
b
b
0
10-4
10-3
10-2
10-1
1.0
2.5
TGF-β1 (ng/mL)
α-CD3 + α-CD28

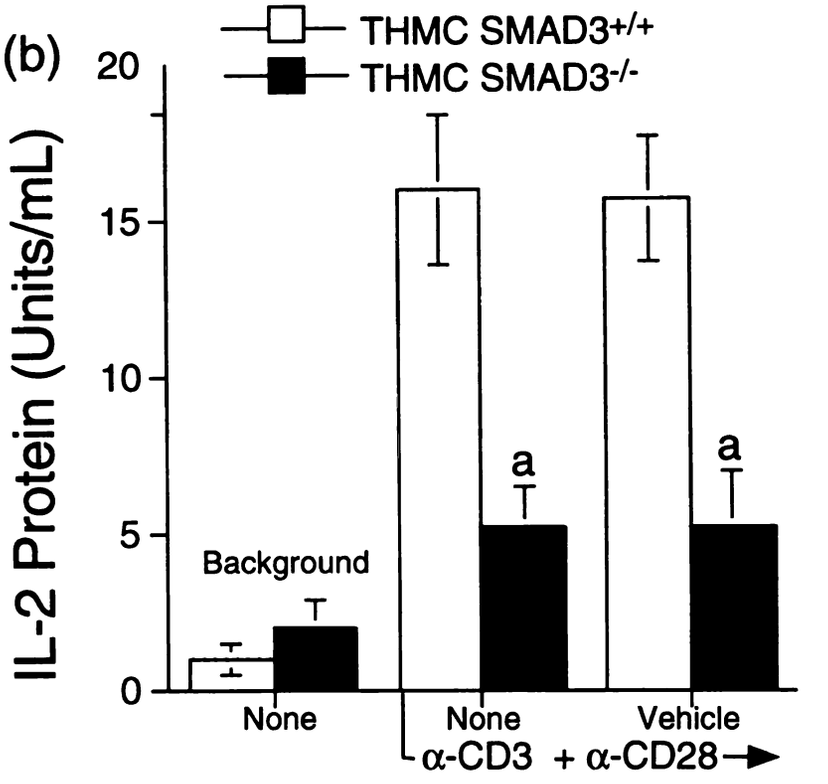
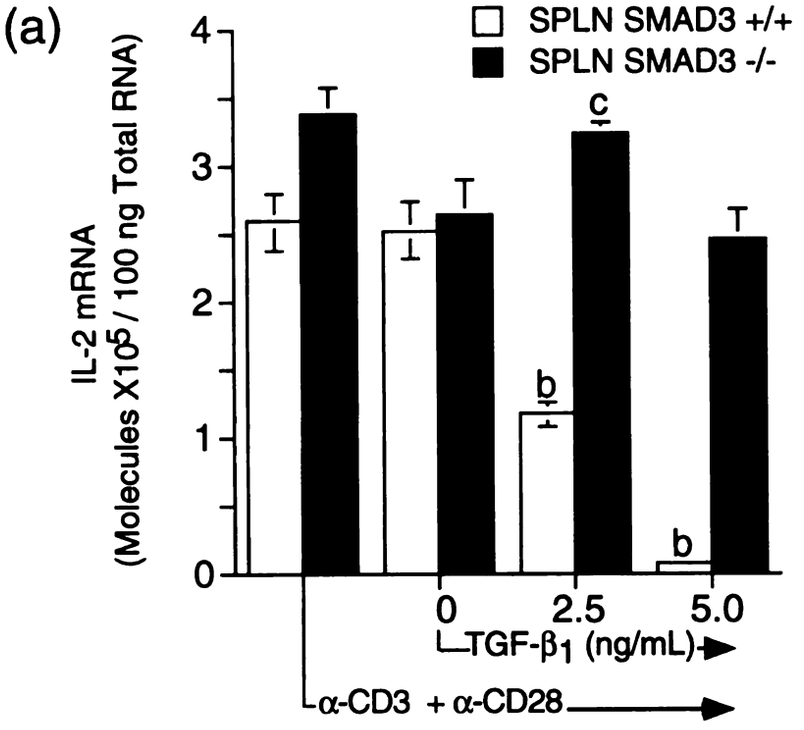

(b)
THMC SMAD3+/+
THMC SMAD3-/-
IL-2 Protein (Units/mL)
20
15
10
5
0
a
a
Background
None
None
Vehicle
α-CD3 + α-CD28

Figure 37. Inhibition of steady state IL-2 mRNA by TGF-β_1 is attenuated in activated Smad3-null splenic T cells. Splenocytes were activated with immobilized α-CD3 + α-CD28 in the presence (a) or absence (b) of TGF-β_1, as indicated for 2 hours at 37^0C. IL-2 mRNA was analyzed by quantitative RT-PCR. The data are expressed as the mean $\pm$ SE of triplicate cultures from two separate experiments. "a" denotes $p < 0.05$, $Smad3^{-/-}$ significantly different from $Smad3^{+/+}$. "b" denotes $p < 0.05$, $Smad3^{+/+}$ significantly different from vehicle. "c" denotes $p < 0.05$, $Smad3^{-/-}$ significantly different from vehicle.


(a)
SPLN SMAD3 +/+
SPLN SMAD3 -/-
IL-2 mRNA
(Molecules X10^5 / 100 ng Total RNA)
4
3
2
1
0
a
b
c
b
0
2.5
5.0
TGF-β$_1$ (ng/mL)
α-CD3 + α-CD28

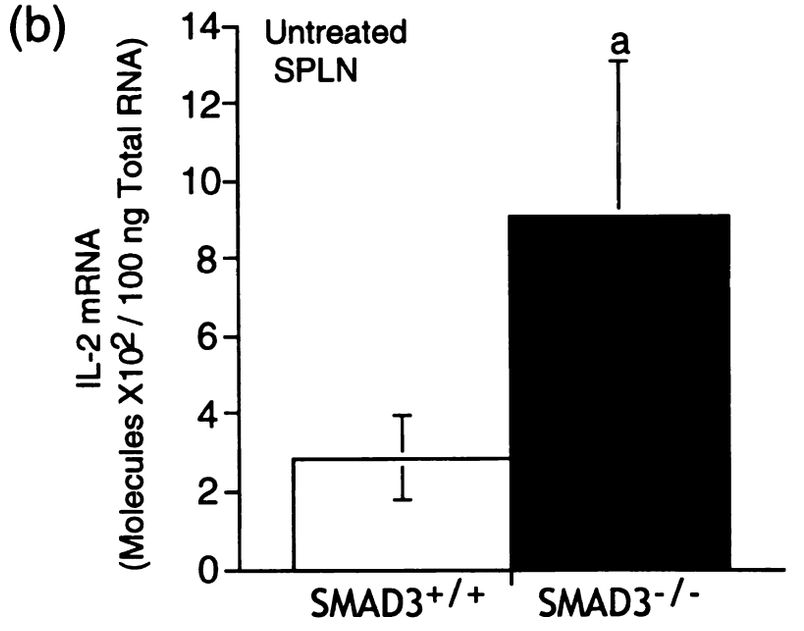
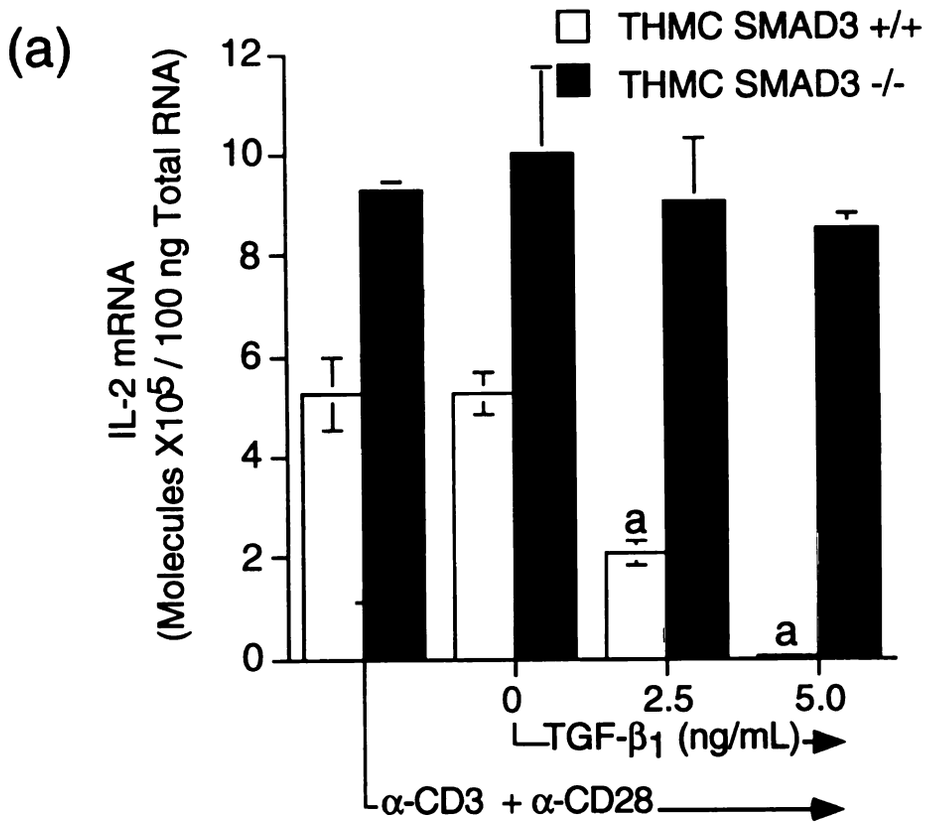
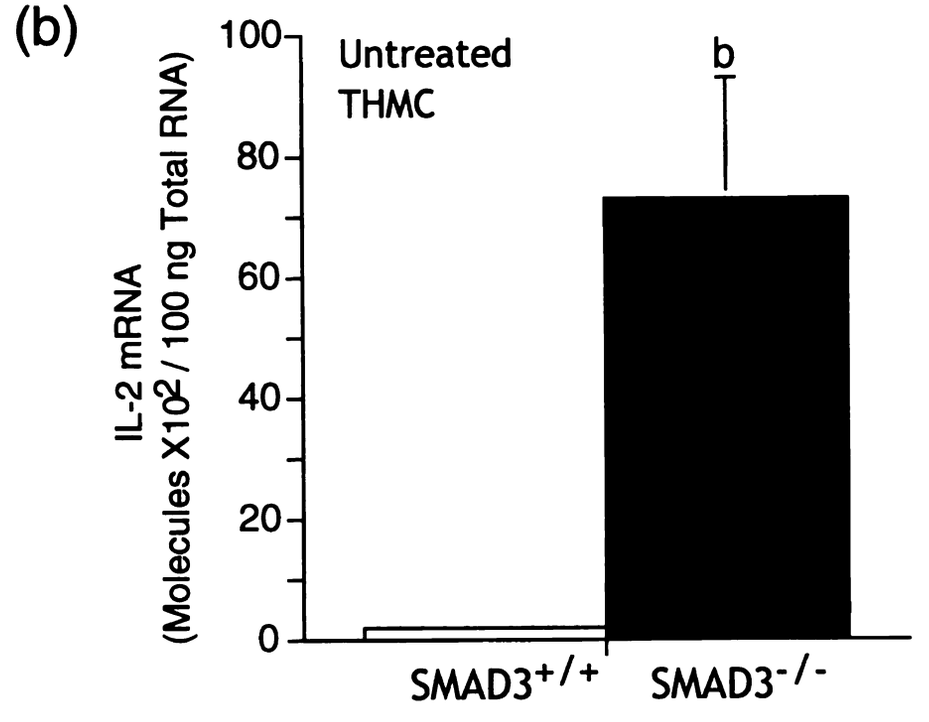

(b)
Untreated
SPLN
IL-2 mRNA
(Molecules X10^2 / 100 ng Total RNA)
14
12
10
8
6
4
2
0
a
SMAD3$^{+/+}$
SMAD3$^{-/-}$

Figure 38. Inhibition of steady state IL-2 mRNA by TGF-β_1 is attenuated in activated Smad3-null thymocytes. Thymocytes were activated with immobilized α-CD3 + α-CD28 in the presence (a) or absence (b) of TGF-β_1, as indicated for 2 hours at 37^0C. IL-2 mRNA was analyzed by quantitative RT-PCR. The data are expressed as the mean $\pm$ SE of two triplicate cultures from two separate experiments. "a" denotes $p < 0.05$, $Smad3^{+/+}$ significantly different from vehicle. "b" denotes $p < 0.05$, $Smad3^{-/-}$ significantly different from $Smad3^{+/+}$. "c" denotes $p < 0.05$, $Smad3^{-/-}$ significantly different from vehicle.


(a)
THMC SMAD3 +/+
THMC SMAD3 -/-
IL-2 mRNA
(Molecules X10^5 / 100 ng Total RNA)
12
10
8
6
4
2
0
a
a
0
2.5
5.0
TGF-β1 (ng/mL)
α-CD3 + α-CD28

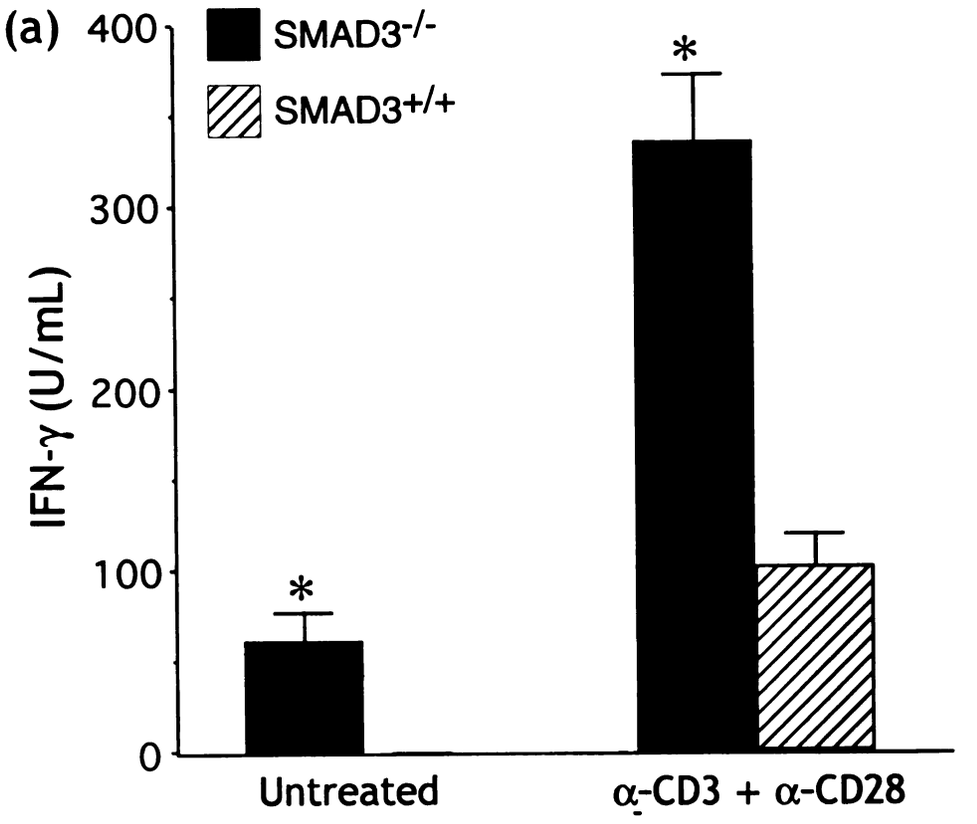

(b)
Untreated
THMC
b
IL-2 mRNA
(Molecules X10^2 / 100 ng Total RNA)
100
80
60
40
20
0
SMAD3+/+
SMAD3-/-

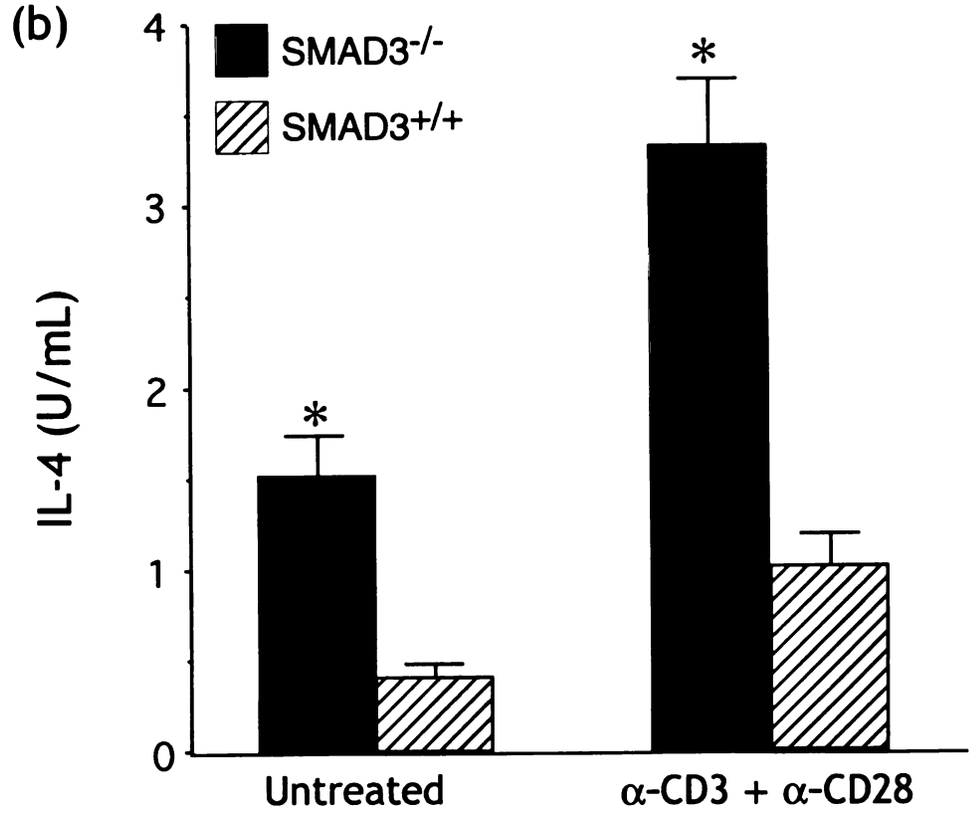
in untreated Smad3$^{-/-}$ splenocytes and Smad3$^{-/-}$ thymocytes, (**Figure 37b** and **38b,** respectively). The magnitude of α-CD3 + α-CD28-induced steady state IL-2 mRNA expression was greater in splenocytes and thymocytes from Smad3$^{-/-}$ mice relative to Smad3$^{+/+}$ mice (**Figure 37a** and **38a,** respectively). Collectively, these results suggest that autocrine TGF-β_1 may regulate both the baseline and activation-induced IL-2 expression in a manner that is dependent on Smad 3 expression.

B. Elevated IL-4 and IFN-γ expression in Smad3-null splenocytes

Elevated IL-2 expression in untreated Smad3$^{-/-}$ splenocytes relative to Smad3$^{+/+}$ splenocytes (**Figure 37** and **Figure 38**) suggest an activated *in vivo* T cell phenotype in Smad3$^{-/-}$ mice. In light of this observation, the objective of this series of experiments was to investigate T cell differentiation in Smad3$^{-/-}$ mice as determined by cytokine production. Splenocytes were isolated from Smad3$^{-/-}$ mice, cultured *in vitro* in the presence or absence of plate-bound α-CD3 + α-CD28 for 24 hours, and supernatants were collected for ELISA analyses. IFN-γ protein was quantified as a marker of T_H1 differentiation and IL-4 protein was quantified to assess T_H2 differentiation. The magnitude of IL-4 and especially IFN-γ protein expression in untreated splenocytes was greater in Smad3$^{-/-}$ mice than Smad3$^{+/+}$ mice (**Figure 39**). Moreover, the magnitude of α-CD3 + α-CD28-induced IFN-γ and IL-4 protein was increased in Smad3$^{-/-}$ relative to Smad$^{+/+}$ splenocytes. These results indicate that Smad3 may play a regulatory role in T cell differentiation and further establish that Smad3$^{-/-}$ splenic T cells can be induced to secrete IFN-γ as well as IL-4 *in vitro.*

Figure 39. Elevated basal IL-4 and IFN-γ expression in Smad3-null splenocytes. Splenocytes were cultured in uncoated (Untreated) or α-CD3 + α-CD28-precoated tissue culture plates, as indicated, for 24 hours at 37°C. (a) IFN-γ and (b) IL-4 were determined by ELISA. The data are expressed as the mean ± SE (n =8). *,$p < 0.05$, $Smad3^{-/-}$ significantly different from $Smad3^{+/+}$.


(a)
400
300
200
100
0
IFN-γ (U/mL)
SMAD3-/-
SMAD3+/+
*
*
Untreated
α-CD3 + α-CD28


(b)
4
3
2
1
0
IL-4 (U/mL)
SMAD3-/-
SMAD3+/+
*
*
Untreated
α-CD3 + α-CD28

C. TGF-β_1-induced phosphorylation of Smad2 is not disrupted in Smad3 null splenocytes

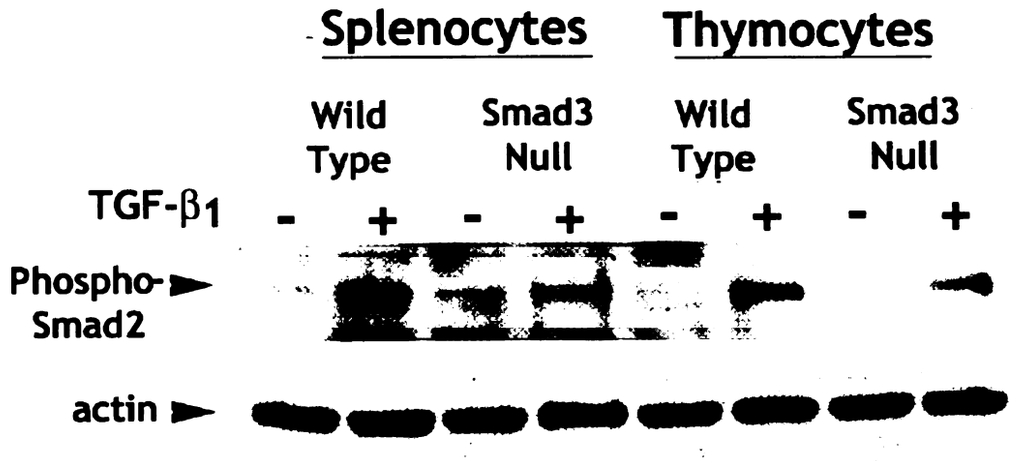
It has been demonstrated that IFN-γ negatively regulates Smad signaling by increasing the activity of the inhibitor Smad, Smad7 (Ulloa *et al.*, 1999). In light of the observed elevated IFN-γ expression in Smad3$^{-/-}$ splenocytes and thymocytes, the objective of these experiments was to verify the 'functionality' of Smad2 signaling in Smad3$^{-/-}$ T cells. Splenocytes and thymocytes were isolated from Smad3$^{-/-}$ mice and treated *in vitro* with TGF-β_1 (10^{-3} ng/mL) for 60 minutes. Western blot analysis of whole cell lysates demonstrated TGF-β_1-induced Smad2 phosphorylation in Smad3$^{-/-}$ splenocytes as well as thymocytes and thus verified Smad2 responsiveness to TGF-β_1 (**Figure 40a**). Moreover, Smad3 protein expression was not detected in whole cell lysates from Smad3$^{-/-}$ splenocytes or thymocytes (**Figure 40b**).

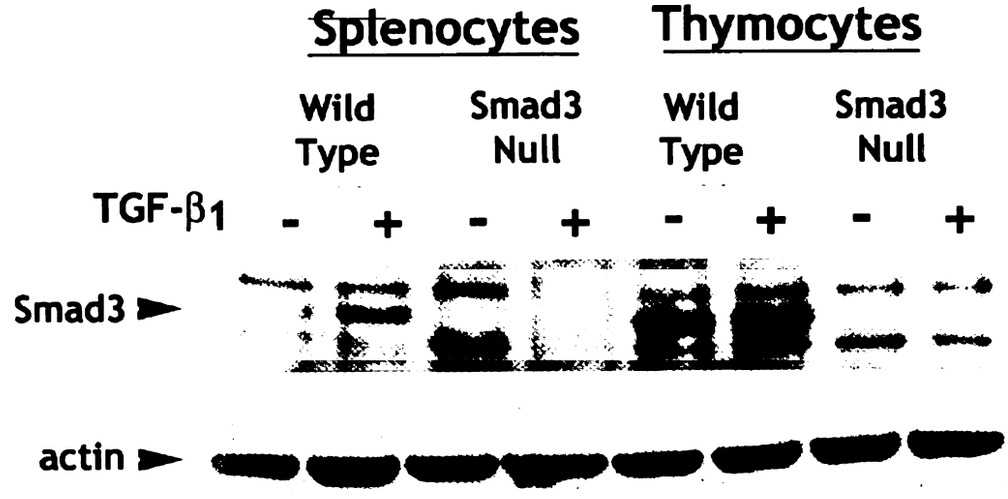
VI. A role for Smad3 in the inhibition of lymphocyte proliferation by TGF-β_1

A. Growth inhibition by TGF-β_1 is attenuated in activated Smad3-null splenic T cells and thymocytes

Having established that Smad3 is critical for regulating IL-2 expression, this series of studies was conducted to investigate a role for Smad3 in T cell growth inhibition by TGF-β_1. Splenocytes and thymocytes were isolated from Smad3$^{-/-}$ mice and activated *in vitro* with α-CD3 + α-CD28. Following 72 hours in culture, [^{3}H]-thymidine incorporation was quantified to assess T cell proliferation. TGF-β_1 attenuated [^{3}H]-thymidine incorporation in α-CD3 + α-CD28-activated splenocytes and thymocytes from Smad3$^{+/+}$ mice in a concentration-dependent manner (**Figure 41a** and **42a**, respectively).

Figure 40. TGF-β_1-induced phosphorylation of Smad2 is not disrupted in Smad3 null splenocytes. (a) Phosphorylated Smad2 expression in Smad3$^{-/-}$ and Smad3$^{+/+}$ splenocytes and thymocytes. Whole cell lysates (50 μg) were separated on SDS-PAGE (10% polyacrylamide) under reducing conditions. Membranes were probed with a polyclonal rabbit Ab recognizing the phosphorylated form of Smad2. Following ECL detection, the membranes were washed, and reprobed with a monoclonal mouse antibody recognizing actin (as a loading control). (b) Smad3 expression in Smad3$^{-/-}$ and Smad3$^{+/+}$ splenocytes and thymocytes. Whole cell lysates (25 μg) were separated on SDS-PAGE (10% polyacrylamide) under reducing conditions. Membranes were probed with a polyclonal rabbit Ab recognizing Smad3. A corresponding Ig horseradish peroxidase-linked secondary antibodies were used for protein detection using the ECL system


Splenocytes
Thymocytes
Wild Type
Smad3 Null
Wild Type
Smad3 Null
TGF-β1
- + - + - + - +
Phospho-Smad2
actin

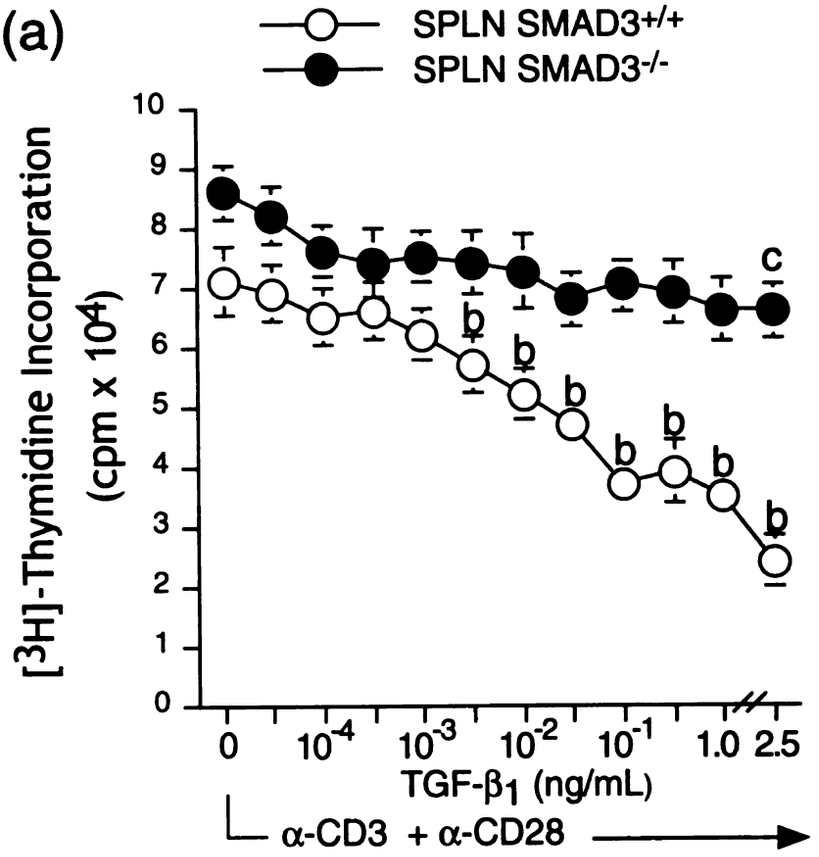

Splenocytes
Thymocytes
Wild Type
Smad3 Null
Wild Type
Smad3 Null
TGF-β1
- + - + - + - +
Smad3
actin

Growth inhibition by TGF-β_1 was significantly diminished, but not abrogated in α-CD3 + α-CD28-activated splenic T cells (**Figure 41a**) and thymocytes (**Figure 42a**). In comparison to Smad3$^{+/+}$ mice, [^{3}H]-thymidine incorporation was elevated in untreated Smad3$^{-/-}$ splenocytes but not Smad3$^{-/-}$ thymocytes (**Figure 41** and **42,** respectively). The magnitude of α-CD3 + α-CD28-induced [^{3}H]-thymidine incorporation was also greater in Smad3$^{-/-}$ splenocytes than Smad3$^{+/+}$ splenocytes (**Figure 41**). Collectively, these observations demonstrate that Smad3 is important for TGF-β_1-induced growth arrest of α-CD3 + α-CD28-activated T cells *in vitro*. These data also suggest that additional Smad3-independent mechanism(s) of growth inhibition may be activated following exposure to high concentrations of TGF-β_1.

B. TGF-β_1 inhibits LPS-induced B cell proliferation in Smad3-null splenocytes

A role for Smad3 in B cell growth inhibition by TGF-β_1 was investigated. Splenocytes were isolated from Smad3$^{-/-}$ mice and activated in culture with LPS for 48 hours prior to quantifying [^{3}H]-thymidine incorporation. TGF-β_1 attenuated [^{3}H]-thymidine incorporation in LPS-stimulated Smad3$^{+/+}$ splenocytes in a concentration-dependent manner (**Figure 43a**). In contrast to T cells, inhibition of LPS-induced B cell growth by TGF-β_1 was not disrupted in Smad3$^{+/+}$ mice (**Figure 43a**). In a manner similar to that observed in T cells, the magnitude of LPS-induced [^{3}H]-thymidine incorporation was elevated in Smad3$^{-/-}$ relative to Smad3$^{+/+}$ B cells (**Figure 43b**). In summary, these results suggest that TGF-β_1 inhibits LPS-induced B cell growth *in vitro* through a Smad3-

Figure 41. Growth inhibition by TGF-β_1 is attenuated in activated Smad3-null splenic T cells. Splenocytes were activated with immobilized α-CD3 + α-CD28 in the presence (a) or absence (b) of TGF-β_1, as indicated for 72 hours at 37^{0}C. Cell proliferation was quantified by incorporation of [^{3}H]-thymidine. The data are expressed as the mean $\pm$ SE of three separate experiments in quadruplicate. "a" denotes $p < 0.05$, Smad3$^{+/+}$ significantly different from Smad3$^{-/-}$. "b" denotes $p < 0.05$, Smad3$^{+/+}$ significantly different from vehicle. "c" denotes $p < 0.05$, Smad3$^{-/-}$ significantly different from vehicle.


(a)
SPLN SMAD3+/+
SPLN SMAD3-/-
[3H]-Thymidine Incorporation
(cpm x 10^4)
10
9
8
7
6
5
4
3
2
1
0
a
b
c
0
10-4
10-3
10-2
10-1
1.0
2.5
TGF-β1 (ng/mL)
α-CD3 + α-CD28

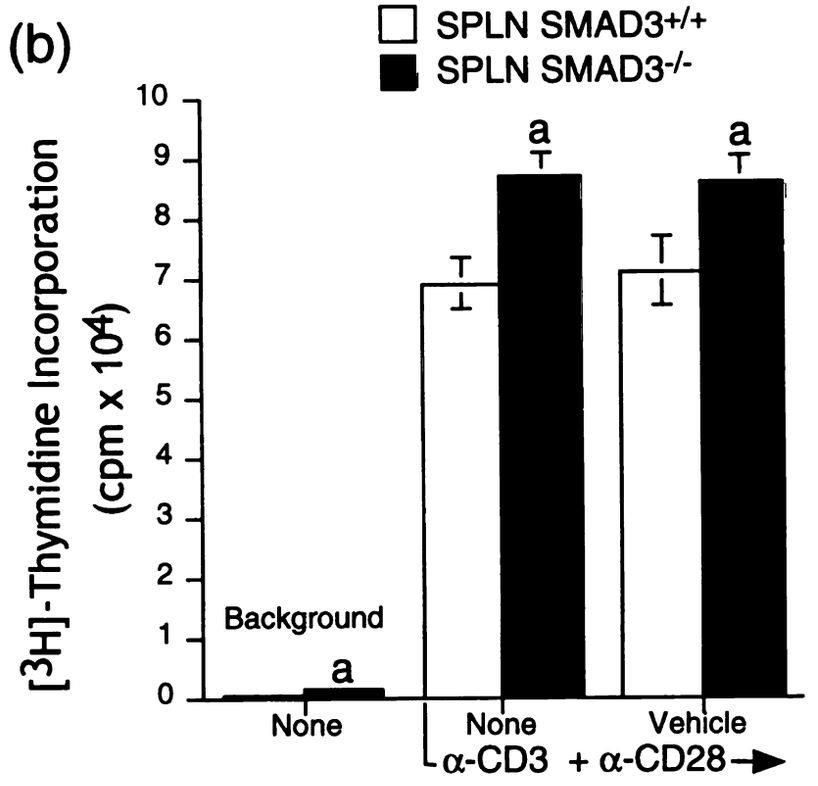
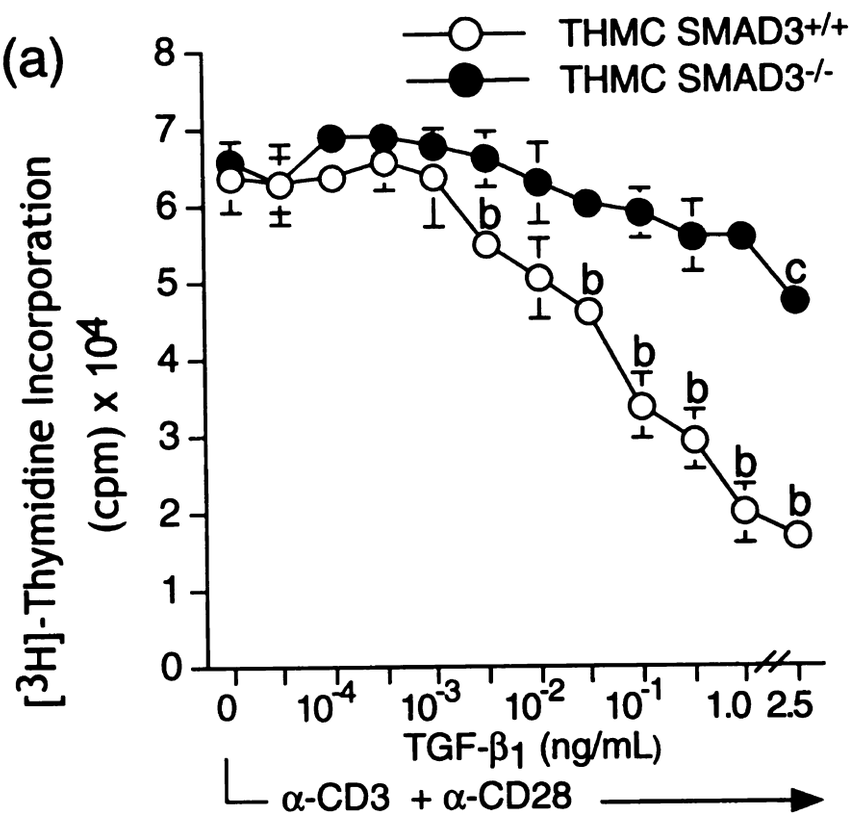

(b)
SPLN SMAD3+/+
SPLN SMAD3-/-
[3H]-Thymidine Incorporation
(cpm x 10^4)
10
9
8
7
6
5
4
3
2
1
0
Background
a
a
a
None
None
Vehicle
α-CD3 + α-CD28

Figure 42. Growth inhibition by TGF-β_1 is attenuated in activated Smad3-null thymocytes. Thymocytes were activated with immobilized α-CD3 + α-CD28 in the presence (a) or absence (b) of TGF-β_1, as indicated for 72 hours at 37^{0}C. Cell proliferation was quantified by incorporation of [^{3}H]-thymidine. The data are expressed as the mean $\pm$ SE for three separate experiments in quadruplicate. "a" denotes $p < 0.05$, Smad3$^{-/-}$ significantly different from Smad3$^{+/+}$. "b" denotes $p < 0.05$, Smad3$^{+/+}$ significantly different from vehicle. "c" denotes $p < 0.05$, Smad3$^{-/-}$ significantly different from vehicle.


(a)
THMC SMAD3+/+
THMC SMAD3-/-
[3H]-Thymidine Incorporation (cpm) x 10^4
8
7
6
5
4
3
2
1
0
b
b
b
b
b
c
0
10^-4
10^-3
10^-2
10^-1
1.0
2.5
TGF-β1 (ng/mL)
α-CD3 + α-CD28

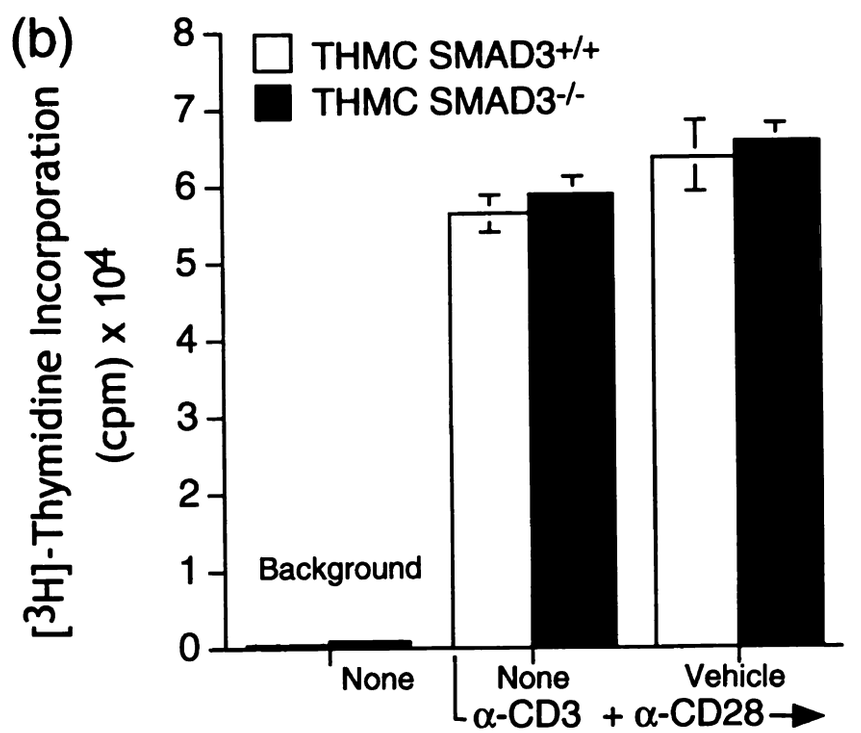
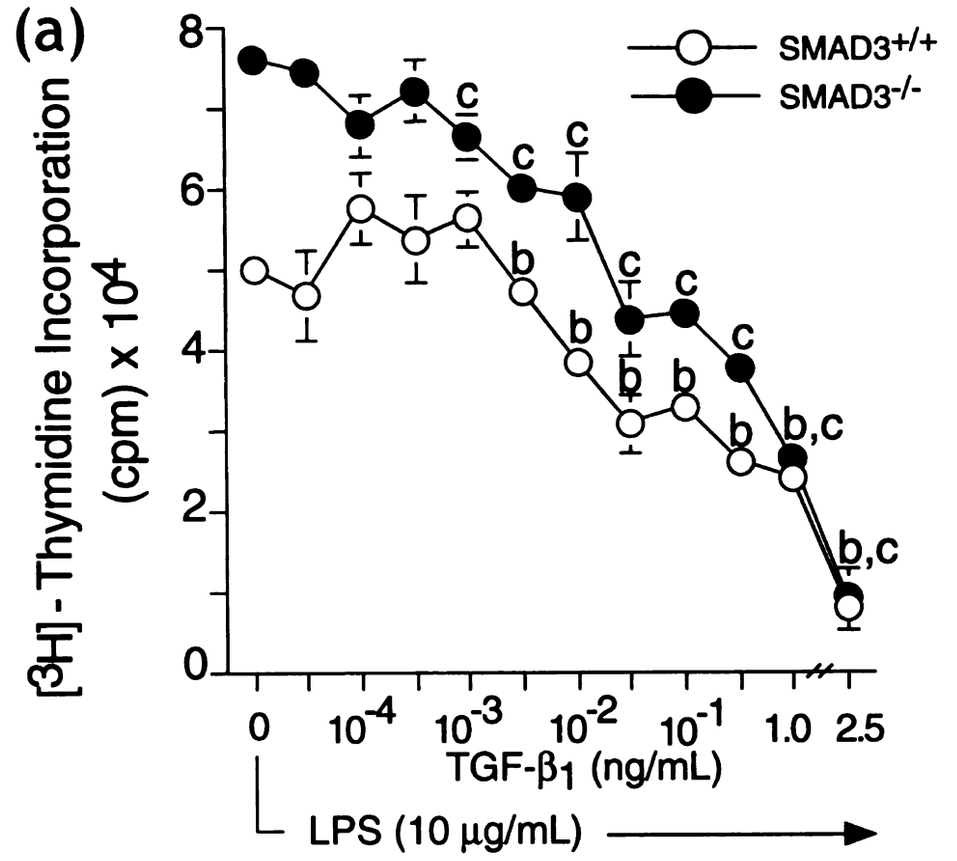
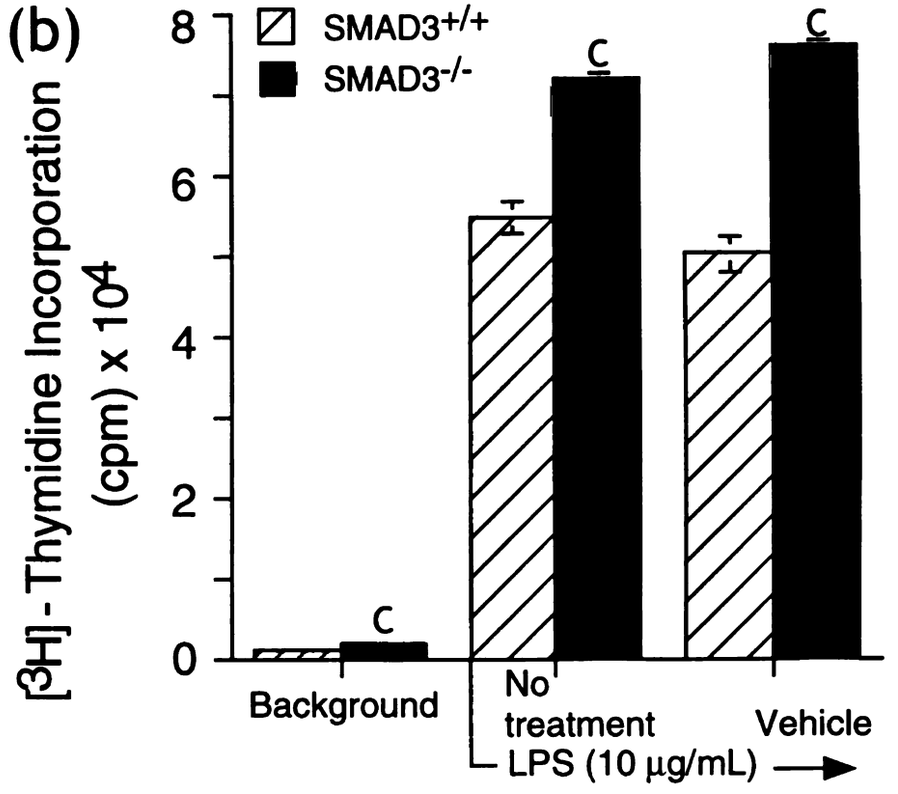

(b)
THMC SMAD3+/+
THMC SMAD3-/-
[3H]-Thymidine Incorporation (cpm) x 10^4
8
7
6
5
4
3
2
1
0
Background
None
None
Vehicle
α-CD3 + α-CD28

Figure 43. TGF-β_1 inhibits LPS-induced B cell proliferation in Smad3-null splenocytes. Splenocytes were stimulated with LPS in the presence (a) or absence (b) of TGF-β_1, as indicated for 48 hours at 37^{0}C. Cell proliferation was quantified by incorporation of [^{3}H]-thymidine. The data are expressed as the mean $\pm$ SE of three separate experiments in quadruplicate. "a" denotes $p < 0.05$, Smad3$^{-/-}$ significantly different from Smad3$^{+/+}$. "b" denotes $p < 0.05$, Smad3$^{+/+}$ significantly different from vehicle. "c" denotes $p < 0.05$, Smad3$^{-/-}$ significantly different from vehicle.


(a)
SMAD3+/+
SMAD3-/-
[3H]- Thymidine Incorporation (cpm) x 10^4
TGF-β1 (ng/mL)
LPS (10 µg/mL)


(b)
SMAD3+/+
SMAD3-/-
[3H]- Thymidine Incorporation (cpm) x 10^4
Background
No treatment
Vehicle
LPS (10 µg/mL)

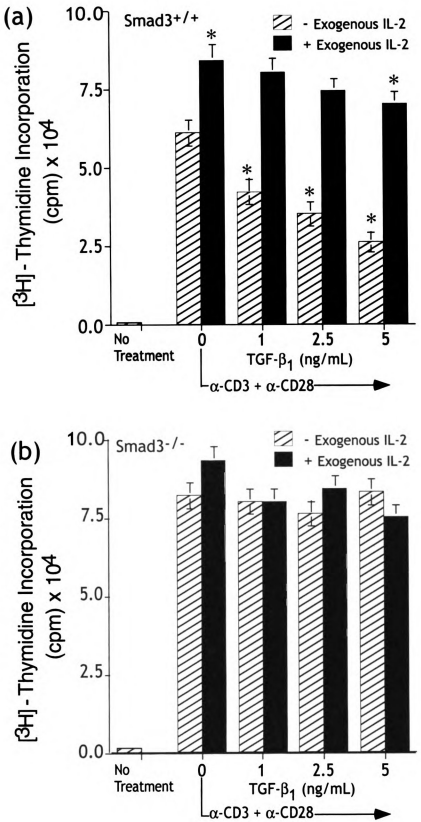
independent mechanism of action and suggests a cell-type specificity for Smad3-mediated regulation of lymphoid proliferation.

C. Exogenous IL-2 reverses TGF-β_1-induced inhibition of T cell growth

IL-2 is essential for α-CD3 + α-CD28-induced splenic T cell proliferation *in vitro*. On-the-other-hand, *in vitro* LPS-stimulated splenic B cell proliferation is not dependent upon IL-2. In light of these observations, a series of experiments were undertaken to determine whether inhibition of α-CD3 + α-CD28-induced T cell growth by TGF-β_1 was due to a direct inhibition of IL-2. [^{3}H]-thymidine incorporation was quantified in α-CD3 + α-CD28-activated splenic T cells in the presence of exogenous IL-2.

Exogenous recombinant IL-2 (50 U/mL) reversed the growth inhibitory effect of TGF-β_1 in activated SMAD3$^{+/+}$ T cells (**Figure 44a**). These results indicate that IL-2 can overcome TGF-β_1-induced inhibition of T cell growth and support a hypothesis that inhibition of α-CD3 + α-CD28-induced T cell growth by TGF-β_1 *in vitro* is mediated through a direct effect of TGF-β_1 on IL-2 production. Thus, it is tempting to speculate that attenuated TGF-β_1-induced inhibition of SMAD3$^{-/-}$ T cell growth by TGF-β_1 to due to a direct effect of Smad3 on IL-2 expression. Moreover, augmentation of proliferation by exogenous IL-2 was demonstrated in activated SMAD3$^{+/+}$ splenocytes (**Figure 44a**), but not SMAD3$^{-/-}$ splenocytes (**Figure 44b**). These results are consistent with elevated IL-2 expression in α-CD3 plus α-CD28-activated SMAD3$^{-/-}$ splenocytes relative to SMAD3$^{+/+}$ splenocytes, as discussed previously.

Figure 44. Reversal of TGF-β_1-induced inhibition of peripheral T cell proliferation by exogenous IL-2. Splenocytes obtained from (a) wild type or (b) Smad3-null splenocytes were activated with immobilized α-CD3 + α-CD28 with or without 50 U/mL IL-2 in the presence or absence of TGF-β_1, as indicated for 72 hours at 37^0C. Cell proliferation was quantified by incorporation of [^{3}H]-thymidine. The data are expressed as the mean $\pm$ SE of two separate experiments in quadruplicate. *,$p < 0.05$, Smad3$^{-/-}$ significantly different from vehicle.


(a)
Smad3+/+
- Exogenous IL-2
+ Exogenous IL-2
[3H]- Thymidine Incorporation (cpm) x 10^4
10.0
7.5
5.0
2.5
0.0
No Treatment
0
1
2.5
5
TGF-β1 (ng/mL)
α-CD3 + α-CD28
(b)
Smad3-/-
- Exogenous IL-2
+ Exogenous IL-2
[3H]- Thymidine Incorporation (cpm) x 10^4
10.0
7.5
5.0
2.5
0.0
No Treatment
0
1
2.5
5
TGF-β1 (ng/mL)
α-CD3 + α-CD28

VII. Modulation of protein binding to the IL-2 promoter by TGF-β_1

A. CAGA elements in the regulatory region of the mouse IL-2 promoter

In further investigating the mechanism(s) by which Smad3 regulates IL-2 gene transcription in response to TGF-β_1, five CAGA sequences were identified in the minimal essential regulatory region of the mouse IL-2 promoter. The four bp CAGA sequence functions as a Smad response element in the promoter of numerous TGF-β_1-responsive genes (Chen *et al.* 1999; Dennler *et al.* 1998; Jonk *et al.* 1998; Wu *et al.* 1997; Zhang *et al.* 2000; Zhang and Derynck 2000). The five CAGA sequences identified in the IL-2 promoter are located -102, -117, -145, -156, and -268 bp upstream (5') of the transcriptional start site, and lie adjacent to or overlap the distal -285 distal NFAT site, the -168 CD28RE, the -153 proximal AP-1 site, or the -113 NRE-A. The close proximity of the CAGA sequence with transcription factor binding sites in the IL-2 promoter is consistent with the cooperative interaction of Smad proteins with other transcription factors (Brodin *et al.* 2000; Chen *et al.* 1999; Kon *et al.* 1999; Liberati *et al.* 1999; Qing *et al.* 2000; Wong *et al.* 1999).

To investigate the importance of CAGA sequences for DNA binding activity in the IL-2 promoter, a pair of oligonucleotides were constructed for each of the four aforementioned DNA binding elements (i.e., the -285 NFAT site, the -168 CD28RE, the -153 proximal AP-1 site, and the -113 NRE-A) with each pair consisting of a wild type oligonucleotide containing the naïve CAGA sequence and a mutant oligonucleotide containing a mutated CAGA sequence. The sequences for each of these eight oligonucleotides are provided in **Table 2**. Splenocytes obtained from naïve B6C3F1 mice, activated in culture with α-CD3 + α-CD28 for two hours, and nuclear lysates were

obtained for EMSA analyses. Mutation of the CAGA sequence markedly shifted DNA binding complexes at the proximal AP-1 site and the CD28RE (**Figure 45**; compare lanes 1 and 3, lanes 2 and 4, lanes 5 and 7, and lanes 6 and 8). A similar shift was not observed in DNA binding complexes at the distal NFAT site or NRE-A (**Figure 45**; compare lanes 9 and 11, lanes 10 and 12, and lanes 13 and 14).

B. Presence of Smad3 in the transcription factor complex that binds to the proximal AP-1 site in the mouse IL-2 promoter

EMSA supershift analyses were performed to investigate whether Smad3 protein was a component of the transcription factor complex binding to the proximal AP-1 site. Splenocytes obtained from naïve B6C3F1 mice were activated in culture with α-CD3 + α-CD28 for 60 minutes in the presence or absence of 10 ng/mL TGF-β_1. Nuclear lysates were obtained for analyses of DNA binding activity. For supershift studies, nuclear lysates were incubated with an antibody specific for Smad3. Modest Smad3 binding was present only with nuclear extracts from TGF-β_1-treated cells as demonstrated by inhibition of DNA binding (**Figure 46**, compare lanes 3 and 5) and supershift (**Figure 46,** compare lanes 8 and 10). Importantly, intact CAGA sequences were demonstrated to be non-essential for TGF-β_1-induced Smad3 binding (**Figure 46,** lane 10) and suggest that Smad3 may bind to the proximal AP-1 site as a component of a protein complex, for example a Smad3•AP-1 heteromer.

C. Smad3 is not essential for TGF-β_1-induced binding to the proximal AP-1 proximal site in the mouse IL-2 promoter

Figure 45. A functional role for CAGA sequences in DNA binding activity in the mouse IL-2 promoter. Splenocytes were either untreated or stimulated with α-CD3 + α-CD28 for 2 hours at 37°C. Nuclear proteins were isolated and incubated (5 μg/lane) with a ^{32}P-labeled probe (proximal AP-1 site, CD28RE, distal NFAT site, or NRE-A; as indicated) containing native CAGA (W) or mutated CAGA (M) sequences as indicated. Protein binding complexes were resolved on a 4% polyacrylamide gel.

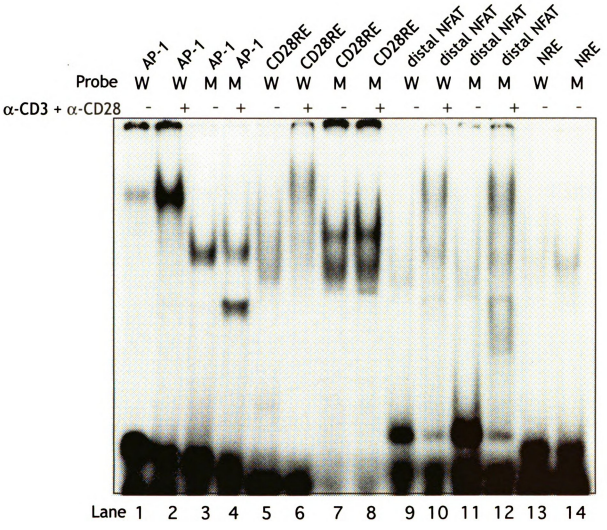
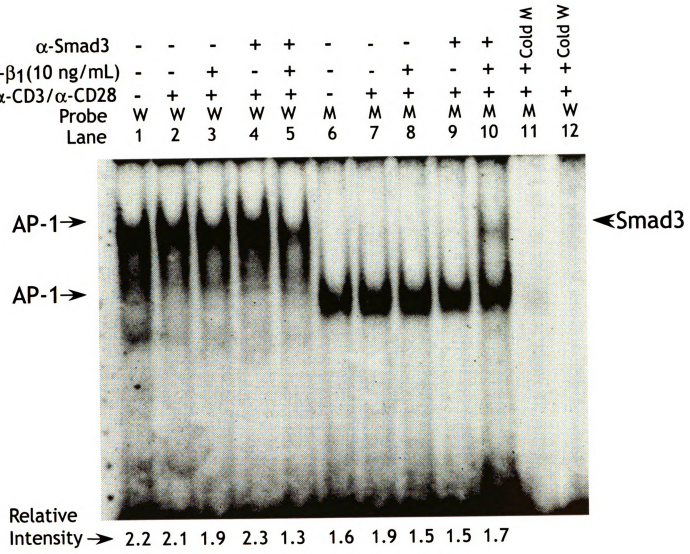

AP-1
AP-1
AP-1
AP-1
CD28RE
CD28RE
CD28RE
CD28RE
distal NFAT
distal NFAT
distal NFAT
distal NFAT
NRE
NRE
Probe W W M M W W M M W W M M W M
α-CD3 + α-CD28 - + - + - + - + - + - + - -
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 46. Smad3 is a component of the transcription factor complex that binds to the proximal AP-1 site in the mouse IL-2 promoter. Splenic T cells were isolated from B6C3F1 mice and treated as indicated. Nuclear proteins were isolated and incubated (5 μg/lane) with a ^{32}P-labeled probe on a 4% polyacrylamide gel. The oligonucleotides used were synthesized to correspond to the native proximal AP-1 site (W) or to a mutated AP-1 oligonucleotide containing disrupted CAGA Smad binding elements (M) was compared. Where indicated the DNA-binding protein complex was supershifted with anti-Smad3 (Zymed) or competed with excess unlabeled (1 pmol) probe.


α-Smad3 - - - + + - - - + + + Cold M + Cold W
TGF-β1(10 ng/mL) - - + - + - - + - + + +
α-CD3/α-CD28 - + + + + - + + + + + +
Probe W W W W W M M M M M M W
Lane 1 2 3 4 5 6 7 8 9 10 11 12
AP-1
AP-1
Smad3
Relative Intensity
2.2 2.1 1.9 2.3 1.3 1.6 1.9 1.5 1.5 1.7

A role for Smad3 in TGF-β_1-induced binding to the proximal AP-1 site was further investigated using nuclear lysates from a purified preparation of T cells. Thy1.2$^+$ purified Smad3$^{-/-}$ and Smad3$^{+/+}$ splenic T cells were activated in culture with α-CD3 + α-CD28 for 60 minutes in the presence or absence of 10 ng/mL TGF-β_1. Nuclear lysates were obtained to determine the influence of Smad3 on TGF-β_1-induced DNA binding activity at the proximal AP-1 site. As illustrated in **Figure 47**, TGF-β_1-induced DNA binding activity was demonstrated in nuclear lysates from activated Smad3$^{+/+}$ as well as Smad3$^{-/-}$ splenic T cells (**Figure 47,** compare lanes 3 and 4 and compare lanes 6 and 7). Moreover, basal DNA binding to the proximal AP-1 site is markedly reduced in nuclear lysates from Smad3$^{+/+}$ mice relative to Smad3$^{-/-}$ mice (**Figure 47**, compare lanes 2 and 5). These results are in consistent with differential basal expression of IL-2 mRNA and protein in untreated Smad3$^{+/+}$ and Smad3$^{-/-}$ splenocytes and support a role for Smad3 in regulating IL-2 gene transcription.

D. <u>CAGA</u> sequences are not essential for TGF-β_1-induced binding to the proximal AP-1 proximal site in the mouse IL-2 promoter

A role for <u>CAGA</u> sequences in TGF-β_1-induced binding to the proximal AP-1 site was also investigated using nuclear lysates from purified T cells. Thy1.2$^+$ purified Smad3$^{-/-}$ and Smad3$^{+/+}$ splenic T cells were activated in culture with α-CD3 + α-CD28 for 60 minutes in the presence or absence of 10 ng/mL TGF-β_1. Nuclear lysates were obtained to determine the influence of Smad3 on TGF-β_1-induced DNA binding activity to proximal AP-1 site containing mutated <u>CAGA</u> sequences. As illustrated in **Figure 48**, TGF-β_1-induced DNA binding activity was evident in nuclear lysates from activated

Figure 47. Smad3 is not essential for TGF-β_1-induced binding to the proximal AP-1 site in the mouse IL-2 promoter. Thy1.2$^+$ splenic T cells were isolated from Smad3$^{+/+}$ or Smad3$^{-/-}$ mice and activated *in vitro* with plate-bound α-CD3 + α-CD28 for 60 minutes in the presence or absence of 10 ng/mL TGF-β_1. Nuclear proteins were isolated and incubated (5 μg/lane) with a ^{32}P-labeled probe corresponding to the naive proximal AP-1 site. DNA binding complexes were resolved on a 4% polyacrylamide gel.

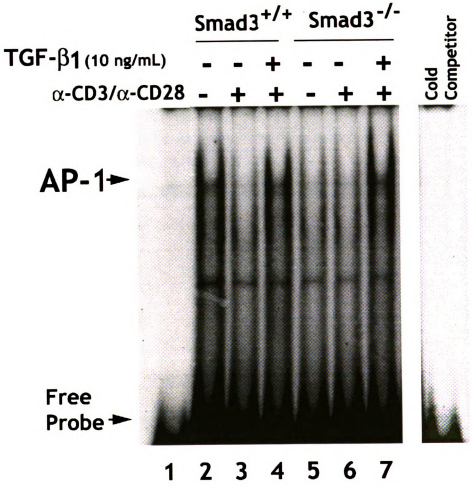
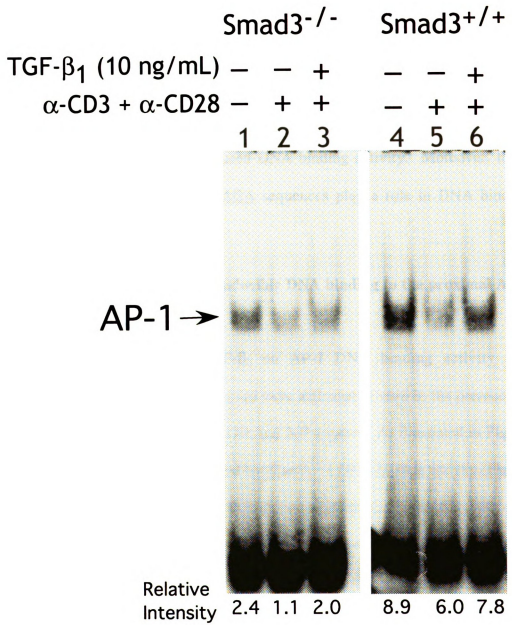

Smad3+/+
Smad3-/-
TGF-β1 (10 ng/mL)
- - + - - +
α-CD3/α-CD28
- + + - + +
Cold Competitor
AP-1
Free Probe
1 2 3 4 5 6 7

Figure 48. CAGA sequences are not essential for TGF-β_1-induced binding to the proximal AP-1 site in the mouse IL-2 promoter. Thy1.2$^+$ splenic T cells were isolated from Smad3$^{+/+}$ or Smad3$^{-/-}$ mice and activated *in vitro* with plate-bound α-CD3 + α-CD28 for 60 minutes in the presence or absence of 10 ng/mL TGF-β_1. Nuclear proteins were isolated and incubated (5 μg/lane) with a ^{32}P-labeled probe corresponding to the CAGA-mutated proximal AP-1 site. DNA binding complexes were resolved on a 4% polyacrylamide gel.


Smad3-/-
Smad3+/+
TGF-β1 (10 ng/mL) – – + – – +
α-CD3 + α-CD28 – + + – + +
1 2 3 4 5 6
AP-1
Relative Intensity
2.4 1.1 2.0 8.9 6.0 7.8

Smad3$^{+/+}$ *and* Smad3$^{-/-}$ splenic T cells (**Figure 48,** compare lanes 2 and 3 and compare lanes 5 and 6). In addition, basal DNA binding is markedly suppressed in the absence of Smad3 (**Figure 48**, compare lanes 1 and 4). These results are consistent with the differential basal DNA binding activity by untreated Smad3$^{+/+}$ and Smad3$^{-/-}$ nuclear lysates at the proximal AP-1 site containing the naïve CAGA sequences. Collectively, these results suggest that while neither Smad3 nor the CAGA sequences are essential for TGF-β_1-induced DNA binding activity at the proximal AP-1 site, each of these factors plays a modulatory role in TGF-β_1-induced DNA binding activity. Moreover, these results also suggest that Smad3 and CAGA sequences play a role in DNA binding activity in naïve resting cells.

E. Temporal response of TGF-β_1-inducible DNA binding to the proximal AP-1 site in the mouse IL-2 promoter.

The temporal response of TGF-β_1 on AP-1 DNA binding activity was investigated. Splenocytes from B6C3F1 mice were activated *in vitro* in the presence or absence of 10 ng/mL TGF-β_1 for 30, 60, 120, and 240 minutes. As illustrated in **Figure 49,** TGF-β_1 (10 ng/mL) effectively impaired baseline AP-1 DNA binding activity (**Figure 49,** compare lanes 2 and 12). However, TGF-β_1 only modestly attenuated α-CD3 + α-CD28-induced binding to the proximal AP-1 site (**Figure 49**). Importantly, these experiments do not address the composition of the DNA binding complex. The temporal response of TGF-β_1 on DNA binding activity to the *mutan*t proximal AP-1 probe was also investigated. TGF-β_1 (10 ng/mL) attenuated basal DNA binding (**Figure 50,** compare lane 3 and 12) and only modestly attenuated α-CD3 + α-CD28-induced proximal AP-1 binding activity at 60, 120, and 240 minutes. It has recently been

Figure 49. Temporal response of TGF-β_1-induced protein binding to the proximal AP-1 site of the IL-2 promoter. Splenocytes were isolated from B6C3F1 mice and activated *in vitro* with α-CD3 + α-CD28 in the presence or absence of 10 ng/mL TGF-β_1 for 0, 30, 60, 120, or 240 minutes. Nuclear proteins were isolated and incubated (5 μg/lane) with a ^{32}P-labeled probe corresponding to the native proximal AP-1 site. DNA binding complexes were resolved on a 4% polyacrylamide gel. Where indicated, cold competitor studies were conducted with 1 pmol unlabeled probe.

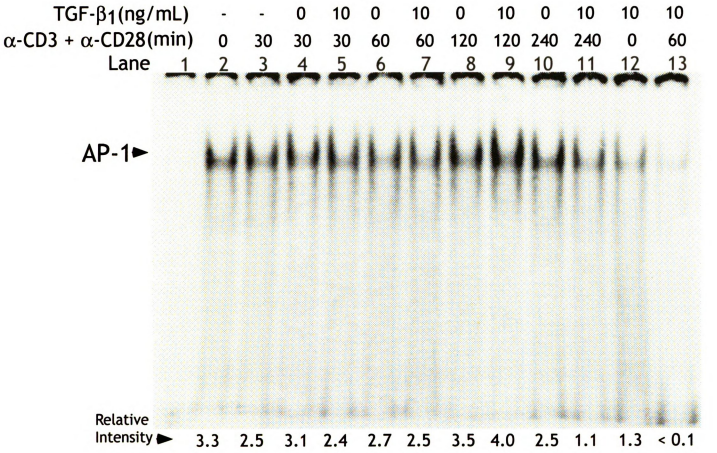
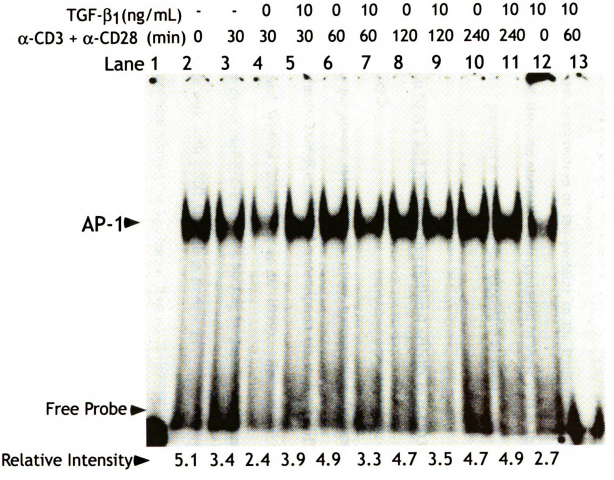

TGF-β1(ng/mL) - - 0 10 0 10 0 10 0 10 10 10
α-CD3 + α-CD28(min) 0 30 30 30 60 60 120 120 240 240 0 60
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13
AP-1
Relative Intensity 3.3 2.5 3.1 2.4 2.7 2.5 3.5 4.0 2.5 1.1 1.3 < 0.1

Figure 50. Temporal response of TGF-β_1-induced protein binding to a CAGA-mutated proximal AP-1 site. Splenocytes were isolated from B6C3F1 mice and activated *in vitro* with α-CD3 + α-CD28 in the presence or absence of 10 ng/mL TGF-β_1 for 0, 30, 60, 120, or 240 minutes. Nuclear proteins were isolated and incubated (5 μg/lane) with a ^{32}P-labeled probe corresponding to a CAGA-mutated proximal AP-1 site. DNA binding complexes were resolved on a 4% polyacrylamide gel. Where indicated, cold competitor studies were conducted with 1 pmol unlabeled probe.


TGF-β1(ng/mL) - - 0 10 0 10 0 10 0 10 10 10
α-CD3 + α-CD28 (min) 0 30 30 30 60 60 120 120 240 240 0 60
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13
AP-1
Free Probe
Relative Intensity 5.1 3.4 2.4 3.9 4.9 3.3 4.7 3.5 4.7 4.9 2.7

demonstrated that AP-1•AP-1 and AP-1•Smad complexes migrate as a single band and are undistinguishable on a 4% PAGE (Verrecchia *et al.*, 2001b). In light of this observation, one interpretation of the present study is that TGF-β_1 modulates IL-2 gene transcription by altering the composition of the proteins in the transcription factor complex binding to the AP-1 site. An alternative interpretation of these data is that the proximal AP-1 site in the IL-2 promoter is not a TGF-β_1-responsive element.

VIII. Effect of TGF-β_1 on NFAT and NF-κB transcriptional activity in α-CD3 + α-CD28-activated splenic T cells

Smad proteins cooperatively interact with other transcription factors and accessory proteins to modulate TGF-β_1-meditated transcription via multiple different mechanisms (Massague and Wotton 2000; Piek *et al.* 1999). Several of these mechanisms do not involve direct modulation of DNA binding by Smad3. For example, Smad3 cooperatively interacts with p300 through a protein•protein interaction to effectively enhance transcriptional activity through the remodeling of chromatin structure (Feng *et al.* 1998; Itoh *et al.* 2000; Shen *et al.* 1998).

In light of the numerous protein binding-independent mechanisms of Smad-mediated transcription, the putative mechanisms whereby TGF-β_1 regulates IL-2 gene transcription were further investigated using reporter gene assays. Mouse EL-4 and human Jurkat T cell lymphoma cells were transiently transfected with pNFAT or pNF-κB reporter plamsids. These reporter plasmids were selected on the basis that each is regulated by a promoter containing multiple copies of CAGA sequences upstream of the transcriptional start site. The transfected cells were activated with α-CD3 + α-CD28 in

the presence of TGF-β_1 (10^{-3} 10^{-1}, and 10 ng/mL) for 24 hours. The pSV-β-gal control vector was co-transfected in each experiment to monitor transfection efficiency. SEAP activity and IL-2 protein were measured from supernatants and β-gal activity was measured from cell lysates. Anti-CD3 + α-CD28-induced p-NFAT-SEAP activity in EL-4 and Jurkat cells was attenuated by TGF-β_1 in a concentration-dependent manner (**Figure 51a** and **Figure 53a** respectively). In contrast, TGF-β_1 stimulated α-CD3 + α-CD28-induced p-NF-κB-SEAP activity in EL-4 as well as Jurkat cells in a concentration-dependent manner (**Figure 52a** and **Figure 53b,** respectively). Interestingly, TGF-β_1 attenuated α-CD3 + α-CD28- induced IL-2 protein secretion in p-NF-κB-SEAP as well as p-NFAT-SEAP-transfected EL-4 cells (**Figure 51b** and **Figure 52b,** respectively).

IX. Involvement of MAPK in the regulation of Smad/TGF-β_1 signaling in T cells

It is well established that MAPK signaling is activated upon TcR ligation (Whitehurst and Geppert 1996). TGF-β_1 also activates MAPK signaling cascades through the TβR (Choi 2000; Hartsough and Mulder 1997; Hu *et al.* 1999; Mulder 2000; Visser and Themmen 1998). Furthermore regulatory cross talk between Smad and MAPK signaling has been established in several epithelial and fibroblast cell lines (Kretzschmar *et al.* 1999; Mulder 2000; Stroschein *et al.* 1999b; Watanabe *et al.* 2001; Yue and Mulder 2000). In light of these observations, the objective of this series of experiments was to investigate a putative role for MAPK signaling in regulating TGF-β_1-induced Smad signaling in T cells.

Figure 51. Effect of TGF-β_1 on NFAT transcriptional activity in α-CD3 + α-CD28-activated T cells. EL-4 cells (2 X 10^5 c/mL) were transiently transfected using Cytofectene transfection reagent with 8 μg pNFAT-SEAP reporter construct and pGL3 β-galactosidase reporter plasmid (1μg) in RPMI for 1 hour at 37°C. The cells were then transferred to un-coated or α-CD3 + α-CD28-precoated 96-well plates and either left untreated (NA) or treated with TGF-β_1 (0.001 – 10 ng/mL) or VH (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture) for 24 hours at 37°C. (a) SEAP activity was normalized for transfection efficiency using β-gal activity from cell lysates. (b) Aliquots of the supernatants were removed for IL-2 protein secretion (ELISA). Data are reported as the mean ± SE of quadruplicate cultures. *,$p < 0.05$ as compared to the matched vehicle control. Results are representative of two experiments.

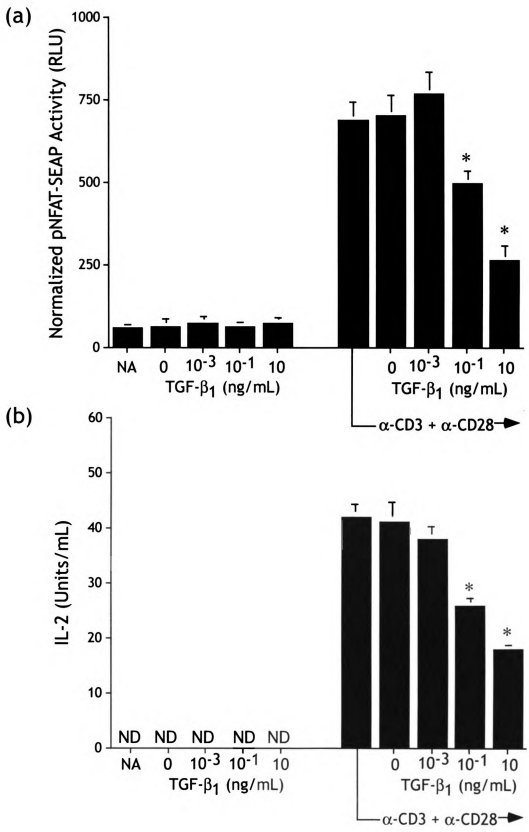
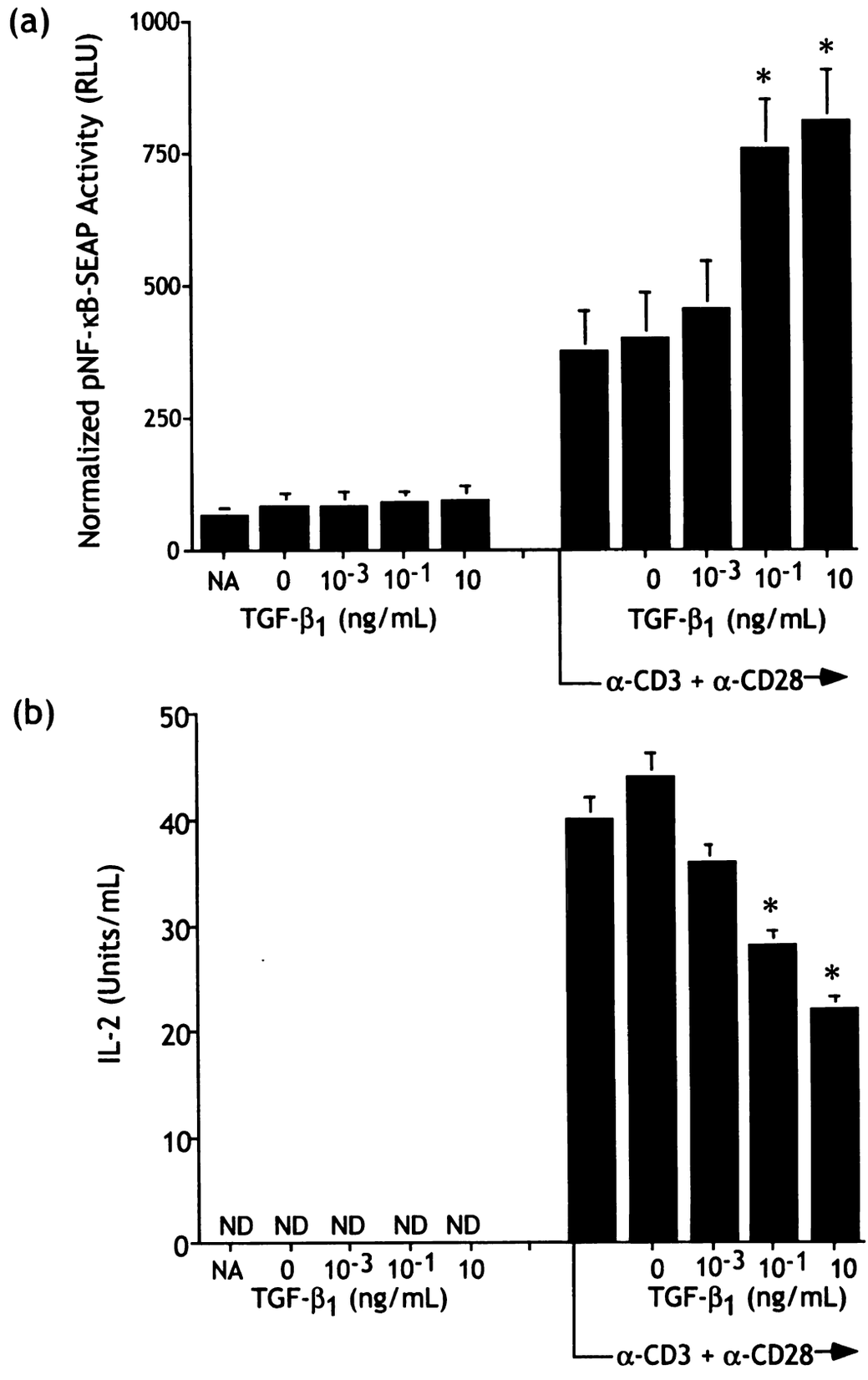
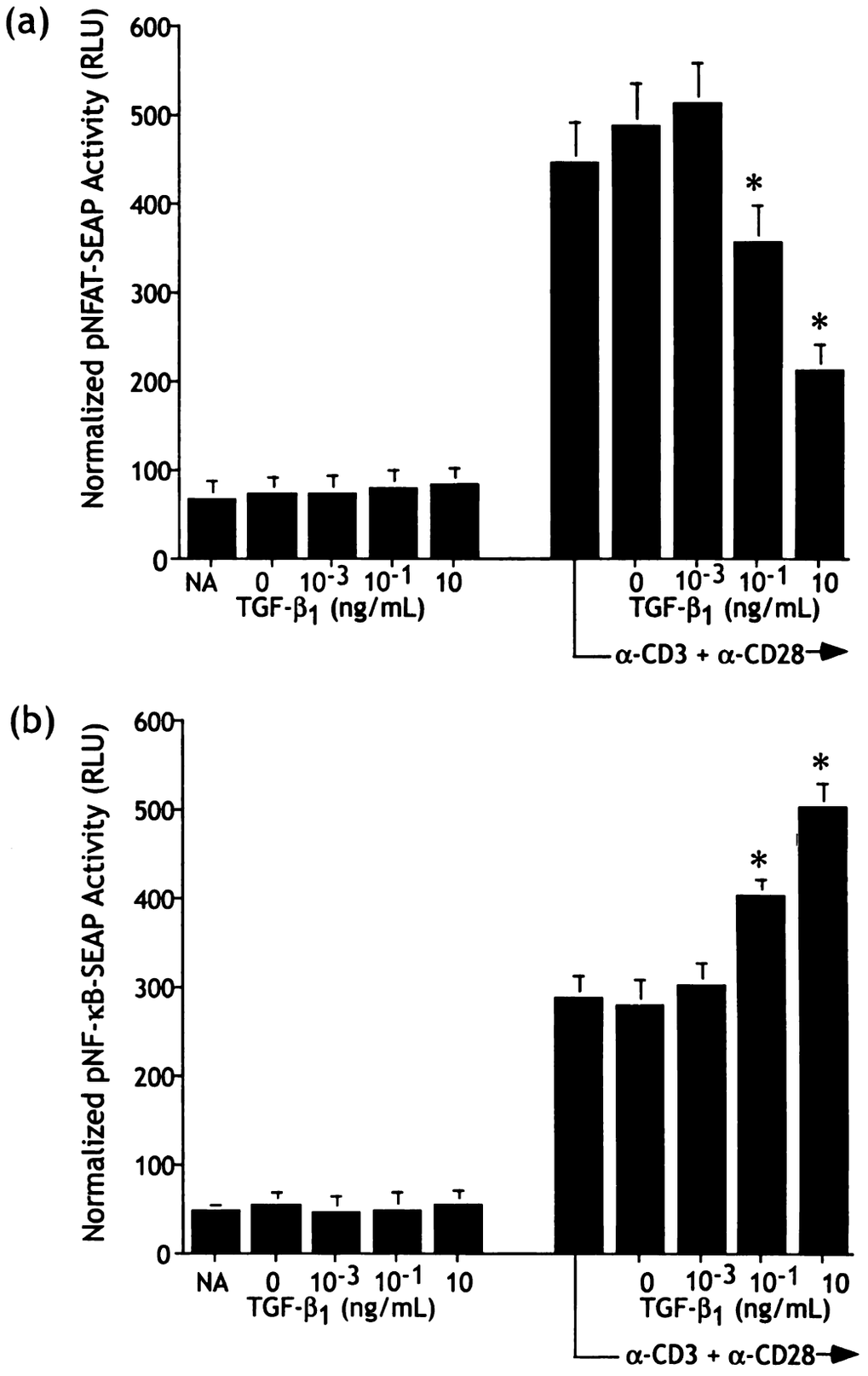

(a)
Normalized pNFAT-SEAP Activity (RLU)
1000
750
500
250
0
NA
0
10-3
10-1
10
TGF-β1 (ng/mL)
0
10-3
10-1
10
TGF-β1 (ng/mL)
α-CD3 + α-CD28
(b)
IL-2 (Units/mL)
60
50
40
30
20
10
0
ND
ND
ND
ND
ND
NA
0
10-3
10-1
10
TGF-β1 (ng/mL)
0
10-3
10-1
10
TGF-β1 (ng/mL)
α-CD3 + α-CD28

Figure 52. Effect of TGF-β_1 on NF-κB transcriptional activity in α-CD3 + α-CD28-activated T cells. EL-4 cells (2 X 10^5 c/mL) were transiently transfected using Cytofectene transfection reagent with 8 μg pNF-κB-SEAP reporter construct and pGL3 β-galactosidase reporter plasmid (1μg) in RPMI for 1 hour at 37°C. The cells were then transferred to un-coated or α-CD3 + α-CD28-precoated 96-well plates and either left untreated (NA) or treated with TGF-β_1 (0.001 – 10 ng/mL) or VH (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture) for 36 hour at 37°C. (a) SEAP activity was normalized for transfection efficiency using β-gal activity from cell lysates. (b) Aliquots of the supernatants were removed for IL-2 protein secretion (ELISA). Data are reported as the mean ± SE of quadruplicate cultures. *,$p < 0.05$ as compared to the matched vehicle control. Results are representative of a two separate experiments.



α-CD3 + α-
nsfected usin
ruct and pGL
ells were the
and either le
PBS, pH 5.
C. (a) SEAP
n cell lysates
ELISA). Data
mpared to the
ments.

Figure 53. Effect of TGF-β_1 on NFAT and NF-κB transcriptional activity in α-CD3 + α-CD28-activated Jurkat cells. Jurkat cells (5 X 10^6 c/mL) were transiently transfected using Cytofectene transfection reagent with (a) pNFAT-SEAP or (b) pNF-κB-SEAP reporter constructs and co-transfected with a pGL3 β-galactosidase reporter plasmid in RPMI for 1 hour at 37°C. The cells were then transferred to un-coated or α-CD3 + α-CD28-precoated 96-well plates and either left untreated (NA) or treated with TGF-β_1 (0.001 – 10 ng/mL) or VH (0.02% PBS, pH 3.5, containing 0.1% BSA, final concentration in culture) for 24 hours at 37°C. SEAP activity was normalized for transfection efficiency using β-gal activity from cell lysates. Data are reported as the mean ± SE of quadruplicate cultures. *, $p < 0.05$ as compared to the matched vehicle control. Results are representative of two separate experiments.


(a)
Normalized pNFAT-SEAP Activity (RLU)
600
500
400
300
200
100
0
NA
0
10-3
10-1
10
TGF-β1 (ng/mL)
0
10-3
10-1
10
TGF-β1 (ng/mL)
α-CD3 + α-CD28
*
*
(b)
Normalized pNF-κB-SEAP Activity (RLU)
600
500
400
300
200
100
0
NA
0
10-3
10-1
10
TGF-β1 (ng/mL)
0
10-3
10-1
10
TGF-β1 (ng/mL)
α-CD3 + α-CD28
*
*
*

A. Concentration-dependent effect of TGF-β_1 on ERK MAPK activity in mouse splenocytes

ERK MAPK has been previously shown to negatively regulate Smad3 nuclear translocation by phosphorylation of serine residues within the linker region of the Smad3 protein (Kretzschmar *et al.* 1997; Kretzschmar *et al.* 1999). TGF-β_1 reportedly activates ERK1 MAPK and ERK2 MAPK in numerous cell lines (Axmann *et al.* 1998; Reimann *et al.* 1997). The effects of TGF-β_1 on ERK MAPK activity in myeloid and/or lymphoid cells has not yet been studied. The objective of the present experiments was to investigate ERK1 MAPK and ERK2 MAPK activity in response to TGF-β_1 in mouse splenocytes. Towards this end, splenocytes were isolated from naïve B6C3F1 mice, treated with TGF-β_1 for 15 minutes, and nuclear lysates were isolated for Western blot analyses. Expression of phosphorylated MAPK is indicative of kinase activity (Cobb and Goldsmith 1995), therefore an antibody specifically recognizing phosphorylated ERK1 MAPK and ERK2 MAPK was utilized. A concentration-dependent augmentation of ERK1 MAPK and ERK2 MAPK phosphorylation by TGF-β_1 was demonstrated **(Figure 54).**

B. Effect of UO126 and PD98059 on TGF-β_1-induced inhibition of IL-2 protein secretion in activated splenic T cells

Having established that TGF-β_1 increases splenic ERK MAPK activity, a role for ERK MAPK in regulating TGF-β_1-induced inhibition of IL-2 secretion was investigated in α-CD3 + α-CD28-activated splenic T cells. Initial experiments investigated the effects of UO126, a MEK1/2-specfic inhibitor, on TGF-β_1-mediated Smad3 nuclear

Figure 54. Concentration-dependent effect of TGF-β_1 on ERK MAPK activity in mouse splenocytes. Naïve splenocytes were treated with TGF-β_1 for 15 minutes. Nuclear proteins (25 μg) were isolated and resolved on a 10% denaturing polyacrylamide gel. Proteins were transferred to nitrocellulose and incubated with antibodies for the phosphorylated ERK and ERK2. Nuclear lysates for splenocytes treated with PMA/Io (80 nM/1 μM) for 15 minutes was incorporated as a positive control for ERK activity.

TGF-β1
(ng/mL) NA 0 10-4 10-3 10-2 10-1 1 10 PMA/Io
p-ERK1
p-ERK2

expression. Splenocytes isolated from naïve B6C3F1 mice were pretreated with UO126 (1.25, 2.5, 5, 10, or 20 μM) for 30 minutes followed by stimulation with 10 ng/mL TGF-β_1 for 60 minutes. As illustrated, UO126 effectively augmented TGF-β_1-induced Smad3 nuclear translocation (**Figure 55**). Next, splenocytes isolated from naïve B6C3F1 mice were pretreated with either PD98059 (25 μM) or UO126 (10 μM) for 30 minutes prior to α-CD3 + α-CD28-induced activation in the presence or absence of TGF-β_1. Following a 24 hour incubation, supernatants were collected for ELISA analyses. As illustrated in **Figure 56**, PD98059 and UO126 attenuated TGF-β_1-induced inhibition of IL-2 secretion. These results suggest that ERK MAPK signaling plays a role in Smad3-dependent TGF-β_1-mediated inhibition of IL-2 protein production.

C. Concentration-dependent effects of TGF-β_1 on JNK MAPK activity in mouse splenocytes

JNK MAPK also plays a regulatory role in T cell activation, and is of particular importance in CD28-associated signaling (Kempiak *et al.* 1999). An inter-dependent relationship between the JNK and SMAD signaling by TGF-β_1 has been established (Engel *et al.* 1999). Activation of JNK MAPK by TGF-β_1 in T cells has not yet been investigated. The objective of these studies was to determine whether TGF-β_1 enhances JNK MAPK activity in splenocytes. Splenocytes were isolated from naïve B6C3F1 mice, treated with TGF-β_1 for 15 minutes, and nuclear lysates were isolated for Western blot analyses. In contrast to ERK MAPK, TGF-β_1 (10^{-5}, 10^{-3}, 10^{-1}, 1, and 10 ng/mL) did not induce phosphorylation of either JNK1 or JNK2 MAPK at any concentration tested (**Figure 57**).

Figure 55. The effect of UO126 on TGF-β_1-induced Smad3 activation Naïve or α-CD3 + α-CD28-activated splenocytes were pretreated with UO126 (10 μM) for 30 minutes and then with TGF-β_1 for 15 minutes. Nuclear proteins (25 μg) were isolated and resolved on a 10% denaturing polyacrylamide gel. Proteins were transferred to nitrocellulose and incubated with an antibody specific for Smad3. The fold induction is reported as intensity relative to naive.

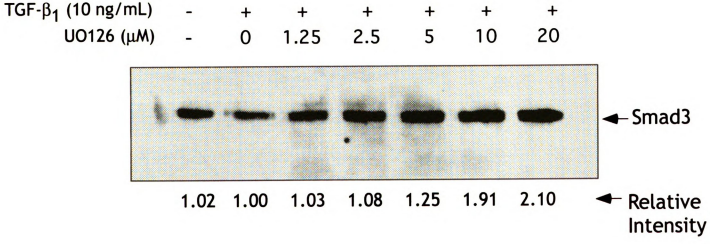

TGF-β1 (10 ng/mL) - + + + + + +
UO126 (μM) - 0 1.25 2.5 5 10 20
Smad3
1.02 1.00 1.03 1.08 1.25 1.91 2.10
Relative Intensity

Figure 56. Effect of UO126 and PD98059 on TGF-β_1-induced inhibition of IL-2 protein secretion in activated splenic T cells. Spleens from B6C3F1 mice were isolated aseptically and made into single cell suspensions. The splenocytes were washed and adjusted to 5 X 10^6 c/mL. Naïve splenocytes were pretreated with UO126 (10 μM) or PD98059 (25 μM) for 30 minutes followed by α-CD3 + α-CD28-induced activation in the presence or absence of TGF-β_1 for 24 hours. Supernatants were collected and analyzed for secreted IL-2 protein via ELISA as described in "Materials and Methods". Data are expressed as the mean ± of quadruplicate samples, and are representative of two separate experiments. *$p < 0.05$ as determined by Dunnett's t test as compared to the relevant vehicle control.

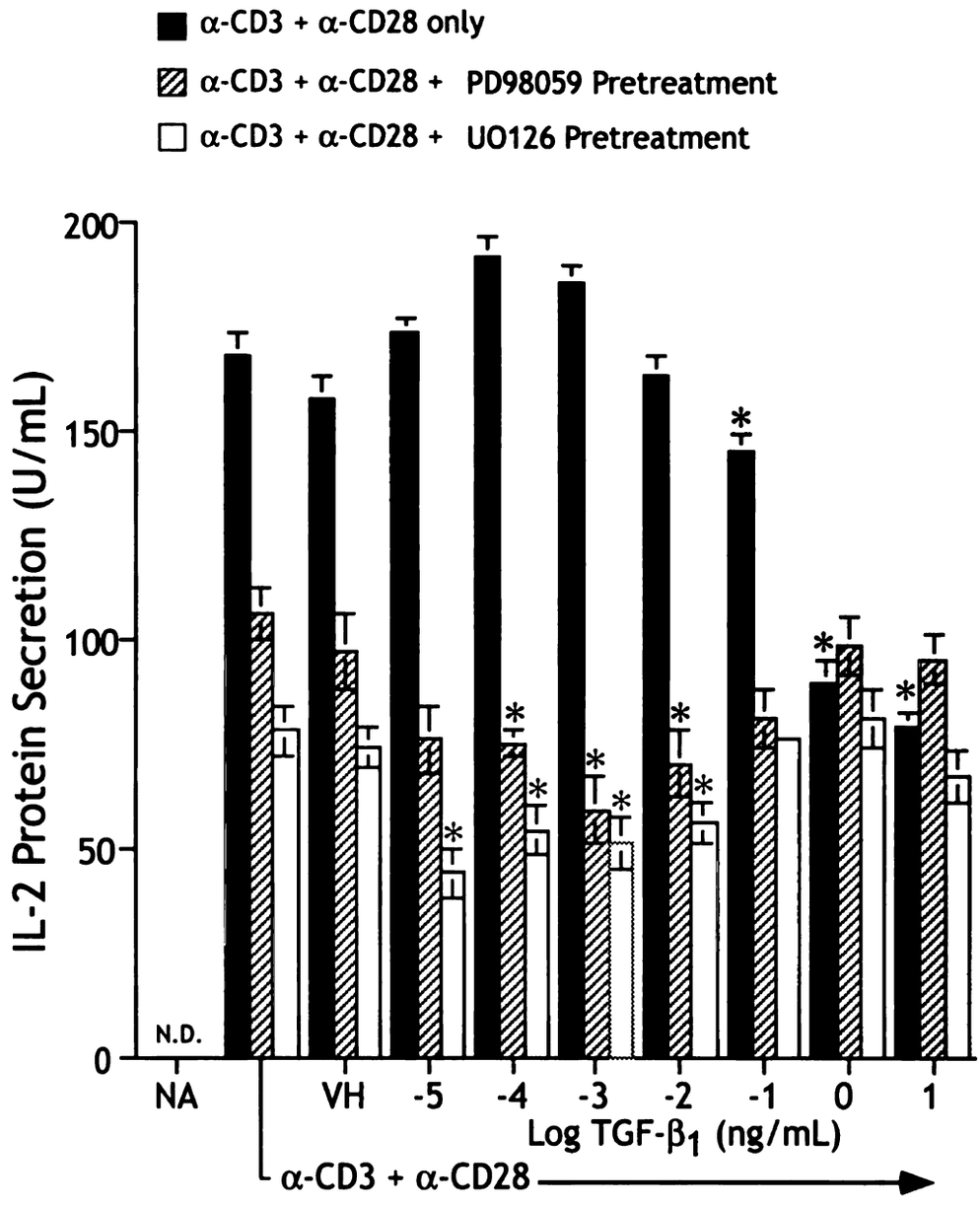
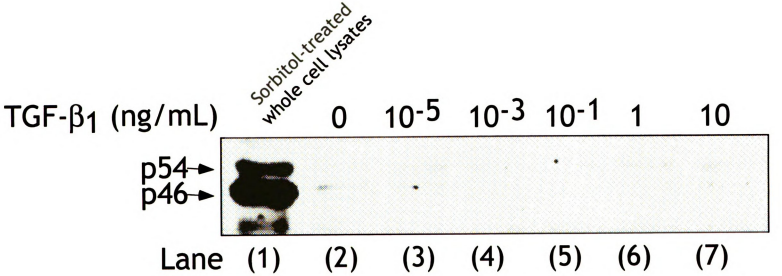

α-CD3 + α-CD28 only
α-CD3 + α-CD28 + PD98059 Pretreatment
α-CD3 + α-CD28 + UO126 Pretreatment
IL-2 Protein Secretion (U/mL)
200
150
100
50
0
N.D.
NA
VH
-5
-4
-3
-2
-1
0
1
Log TGF-β1 (ng/mL)
α-CD3 + α-CD28

Figure 57. The effect of TGF-β_1 on JNK1 and JNK2 activation. Naïve splenocytes were treated with TGF-β_1 (10 ng/mL) for 15 minutes. Nuclear proteins (25 μg) were isolated and resolved on a 10% denaturing polyacrylamide gel. Proteins were transferred to nitrocellulose and incubated with a polyclonal rabbit anti-phospho antibody for JNK1/2 as described in "Materials and Methods". Data are representative of two separate experiments


Sorbitol-treated whole cell lysates
TGF-β1 (ng/mL)
0
10-5
10-3
10-1
1
10
p54
p46
Lane
(1)
(2)
(3)
(4)
(5)
(6)
(7)

X. A role for Smad3 in immunoglobulin production by TGF-β_1 *in vitro*

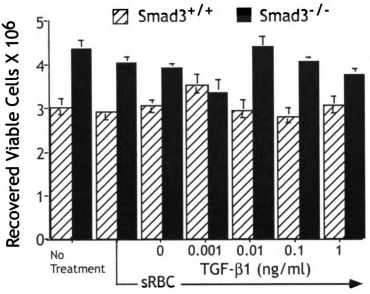
Smad3$^{-/-}$ mice are viable, but succumb at an early age to a deficiency in mucosal immunity (Yang *et al.* 1999). Serum IgA levels and the number of intestinal IgA$^+$ B cells are normal in Smad3-null mice (Datto *et al.* 1999; Yang *et al.* 1999). Having established that inhibition of LPS-induced B cell growth by TGF-β_1 is unaffected in Smad3$^{-/-}$ B cells, the present studies investigated whether Smad3 is essential for modulation of humoral immune responses by TGF-β_1 *in vitro*.

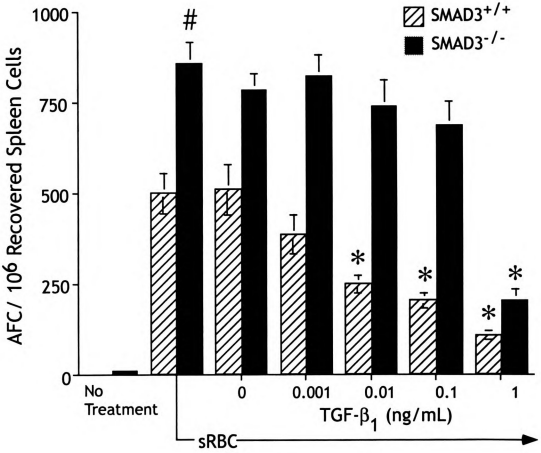
A. Inhibition of the *in vitro* T cell-dependent sRBC IgM AFC response by TGF-β_1 is augmented in Smad3-null splenic B cells

Class II major histocompatability complex (MHC) molecules function to present processed antigens to T helper cells and are thus essential for T cell-dependent humoral immune responses. It is well established that TGF-β_1 attenuates humoral immune responses, in part, through down-regulation of major MHC molecules (Kobayashi *et al.* 1999; Letterio *et al.* 1996; Nakabayashi *et al.* 1997). Smad3 is essential for down-regulation of MHC II expression by TGF-β_1 in astrocytes (Dong *et al.* 2001).

In light of these observations, a role for Smad3 in mediating TGF-β_1-induced inhibition of the T cell-dependent sRBC IgM AFC response was investigated. Splenocytes were isolated from Smad3$^{-/-}$ mice and sRBC-sensitized for 5 days in culture. The hemolytic plaque assay was used to quantify antibody producing cells. TGF-β_1 attenuated the AFC response in Smad3$^{+/+}$ B cells in a concentration-dependent manner (**Figure 58**). In contrast, Smad3$^{-/-}$ B cells were less sensitive to the inhibitory effects of TGF-β_1 as illustrated by a significant ($p < 0.05$) inhibition of antibody producing cells by TGF-β_1 at the 1 ng/mL concentration only. The total number of viable splenocytes

Figure 58. Inhibition of the T cell-dependent sRBC AFC response by TGF-β_1 is unaffected in Smad3-null splenic B cells *in vitro*. Spleens from Smad3$^{-/-}$ and Smad$^{+/+}$ were isolated aseptically and made into single cell suspensions. The splenocytes were washed and adjusted to 1 X 10^7 c/mL and transferred in 500-µL to the wells of a 48-well culture plate. Quadruplicate cultures were left untreated (no treatment) or sensitized with sRBC in the presence or absence of TGF-β_1 (0.001, 0.01, 0.1, and 1 ng/mL final concentration in culture) for 5 days. Alternatively cells were treated with the vehicle (VH; 0.02% PBS final concentration in culture, pH 3.5, containing 0.1% BSA) for 3 days. Cultures were subsequently analyzed for a day 5 antibody response by enumerating the number of AFC; spleen cell viability and total recovered cells/culture were also determined. The results from quadruplicate determinations are expressed as the mean AFC per 10^6 recovered viable cells $\pm$ SEM (n=4). *$p < 0.05$ as determined by Dunnett's t test as compared to the vehicle control. "#" denotes $p < 0.05$, Smad3$^{-/-}$ compared to same group Smad3$^{+/+}$ as determined by Dunnett's t test. The results are representative of two separate experiments.


Smad3+/+
Smad3-/-
Recovered Viable Cells X 10^6
No Treatment
0
0.001
0.01
0.1
1
TGF-β1 (ng/ml)
sRBC


SMAD3+/+
SMAD3-/-
AFC/ 10^6 Recovered Spleen Cells
#
*
No Treatment
0
0.001
0.01
0.1
1
TGF-β1 (ng/mL)
sRBC

recovered after 5 days in culture were not markedly altered with targeted deletion of Smad3 (**Figure 58** insert). Noteworthy, the magnitudes of basal and sRBC-induced primary AFC responses were greater in $Smad3^{-/-}$ splenocytes than $Smad3^{+/+}$ splenocytes (**Figure 58**).

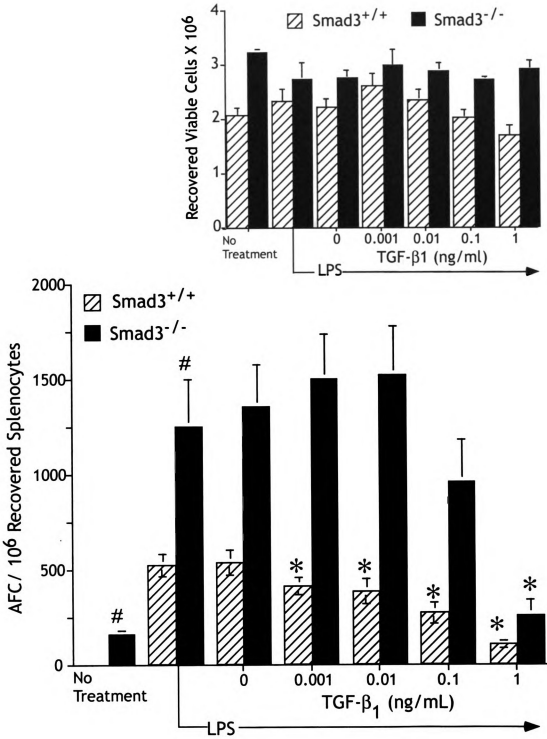
B. Inhibition of the *in vitro* polyclonal antibody response by TGF-β_1 is augmented in Smad3 null splenic B cells

In further defining a role for Smad3 in regulating humoral immunity, the polyclonal response to LPS was also investigated. Splenocytes were isolated from $Smad3^{-/-}$ mice and sensitized with LPS for 3 days in culture. Similar to the T cell-dependent sRBC AFC response, a decreased sensitivity to TGF-β_1-induced inhibition of the polyclonal response to LPS was demonstrated in $Smad3^{-/-}$ B cells relative to $Smad3^{+/+}$ B cells (**Figure 59**). The total number of viable splenocytes recovered after 3 days in culture was unaffected with targeted deletion of Smad3 (**Figure 59** insert) demonstrating that the AFC response was not indirectly confounded by cytotoxic effects of TGF-β_1 treatment. Notably, the magnitude of LPS-induced IgM AFC response was elevated in $Smad3^{-/-}$ splenocytes relative to $Smad3^{+/+}$ splenocytes (**Figure 59**). These results were somewhat surprising as normal numbers of splenic and lymph nodal IgM B cells in $SMAD3^{-/-}$ mice have been reported (Yang *et al.* 1999).

C. Inhibition of LPS-induced IgM production in Smad3-null B cells by TGF-β_1 *in vitro*.

The results of the LPS IgM AFC response were corroborated by measuring IgM production *in vitro*. Splenocytes were isolated from $Smad3^{-/-}$ mice, stimulated with LPS

Figure 59. Inhibition of the T cell-independent LPS AFC response by TGF-β_1 is unaffected in Smad3-null splenic B cells *in vitro*. Spleens from Smad3$^{-/-}$ and SMAD3$^{+/+}$ were isolated aseptically and made into single cell suspensions. The splenocytes were washed and adjusted to 5 X 10^6 c/mL and transferred in 500-µL to the wells of a 48-well culture plate. Quadruplicate cultures were left untreated (no treatment) or activated with LPS (10 µg/mL) in the presence or absence of TGF-β_1 (0.001, 0.01, 0.1, and 1 ng/mL final concentration in culture) for 3 days. Alternatively cells were treated with the vehicle (VH; 0.02% PBS final concentration in culture, pH 3.5, containing 0.1% BSA) for 3 days. Cultures were subsequently analyzed for a day 3 IgM antibody response by enumerating the number of AFC; spleen cell viability and total recovered cells/culture were also determined. The results from quadruplicate determinations are expressed as the mean AFC per 10^6 recovered viable cells $\pm$ SEM (n=4). "a" denotes $p < 0.05$ as determined by Dunnett's t test and compared to the Smad3$^{+/+}$ vehicle control. "b" denotes $p < 0.05$ as determined by Dunnett's t test and compared to the Smad3$^{-/-}$ vehicle control. "#" denotes $p < 0.05$, Smad3$^{-/-}$ compared to same group Smad3$^{+/+}$ as determined by Dunnett's t test. The results are representative of two independent experiments.


Smad3+/+
Smad3-/-
Recovered Viable Cells X 10^6
No Treatment
0
0.001
0.01
0.1
1
TGF-β1 (ng/ml)
LPS
AFC/ 10^6 Recovered Splenocytes
TGF-β1 (ng/mL)

in the presence or absence of TGF-β_1 for 3 days, and supernatants were collected for ELISA. LPS-induced IgM secretion was attenuated by TGF-β_1 in a concentration-dependent manner in SMAD3$^{-/-}$ B cells as well as SMAD3$^{+/+}$ B cells (**Figure 60**). In agreement with the *in vitro* AFC responses, SMAD3$^{-/-}$ B cells were less sensitive to inhibition by TGF-β_1 when compared with wild type littermates. Moreover, a greater magnitude of LPS-induced IgM production in Smad3$^{-/-}$ B cells than SMAD3$^{+/+}$ B cells corresponds to the elevated polyclonal IgM AFC response in Smad3$^{-/-}$ spleen cells.

D. IgA secretion is not enhanced by TGF-β_1 in LPS-stimulated Smad3-null mouse splenic B cells *in vitro*

TGF-β_1 is well established as a positive regulator of IgA production. Recent *in vitro* studies have implicated that Smad proteins physically and functionally interact with transcription factors that regulate IgA transcription in response to TGF-β_1.(Park *et al.* 2001; Shi *et al.* 2001; Zhang and Derynck 2000). The objective of this set of experiments was to investigate the role of Smad3 in IgA production by TGF-β_1 *in vitro*. Splenocytes were isolated from Smad3$^{-/-}$ mice, stimulated with LPS and IL-10 in the presence or absence of TGF-β_1 for 5 days, and supernatants were collected for ELISA analyses. While an expected increase (6X) in IgA production by TGF-β_1 was demonstrated in LPS-stimulated B cells from Smad3$^{+/+}$ mice, the production of IgA in response to TGF-β_1 was ablated in Smad3$^{-/-}$ B cells (**Figure 61a**). A concomitant decrease in total IgM secretion in the LPS-stimulated Smad3$^{+/+}$ B cells by TGF-β_1 is consistent with TGF-β_1-induced IgA class switching (**Figure 61b**). Interestingly, LPS-induced IgM secretion by TGF-β_1 was also attenuated in Smad3$^{-/-}$ spleen cells (**Figure 61b**). One interpretation of these results is that TGF-β_1 induces IgA class switching in Smad3$^{-/-}$ B cells; however, the mature

Figure 60. Inhibition of LPS-induced IgM secretion by TGF-β_1 *in vitro* is unaffected by Smad3-null splenic B cells *in vitro*. Spleens from Smad3$^{-/-}$ and Smad3$^{+/+}$ were isolated aseptically and made into single cell suspensions. The splenocytes were washed and adjusted to 5 X 10^6 c/mL and transferred in 500-µL to the wells of a 48-well culture plate. Quadruplicate cultures were left untreated (no treatment) or activated with LPS (10 µg/mL) in the presence or absence of TGF-β_1 (0.001, 0.01, 0.1, and 1 ng/mL final concentration in culture) for 3 days. Alternatively cells were treated with the vehicle (VH; 0.02% PBS final concentration in culture, pH 3.5, containing 0.1% BSA) for 3 days. Supernatants were collected at 72 hours and analyzed for total IgM by ELISA. The results from quadruplicate determinations are expressed as the mean IgM (µg 10^6 recovered viable cells $\pm$ SEM (n=4). "a" denotes $p < 0.05$ as determined by Dunnett's t test and compared to the Smad3$^{+/+}$ vehicle control. "b" denotes $p < 0.05$ as determined by Dunnett's t test and compared to the Smad3$^{-/-}$ vehicle control. "#" denotes $p < 0.05$, Smad3$^{+/+}$ compared to same group Smad3$^{-/-}$ as determined by Dunnett's t test. The results are representative of two independent experiments.

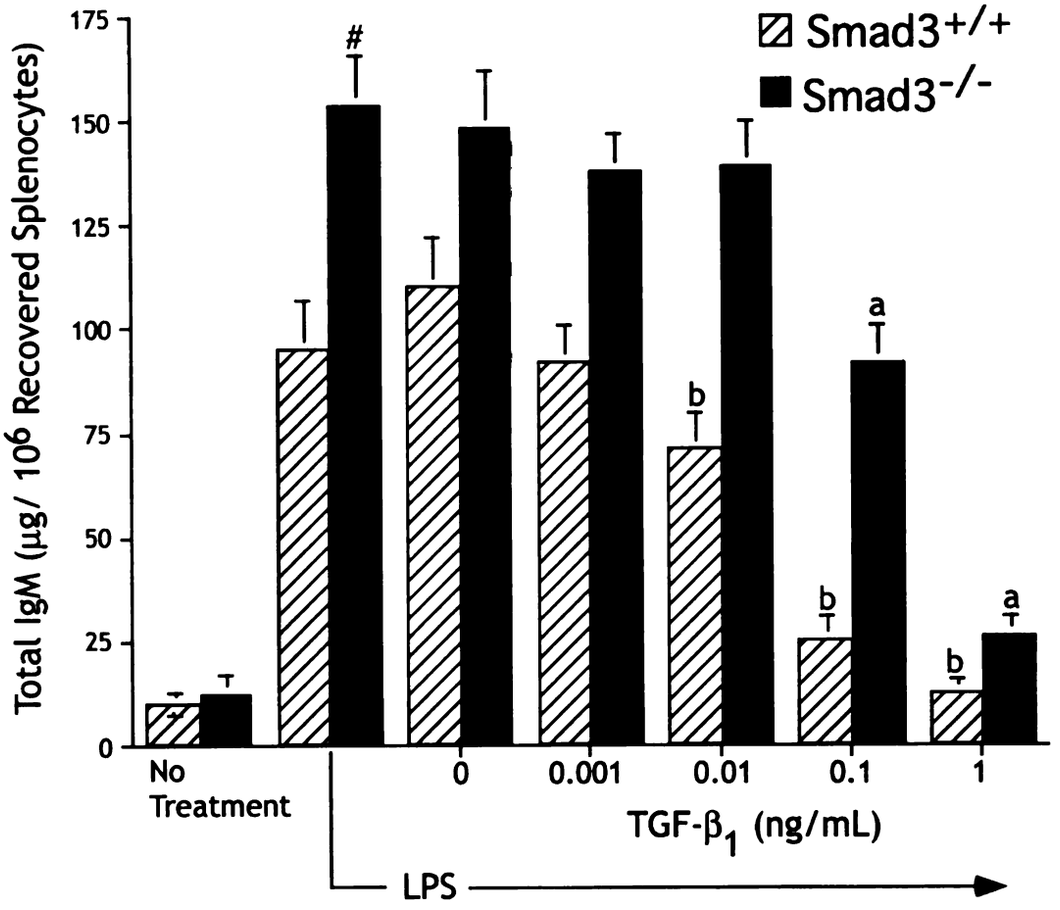
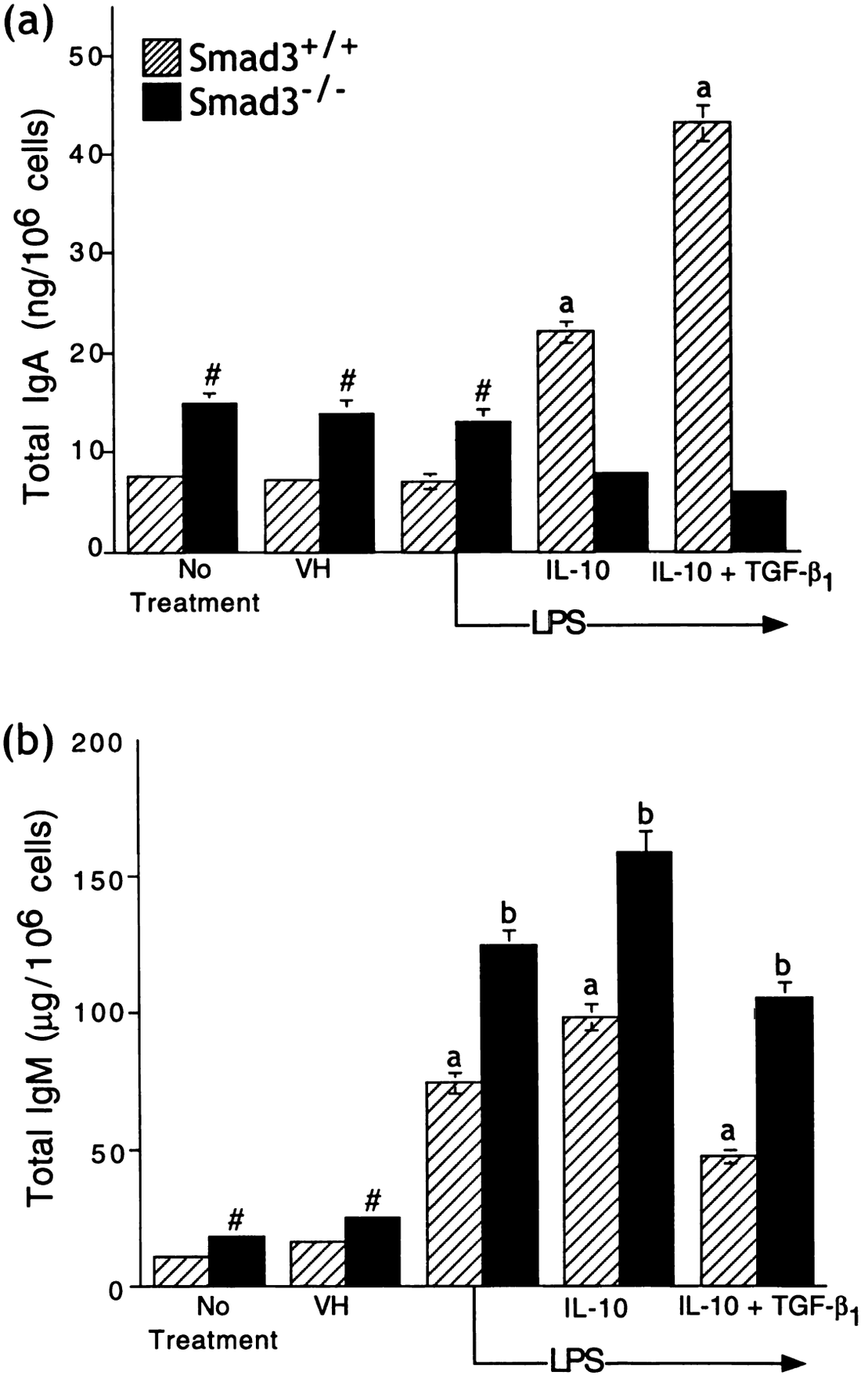

Smad3+/+
Smad3-/-
Total IgM (µg/ 10^6 Recovered Splenocytes)
175
150
125
100
75
50
25
0
#
a
b
No Treatment
0
0.001
0.01
0.1
1
TGF-β1 (ng/mL)
LPS

Figure 61. IgA secretion is not enhanced by TGF-β_1 in LPS-stimulated Smad3-null mouse splenic B cells *in vitro*. Spleens from Smad3$^{-/-}$ and Smad3$^{+/+}$ were isolated aseptically and made into single cell suspensions. The splenocytes were washed and adjusted to 5 X 10^6 c/mL and transferred in 500-µL to the wells of a 48-well culture plate. Quadruplicate cultures were left untreated (no treatment) or activated with LPS (10 µg/mL) in the presence or absence of TGF-β_1 (1 ng/mL final concentration in culture) for 5 days. Alternatively cells were treated with the vehicle (VH; 0.02% PBS final concentration in culture, pH 3.5, containing 0.1% BSA) for 5 days. Supernatants were harvested and subsequently analyzed for total (a) IgA and (b) IgM secretion by ELISA. The results from quadruplicate determinations are expressed as the mean IgA (nanogram per 10^6 recovered viable cells ± SEM) or mean IgM (microgram per 10^6 recovered viable cells ± SEM) (n=4). "a" denotes $p < 0.05$ as determined by Dunnett's t test and compared to the SMAD3$^{+/+}$ vehicle control. "b" denotes $p < 0.05$ as determined by Dunnett's t test and compared to the SMAD3$^{-/-}$ vehicle control. "#" denotes $p < 0.05$, SMAD3$^{+/+}$ compared to same group SMAD3$^{-/-}$ as determined by Dunnett's t test. The results are representative of two independent experiments.


(a)
Smad3+/+
Smad3-/-
Total IgA (ng/10^6 cells)
50
40
30
20
10
0
#
a
No
VH
IL-10
IL-10 + TGF-β1
Treatment
LPS
(b)
Total IgM (μg/10^6 cells)
200
150
100
50
0
b
a
#
No
VH
IL-10
IL-10 + TGF-β1
Treatment
LPS

protein product is not generated due to a disruption of an essential T_H2 cytokine environment necessary for the translation of TGF-β_1-induced sterile mRNA transcripts with Smad3 deficiency. This interpretation is supported by elevated production of IFN-γ, a characteristic T_H1 cytokine, by $Smad3^{-/-}$ T cells (**Figure 39a**). Moreover, the magnitudes of basal and LPS-induced IgA protein are greater in $SMAD3^{-/-}$ than $SMAD3^{+/+}$ splenocytes (**Figure 61a**). These results demonstrate that $SMAD3^{-/-}$ B cells can produce IgA *in vitro*.

DISCUSSION

It has long been established that TGF-β_1 is essential for maintaining immune homeostasis. More recently an essential role for TGF-β signaling in T cells has been established. Probably the most compelling supportive evidence is exemplified by *in vivo* spontaneous differentiation of T cells into effector cytokine producing cells and autoimmune manifestations in transgenic mice that have impaired TGF-β_1 signaling specifically and exclusively in T cells (Gorelik and Flavell 2000).

Over the past several years, numerous *in vitro* studies have delineated a rather confusing and sometimes contradictory role for TGF-β_1 in regulating T cell immune homeostasis. For example, T cell growth is impaired by high concentrations of TGF-β_1 (Stoeck *et al.* 1989a), while low concentrations of TGF-β_1 reportedly stimulate T cell growth (Kondo *et al.* 1993). In one report, TGF-β_1 down-regulated IL-2 receptors (Kehrl *et al.* 1986c), while in a second report had no affect on IL-2 receptor expression (Smyth *et al.* 1991). TGF-β_1 also paradoxically induces (Weller *et al.* 1994) as well as suppresses T cell apoptosis (Cerwenka *et al.* 1996). Moreover, TGF-β_1-null mice succumb to a $CD4^+$ T cell-mediated multi-organ autoimmune inflammatory disease (Diebold *et al.* 1995; Shull *et al.* 1992). Transgenic mice overproducing TGF-β_1 also succumb to chronic inflammation (Kim *et al.* 1991).

TGF-β_1 acts on all immune cells, and elicits its effects through multiple mechanisms, thus it is not surprising that many of the reported effects of TGF-β_1 using completely different experimental models are complicated and disparate. The toxicological and pharmacological implications of TGF-β_1 on T cell homeostasis and

tolerance are profound and resoundingly establish a necessity for delineating the mechanisms whereby (1) TGF-β_1 modulates T cell function and (2) TGF-β_1 signaling in T cells regulates immune homeostasis . . . hence the relevance of this research.

The overall working hypothesis that was put forth to test in this dissertation research is as follows. '*TGF-β_1 acts directly on T cells in a receptor-dependent manner to regulate IL-2 expression through Smad-mediated intracellular signaling.*' The overall specific aims for this research were three-fold: (1) Develop and validate an *in vitro* model to characterize the effects of TGF-β_1 on T cell growth and IL-2 expression. (2) Determine whether Smad signaling plays a role in the regulation of IL-2 expression by TGF-β_1. (3) Determine whether cross talk between MAPK and Smad signaling plays a role in the regulation of IL-2 expression by TGF-β_1.

This discussion is organized and presented in five sections to address the aforementioned working hypothesis and specific aims. Section I describes the concentration- and time-dependent regulation of T cell growth and IL-2 expression by TGF-β_1. The second section presents evidence that Smad3 is essential for inhibition of T cell growth and IL-2 production by TGF-β_1 *in vitro*. Section III describes a putative role for DNA sequence specific binding of Smad3 in the regulation of IL-2 expression by TGF-β_1. The fourth section discusses evidence for putative MAPK and Smad signaling cross talk in the regulation of TGF-β_1 signaling in T cells. And the last section, section V, discusses a role for Smad3 in the regulation of T-cell dependent and T cell-independent humoral immune responses by TGF-β_1 *in vitro*.

I. Concentration- and time-dependent regulation of T cell growth and IL-2 expression by TGF-β_1

TGF-β_1 mediates its effects through the activation of a heteromeric complex of transmembrane receptors with intrinsic serine threonine kinase activity. Despite an overwhelming amount of newly-acquired mechanistic data regarding the regulation of TGF-β_1-responsiveness through Smad-dependent processes, Smad signaling in T cells remains relatively unexplored. This is probably due, in part, to the pleotropic nature of TGF-β_1-mediated immune responses. In light of these often seemingly paradoxical pleotropic responses by TGF-β_1, one of the initial focuses of this research was to establish an *in vitro* model to investigate a role for Smad-dependent signaling in the regulation of T cell growth and IL-2 secretion by TGF-β_1.

Lymphoid responsiveness to TGF-β_1 depends on the type and state of the cell (e.g., activation, differentiation, and maturation) (Cerwenka *et al.* 1994; Ludviksson *et al.* 2000; Wahl *et al.* 2000; Weller *et al.* 1994; Yates *et al.* 2000). Therefore several experimental variables including TGF-β_1 concentration, CD28 co-stimulation, and the time of addition of TGF-β_1 relative to T cell activation were compared in primary mouse splenocytes and thymocytes to optimize TGF-β_1 responsiveness. Primary thymocytes were incorporated into the study design to investigate the effects of TGF-β_1 on T cells in the absence of antigen presenting cells, i.e., B cells, macrophages, and dendritic cells. Physiological concentrations of TGF-β_1 (Ahmad *et al.* 1997) were employed. Notably, a direct comparison between the biological activity of *in vivo* and *in vitro* TGF-β_1 concentrations is limited due to the secretion and distribution of TGF-β_1 as a latent, biologically inactive precursor molecule (Miyazono and Heldin 1989). Splenocytes and

thymocytes were isolated and activated *in vitro* using antibodies against the TcR/CD3 complex and the CD28 co-stimulatory molecule to pharmacologically mimic antigen-induced complete T cell activation.

A similar concentration-dependent inhibition of splenic T cell and thymocyte growth by TGF-β_1 is suggestive of a direct effect by TGF-β_1 on T cells. The T cell growth inhibitory effect by TGF-β_1 was further demonstrated to be dependent upon the time of addition of TGF-β_1 to the cell cultures relative to when the T cells were activated. This temporal relationship was interpreted to implicate regulatory cross talk between signaling cascades activated downstream of the TcR and the TβR. The markedly varied responsiveness to TGF-β_1 over a relatively short interval (e.g., 30 minutes, in some cases) further suggested the involvement of an early, upstream signaling event. Surprisingly, CD28 co-stimulation did not markedly influence the inhibition of T cell growth by TGF-β_1 implicating a lack of regulatory cross talk between CD28-associated and TβR signaling.

In contrast to T cell growth, concentration-dependent bifunctional stimulation and attenuation of steady state IL-2 mRNA expression as well as IL-2 protein secretion by TGF-β_1 was demonstrated in activated splenocytes. CD28 co-stimulation augmented the stimulatory effect of low concentrations of TGF-β_1 on splenic T cell IL-2 expression, but did not influence the inhibitory effects by high concentrations under conditions of simultaneous addition of TGF-β_1 and T cell activation. Time of addition studies substantiated a regulatory role for CD28-associated as well as TcR signaling in the bifunctional concentration-dependent stimulatory and inhibitory effects of TGF-β_1 on IL-2 secretion. Importantly, quantitative RT-PCR analyses provides evidence suggesting

that the bifunctional modulatory effects on IL-2 secretion by TGF-β_1 are mediated though direct effects on IL-2 gene transcription. Collectively, these results support a hypothesis that cross talk among TcR, TβR, and CD28-associated signaling regulates IL-2 expression by TGF-β_1 in activated T cells. In light of these *in vitro* observations, it is tempting to speculate that similar *in vivo* bimodal concentration-dependent effects by TGF-β_1 on immune cell function may also be regulated through direct effects of TGF-β_1 on gene expression.

These studies are the first to describe the concentration-dependent effects of TGF-β_1 on IL-2 expression, at the level of protein secretion as well as mRNA expression, in α-CD3 + α-CD28-activated T cells. In deciphering the observed stimulatory effects of low concentrations of TGF-β_1, putative indirect contributory factors warrant consideration. For example, low concentrations of TGF-β_1 may selectively target populations of T lymphocytes expressing elevated levels of high affinity TGF-β receptors; these T cell 'subpopulations' may be inherently stimulated by TGF-β_1. Accordingly, it has been demonstrated that TβR expression and affinity is dependent upon T cell maturation and differentiation (Wahl 1992).

Alternatively, TGF-β_1 may, in a concentration-dependent manner, differentially activate intracellular signaling cascades, e.g., Smads and MAPKs (Piek *et al.* 1999). Cross talk among these different signaling cascades as well as between TβR, TcR, and CD28-associated signaling may provide putative mechanisms whereby TGF-β_1 is able to differentially regulate IL-2 expression in a concentration-dependent manner. This latter possibility is particularly interesting in light of the observation that TGF-β_1 modulates the activity of several transcription factors critical for regulating IL-2 transcription including

AP-1, CREB, and NF-κB (Brabletz *et al.* 1993). Notably, each of these aforementioned transcription factors has been demonstrated to bind to the CD28RE in the IL-2 promoter (Shapiro *et al.*, 1997).

Upregulation of TβR expression following T cell activation also warrants consideration as a putative contributing factor for the bifunctional T cell responsiveness to TGF-β_1 (Ellingsworth *et al.* 1989). Based upon the observation that a temporal loss of TGF-β_1-induced augmentation of IL-2 secretion occurs only under conditions of CD28 co-stimulation, it is possible that CD28-associated signaling may contribute to the upregulation of TGF-β receptors in activated T cells. Alternatively, CD28 signaling may modulate the effects of TGF-β_1 on α-CD3-activated T cells through direct stimulation of cytokine production, growth survival signals, or stablization of IL-2 mRNA (Boise *et al.* 1995; Shapiro *et al.* 1997). Notably, a requirement for CD28-associated signaling for augmentation of IL-2 secretion by TGF-β_1 is consistent with previous studies (Aoki *et al.* 1991). Probably, the single most important biological significance gleaned from this set of experiments is the demonstration that low concentrations of TGF-β_1 augment steady state IL-2 mRNA and IL-2 secretion in α-CD3 + α-CD28-activated T cells and high concentrations of TGF-β_1 paradoxically attenuate IL-2 expression under similar conditions of T cell activation. Putative mechanisms for this phenomenon have been discussed above.

In light of the observation that intracellular signaling by TGF-β_1 permits interactions among multiple receptors and intracellular signaling intermediates allowing for a diversity of biological responses, it is proposed that the differential regulatory effects of TGF-β_1 on IL-2 expression are mediated through the cooperative interaction of

multiple Smad-interacting signaling cascades that are activated by TGF-β_1, in a concentration-dependent manner.

II. Smad3 is essential for inhibition of T cell growth and IL-2 expression by TGF-β_1 *in vitro*

At the onset of these studies, a role for Smad signaling in TGF-β_1-responsiveness had been established in numerous cell types; however, Smad protein expression had not been established in lymphoid tissue. Western blot analyses confirmed that the TβR activated-Smads, Smad2 and Smad3, as well as the co-Smad, Smad4 are expressed in murine splenocytes and thymocytes. Moreover, these studies demonstrated that TGF-β_1 activated Smad3 in splenocytes in a concentration- and time-dependent manner. These results are in agreement with other studies demonstrating rapid and transient activation of Smad proteins by TGF-β_1 (Shen *et al.* 1998).

Our studies further established a role for Smad3 in mediating the inhibitory effects of TGF-β_1 on IL-2 expression. Anti-CD3 + α-CD28-activated Smad3-null splenic T cells and thymocytes were refractory to the inhibitory effects of TGF-β_1 on steady state IL-2 mRNA and IL-2 secretion. These observations are consistent with reports demonstrating a role for Smad3 in maintaining T cell-dependent immune homeostasis (Datto *et al.* 1999; Yang *et al.* 1999).

While it has been previously reported that T cells from Smad3-null mice are resistant to growth inhibition by TGF-β_1 (Datto *et al.* 1999; Yang *et al.* 1999), our studies were the first to demonstrate this refractory phenomenon in fully activated mature T cells. Moreover, our results are in agreement with others (Datto *et al.* 1999; Yang *et al.* 1999)

demonstrating that inhibition of LPS-induced B cell growth by TGF-β_1 *in vitro* is unaffected by targeted deletion of Smad3. A cause and effect relationship between the inhibition of IL-2 and T cell growth is supported by the ability of exogenous IL-2 to reverse the inhibition of T cell growth by TGF-β_1 in activated T cells. It has previously been established that *in vitro* LPS-stimulated B cell growth does not require B cell autocrine or T cell-derived IL-2 (Hashimoto *et al.* 1986). Collectively, these results suggest that TGF-β_1 may differentially regulate lymphoproliferation in Smad3-null mice through direct regulatory effects on IL-2 expression. In light of these observations, a model is presented that defines a novel mechanism whereby TGF-β_1 may differentially regulate proliferation of B cells and T cells (**Figure 62**).

Characteristically, immune competent naïve T cells in peripheral tissues predominantly secrete IL-2 upon stimulation. In contrast, differentiated effector T cells generated during an immune response secrete a battery of cytokines characteristic of a TH1 or TH2 phenotype. Naïve Smad3-null splenic T cells display a differentiated TH1 phenotype as demonstrated by elevated basal IL-2 and IFN-γ expression. These results are consistent with an *in vivo* activated Smad3-null peripheral T cell phenotype, as demonstrated by increased CD62L surface expression (Yang *et al.* 1999). Moreover, elevated IFN-γ expression in Smad3-null T cells correlates with a phenotype

Activation of IFN-γ/STAT pathways has been shown to antagonize TGF-β_1/Smad signaling, at least in part, by upregulating activity of the inhibitory Smad7 (Ghosh *et al.* 2001; Ulloa *et al.* 1999). In light of the elevated IFN-γ levels observed in naïve and activated Smad3$^{-/-}$ splenocytes and thymocytes, we verified that abrogation of the inhibitory effects of TGF-β_1 on SMAD3$^{-/-}$ T cells was not due to an indirect IFN-γ-

Figure 62. Putative model for Smad3-dependent inhibition of T cell growth by TGF-β_1. This model proposes that TGF-β_1 differentially attenuates T- and B-cell growth through Smad3-dependent and Smad3-independent mechanisms, respectively. Moreover, Smad3-dependent inhibition of T cell growth by TGF-β_1 is through a direct inhibition of IL-2 mRNA expression.

α-CD3
α-CD28
TCR/CD3
CD28
IL-2R
IL-2
IL-2 mRNA
Cyclin mRNA
cdk mRNA
(-)
TGF-β1
SMAD3-
Dependent
TGF-β1
SMAD3-
Independent
cdkI mRNA
cdkI Activity
(-)
(-)
CyclinE•cdk4/cdk6
Complex Kinase
CyclinD•cdk2
Complex Kinase
[3H]-
Thymidine
Incorporation

dependent down-regulation of Smad2 signaling. Smad2 phosphorylation by TGF-β_1 *in vitro* was demonstrated in Smad3-null T cells and implicated functional Smad2 signaling.

In summary, these results provide evidence suggesting that Smad3 is essential for inhibition of IL-2 mRNA and protein secretion by TGF-β_1. These data are also significant as they provide a mechanism whereby TGF-β_1 may selectively inhibit T cell proliferation in a Smad3-dependent manner. These results also provide evidence for a role for Smad3 in regulating T cell-dependent immune homeostasis through the regulation of basal cytokine production *in vivo* as well as the magnitude of stimulated cytokine secretion *in vitro*. Collectively, the evidence presented here provides a novel mechanism whereby TGF-β_1 may directly and selectively target T cells to maintain immune homeostasis and regulation of T cell tolerance.

III. A role for DNA sequence specific binding of Smad3 in the regulation of IL-2 expression by TGF-β_1

In deducing the mechanism(s) whereby TGF-β_1/Smad3 signaling modulates IL-2 expression, five CAGA sequences were identified in the 5' minimal essential regulatory region of the mouse IL-2 promoter. It is hypothesized that these CAGA sequences function as TGF-β_1 responsive elements. In theory, one sequence should appear once every 256 bp in the genome; however, five CAGA sequences reside within a 160 bp span of the IL-2 promoter.

Sequence-specific Smad binding has been demonstrated (Chen *et al.* 2000; Chen *et al.* 1999; Dennler *et al.* 1998; Jonk *et al.* 1998; Poncelet and Schnaper 2000; Zhang *et al.* 2000); however the positioning of CAGA sequences in close proximity to DNA

binding sites for other transcription factors is important for Smad3 transcriptional activity (Dennler *et al.* 1998; Zhang *et al.* 1998). Smads mediate transcriptional activation by interacting with other transcription factors, for example AP-1 responsive elements in the collagenase promoter overlap SBEs (Zhang *et al.* 1998). Consistently, each of the five CAGA sequences in the mouse IL-2 promoter lies adjacent to or overlaps a DNA binding site for another transcription factor known to regulate IL-2 transcription in response to antigen-induced T cell activation (i.e. AP-1, CREB, NFAT, NF-κB, and ZEB) (Jain *et al.* 1995; Jain *et al.* 1992b; Powell *et al.* 1999; Shapiro *et al.* 1997). A regulatory role for CREB•Smad3, AP-1•Smad3, and NF-κB•Smad3 interactions has also been substantiated in numerous TGF-β_1 responsive genes. (Lopez-Rovira *et al.* 2000; Sano *et al.* 1999; Verrecchia *et al.* 2001b; Wong *et al.* 1999).

In the present studies, EMSA analyses demonstrated that mutation of the CAGA sequences in the IL-2 promoter markedly altered the DNA binding profiles at the proximal AP-1 site and the CD28RE and provides evidence supporting a role for CAGA sequences in regulating IL-2 transcription. Moreover, TGF-β_1-induced Smad3 binding to proximal AP-1/CAGA site suggests that Smad3 is a component of the DNA binding transcription factor complex.

Identification of Smad3 as a component of protein complex binding to the proximal AP-1 site containing mutated CAGA sequences suggests that Smad3 may bind to this AP-1 response element as a Smad3•AP-1 heterodimer. We further demonstrated that TGF-β_1-induced binding of Smad3-null nuclear proteins to the proximal AP-1 site does not produce a shift in the DNA binding complex. In light of the observation that Smad3•AP-1 and AP-1•AP-1 DNA binding complexes are indistinguishable on 4%

PAGE resolving gels (Verrecchia *et al.* 2001b), one interpretation of these results is that TGF-β_1 may induce multiple transcription factors to bind to DNA elements, but the presence of Smad3 as a component of the DNA binding complex may play a role in the regulating the transcriptional activity of the transcription factor binding complex.

In summary, these results provide evidence that Smad3 and CAGA sequences may regulate basal as well as TGF-β_1-induced DNA binding activity in the mouse IL-2 promoter; however, neither Smad3 nor CAGA sequences are essential for TGF-β_1 induced binding to the proximal AP-1 response element.

IV. MAPK and Smad signaling cross talk in regulating TGF-β_1 signaling in T cells

The observed bifunctional effect of TGF-β_1 on steady state IL-2 mRNA and IL-2 protein is consistent with the well characterized diverse pleotropic nature by which TGF-β_1 seemingly acts. Our results do not discriminate between mRNA transcription and stability; however, evidence strongly suggests that IL-2 is predominantly regulated at the level of transcription (Powell et al. 1998). The specificity of TGF-β_1-responsiveness is resultant of specific interactions between Smad proteins and other regulatory proteins present within the nucleus at any given time (Massague and Wotton 2000), thus it is tempting to speculate that the bifunctional concentration-dependent effect of TGF-β_1 on IL-2 expression may be mediated through regulatory cross talk between Smad and MAPK signaling. In support, several recent findings have implicated regulatory cross talk between ERK MAPK and Smad signaling in controlling TGF-β_1-responsiveness. For example, TGF-β_1-induced furin gene transactivation in hepatic HepG2 cells is

Smad2- and ERK MAPK-dependent (Blanchette et al. 2001). In addition, Smad and ERK MAPK signaling is essential for TGF-β_1-induced p21 expression in HaCaT cells (Hu et al. 1999).

The present studies demonstrate that TGF-β_1 selectively upregulates ERK MAPK, but not JNK MAPK activity in mouse splenocytes. Pharmacological inhibition of ERK MAPK augments Smad3 nuclear expression and attenuates inhibition of IL-2 protein secretion by high concentrations of TGF-β_1. A negative regulatory role for ERK MAPK on Smad signaling has been previously documented (Kretzschmar *et al.*, 1999). Our results demonstrating that ERK MAPK signaling is essential for inhibition of α-CD3 + α-CD28-induced IL-2 protein secretion by TGF-β_1 is the first evidence of a regulatory interaction between these two pathways in T cells.

Our results implicating regulatory cross talk between Smad and MAPK signaling are of particular interest, as it is well established that activation of the TcR leads to activation of various MAPKs. ERK, JNK, and p38 MAPKs are all critical components of TcR signaling (Berridge 1997). Activated MAPKs phosphorylate cytosolic as well as nuclear proteins in activating downstream targets important for the regulation of IL-2 expression, including Jun, Fos, Elk-1 (a positive regulator of c-fos), and ATF2 (a CREB family member).

Activation of JNK, p38, and ERK MAPKs as well as TGF-β_1-activated kinase (TAK) by TGF-β_1 has been reported in primary epithelial cells, fibroblasts, rat hepatic stellate cells, and breast cancer cell lines (Axmann *et al.* 1998; Reimann *et al.* 1997;Hartsough and Mulder 1997). Regulation of TGF-β_1/Smad signaling in T cells remains to be investigated. Because Fos, Jun, and CREB are all transcriptional

components of the TcR pathway, it is tempting to speculate that cross talk among Smad and MAPK signaling pathways play a critical regulatory role in the overall biological effect of TGF-β_1 on cytokine expression, including IL-2.

In summary, these results have important biological implications in suggesting putative regulatory cross talk between TcR and Smad signaling cascades in T cells. Since MAPKs are functional components of both the TβR and TcR pathways, the possibility arises that MAPKs may mediate cross talk between these two pathways, and may provide a mechanism whereby TGF-β_1 may elicit different responses on IL-2 that are concentration- and context-dependent.

V. TGF-β_1 differentially modulates humoral immune responses *in vitro*

In these studies, the functional significance of Smad3-dependent cellular and biochemical changes that accompany alterations in B cell effector function in response to TGF-β_1 were investigated. It has been previously demonstrated that *in vivo* exposure of B6C3F1 mice to modest hepatotoxic doses of CCl_4 induces potent immune suppression (Kaminski *et al.* 1990; Kaminski *et al.* 1989). Pre-incubation of sera from CCl_4-treated mice with neutralizing antibodies to TGF-β_1 abrogates suppression of the IgM AFC response by TGF-β_1, demonstrating that CCl_4, at least in part, induces immune suppression via upregulation of TGF-β_1 activity. These results are consistent with other experimental and clinical evidence strongly supporting a role for TGF-β_1 in immune suppression following exposure to a range of chemicals and pharmacological agents that induce liver damage and/or diseases, including chemotherapeutics, immunosupressants, alcohol, and acetaminophen (Ahuja *et al.* 1995; Gutierrez-Ruiz *et al.* 2001; He *et al.*

2000; Hori *et al.* 2000; Inoue *et al.* 2000; Lemmer *et al.* 1999; Neuman 2001; Simile *et al.* 2001; Szuster-Ciesielska *et al.* 2000; Vodovotz *et al.* 2000; Yin *et al.* 2001).

In light of these observations, it is conceivable that TGF-β_1-induced systemic immune suppression may be a characteristic secondary consequence of hepatotoxic agents. The mechanisms underlying TGF-β_1-mediated immune suppression following hepatotoxicant exposure have not yet been identified, in part, due to an incomplete understanding of the comprehensive and often seemingly paradoxical immune modulation that ensue upon TGF-β_1 exposure.

TGF-β_1 differentially modulates proliferation, differentiation, apoptosis, cytokine expression, and immunoglobulin production in a concentration- and context-dependent manner. Immune responses are highly dependent upon cell to cell interactions and therefore it is highly conceivable that the overall influence of TGF-β_1 is likely a composite of multiple individual effects on numerous immune cell subpopulations.

In B cells, TGF-β_1 is well recognized for its ability to inhibit proliferation, immunoglobulin secretion, antigen receptor expression, and MHC class II molecules; and alternatively stimulate IgA and IgG2b isotype class switching (Letterio and Roberts 1998). We demonstrated that femtomolar concentrations of TGF-β_1 augmented T cell-dependent and T cell-independent IgM AFC responses. The observed profile of inhibition with respect to humoral responses is highly suggestive of a cell-type specific effect by TGF-β_1. The sRBC response requires both accessory T cells and antigen-presenting cells; conversely, the DNP-Ficoll AFC response is T cell-independent, but requires functional antigen presenting cells. In contrast, LPS induces a direct B cell mitogenic response, and is independent of either accessory T cells or antigen-presenting

cells. Therefore, these observations suggest that TGF-β_1 directly targets accessory T cells and/or antigen presenting cells, but not B cells to augment the IgM AFC response. In contrast, B cells clearly contribute to the inhibitory effects elicited by TGF-β_1.

There are at least two, and not necessarily mutually exclusive, ways in which TGF-β_1 might function to augment the magnitude of the aforementioned AFC responses. First, TGF-β_1 may act directly on accessory T cells to up-regulate a battery of cytokines essential for humoral-mediated responses, for example IL-2, IL-4 and IL-5. This scenario is consistent with our previously discussed results demonstrating that femtomolar concentrations of TGF-β_1 stimulate IL-2 secretion in activated T cells. In addition, it has been demonstrated that TGF-β_1 preferentially induces a shift towards a TH2 cytokine profile as demonstrated by elevated IL-4 and IL-5 expression. A second plausible explanation is that TGF-β_1 might act to enhance antigen presentation by directly or indirectly (e.g. via cytokine production) stimulating antigen presenting cells. In support of this explanation, femtomolar concentrations TGF-β_1 have been shown to induce monocyte chemotaxis (McCartney-Francis *et al.* 1998; Wahl *et al.* 1987).

An important role for Smad3 in maintaining immune homeostasis has been convincingly established as demonstrated by the development of severely impaired mucosal immunity and diminished T cell responsiveness to TGF-β_1 in Smad3-null mice (Yang *et al.* 1999). Defined regulatory roles for Smad3 in cytokine secretion, lymphocyte proliferation, immunoglobulin production, and MHC II expression also have been reported (Datto *et al.* 1999; Dong *et al.* 2001; Yang *et al.* 1999). Our results indicate that the inhibitory effects of TGF-β_1 on T cell-dependent and T cell-independent

IgM AFC responses *in vitro* are not disrupted, but rather augmented in Smad3-null splenic B cells.

Consistently, we further demonstrated that inhibition of LPS-induced IgM secretion by TGF-β_1 is unaffected in Smad3-null splenic B cells. TGF-β_1 is known to suppress IgM secretion at two distinct levels specifically, inhibition of μ heavy chain mRNA transcription and switching from membrane to the secreted form of μ chains. While the role of Smad3 in mediating these two regulatory routes of IgM synthesis has not yet been studied, our data suggest a Smad3-independent mechanism(s) of action. It is important to note that we can not rule out the possibility of a confounding influence by the complement activating immunoglobulin, IgG3, on the AFC response. TGF-β_1 suppresses IgG3 secretion and circulating levels of IgG3 are elevated 3-fold in TGF-β_1 signaling-deficient mice (Cazac and Roes 2000).

During the normal course of differentiation, B cells undergo immunoglobulin class switching, whereby the initial synthesis of IgM is converted to IgD, IgG, IgE, or IgA. Distinct immunoglobulin isotype profiles are essential for development of an optimally protective humoral immunity. TGF-β_1 has been implicated as a positive regulator of IgA synthesis by upregulating germline Igα transcription and directing IgA isotype class switching. Serum IgA and IgA-expressing B cells are severely compromised in mice deficient in TGF-β_1 signaling (Hein *et al.* 1998; Lebman *et al.* 1990; Zhang and Derynck 2000). Transcriptional regulation of Igα by TGF-β_1 is mediated through a proximal promoter/enhancer region located approximately 130 base pairs upstream of the human and mouse intron (I) α transcription start site. This aforementioned sequence contains an array of interspersed Smad, acute myloid leukemia

(AML), and cAMP-response element-binding protein (CREB) response elements that cooperatively, via protein•protein and protein•DNA interactions, mediate TGF-β_1 responsiveness (Pardali *et al.* 2000a; Zhang and Derynck 2000).

EMSA analysis using GST-fusion proteins has revealed direct binding of both Smad3 and Smad4 to the CAGA elements within this region (Zhang and Derynck 2000). The over-expression of Smad3 and Smad4 selectively increases surface IgA expression as well as IgA production by murine B lymphoma cells *in vitro* (Park *et al.* 2001).

Our data further implicate an essential positive regulatory role for Smad3 in IgA synthesis by TGF-β_1, as evidenced by a lack of IgA production by TGF-β_1 in LPS-activated primary splenocytes obtained from Smad3-null mice. In contrast, *in vivo* B cell surface IgA expression and IgA serum levels are not compromised in Smad3-null mice, suggesting that Smad3 is *not* required for IgA synthesis *in vivo* (Yang *et al.* 1999). These *in vivo* observations are in agreement with the elevated IgA levels that we observed in unstimulated and LPS-stimulated splenocytes from Smad3-null mice, implicating a second alternative, negative regulatory role for Smad3.

Perhaps some light may be shed on these seemingly contradictory results by the recent observation that two distinct populations of IgA-producing B cells are differentially regulated by two groups of cytokines (Hiroi *et al.* 2000). Specifically, IL-5, IL-6, IL-15, and TGF-β_1 seemingly play distinct and perhaps somewhat contrasting roles in regulating the differentiation of B cells into IgA-producing cells. Interestingly, IL-5, IL-6, and IL-15 expression are upregulated in Smad3-null mice (Datto *et al.* 1999). Therefore, it is plausible to hypothesize that the seemingly disparate dual roles of Smad3 in regulating IgA production may be due, in part, to a disruption of cytokine homeostasis.

In addition, a role for Smad3 in the regulation of cytokine and MHC II expression may also, at least in part, explain the elevated AFC, Ig secretion, and B cell mitogenesis in Smad3-null mice, which, again, implies a putative negative regulatory role for Smad3.

In conclusion, direct addition of femtomolar concentrations of TGF-β_1 augment T cell-dependent and T cell-independent humoral immune responses in splenocyte cultures. These concentration-dependent responses are mediated through directs effects of TGF-β_1 on accessory T cells and/or antigen presenting cells, and not through a direct influence of TGF-β_1 on B cells, thus further illustrating that the functional humoral immune response achieved by TGF-β_1 is concentration- and context-dependent.

Inhibition of sRBC- and LPS-induced IgM secretion by TGF-β_1 *in vitro* is also dependent on Smad3 signaling. Moreover, Smad3 is essential for IgA production by TGF-β_1 in LPS-activated B cells *in vitro.* These results provide critical functional relevance to previous studies describing Smad3-dependent transcriptional modulation of the IgA promoter/enhancer. Collectively, these results demonstrate enhanced cell growth and antibody secretion in activated Smad3-null B cells and further support a role for Smad3 in maintaining immune homeostasis.

VI. Conclusions

TGF-β_1 is a multifunctional cytokine with potent immune suppressive and pro-inflammatory properties. The molecular mechanisms whereby TGF-β_1 selectively mediates paradoxical responses on immune cell functions remain elusive. The present studies have established that TGF-β_1 bifunctionally augments and attenuates IL-2 expression in activated T cells at femtomolar and picomolar concentrations, respectively.

A similar bifunctional concentration-dependent effect by TGF-β_1 was demonstrated in T cell-dependent and T cell-independent IgM AFC responses. Collectively, these data suggest that concentration is a critical factor in determining the regulatory effect of TGF-β_1 on lymphocyte function and immune homeostasis.

Among the known cytoplasmic intermediates associated with signaling through the TβR, the Smad proteins are of particular importance. Smad3 was demonstrated to be essential for TGF-β_1-mediated inhibition of IL-2 mRNA expression, IL-2 protein secretion, and TcR-induced T cell, but not LPS-induced B cell proliferation. Collectively these results permit a proposed mechanistic model whereby TGF-β_1 may selectively attenuate T cell growth by suppressing IL-2 gene transcription through Smad3 signaling.

In elucidating the mechanism(s) whereby Smad3 regulates IL-2 expression, five putative CAGA Smad3 binding elements were identified in the IL-2 promoter. Each CAGA element is within close proximity of a DNA binding site(s) for other transcription factor(s), including AP-1, CREB, ZEB, and NF-κB, and NFAT. Moreover, evidence is presented suggesting that Smad3 is a component of the transcription factor complex that binds to the proximal AP-1 site in response to TGF-β_1. In addition, mutation of the CAGA sequences at the proximal AP-1 site and CD28RE were demonstrated to markedly alter the DNA binding complexes in naïve as well as activated T cells. These studies further demonstrate that, in addition to Smad signaling, TGF-β_1 also activates the ERK MAPK pathways in splenic T cells. Collectively, on the basis of these observations, it is tempting to speculate that Smad3 signaling may involve cooperative as well as antagonistic interactions with other signaling pathways, including ERK/AP-1, p38/ATF-2, and JNK/c-jun signaling in activating/suppressing IL-2 transcription.

Finally, TGF-β_1 effectively inhibits the *in vitro* IgM AFC response to either T cell-dependent anti-sRBC or T cell-independent LPS, and suppressed IgM secretion in Smad3-null and Smad3$^{+/+}$ splenic B cells. However, in contrast to Smad3$^{+/+}$ B cells, IgA secretion is not enhanced by TGF-β_1 in LPS-activated Smad3-null splenic B cells *in vitro*. These results define a role for Smad3 in mediating enhanced IgA production in response to TGF-β_1 *in vitro*. Collectively, these results further substantiate an essential role for Smad3 signaling in regulating TGF-β_1-mediated immune homeostasis, but also demonstrate that Smad3-independent pathways may contribute to the regulation of B cell responses by TGF-β_1.

In summary, this work has demonstrated the importance of concentration as a critical parameter of the modulatory effects elicited by TGF-β_1 in T cell-dependent as well as T cell-independent immune responses. These studies have also established that Smad3 is essential for the inhibition of IL-2 expression by TGF-β_1 and further demonstrate a role for Smad3 as a mediator for some, but not all of the immune modulatory effects elicited by TGF-β_1 on T cell-dependent as well as T cell-independent effector cell function.

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