Conditional Ablation of the Gene Encoding Transforming Growth Factor-β1 in the Mouse

Inaugural-Dissertation
zur
Erlangung des Doktorgrades
Der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

vorgelegt von

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Tag der mündlichen Prüfung: 14.Mai, 2002

ABBREVIATIONS

Aa	amino acids
Ab	antibody
Ag	antigen
APC	antigen presenting cell
BCR	B cell receptor
BM	bone marrow
Bp	base pair(s)
BSA	bovine serum albumin
CD	cluster of differentiation
cDNA	complementary DNA
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNTP	2'-deoxyribonucleosidetriphosphate
EDTA	ethylene-diaminetetraacetic acid
ES cells	Embryonic stem cells
FACS	Fluorescence Activated Cell Sorter
FCS	fetal calf serum
Fig.	Figure
H/E	hematoxylin/eosin
HSV	Herpes Simplex virus
Ig	immunoglobulin
IFN	interferon
Kb	kilobase (pairs)s
LAH	long arm of homology
LysM	M-lysozyme
MAb	monoclonal antibody
MW	molecular weight
O/N	overnight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerytherine
RAG	recombination activating gene
RT	room temperature
SAH	short arm of homology
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulfate
SPF	Specific Pathogen Free
SSC	standard sodium citrate
SV40	Simian virus 40
TCR	T cell receptor
Tg	transgenic
Th	T helper cell
TI	T-independent
TK	thymidine kinase
Tris	Tris-(hydroxymethyl)aminoethane
Wt	wild type

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Generation of conditional knockout mice for the TGF-β1 gene

A INTRODUCTION

The transforming growth factor beta (TGF-β) superfamily includes secreted signaling molecules that mediate a wide range of physiological processes. Thirty members of the TGF-β family have been described in humans and many orthologs are known in mouse, Xenopus and Drosophila (Massague et al., 2000). These growth factors are structurally related polypeptides that share a cluster of conserved cysteine residues. The residues form a common cysteine knot structure, held together by intramolecular disulfide bonds (Massague, 1998). Members of the family comprise the TGFbetas, activins, inhibins, bone morphogenetic proteins, Mullerian-inhibiting substance, nodal, dorsalin, products of the Drosophila decapentaplegic, Xenopus Vg-1 genes and the growth differentiation factor family of proteins (Kingsley, 1994; Lee, 1990; Massague, 1990; McPherron and Lee, 1993; Roberts et al., 1988; Storm et al., 1994). The family is divided into two general branches: the BMP/GDF and the TGF-β /Activin/Nodal branches.

In mammals, TGF-β has three isoforms TGF-β1, TGF-β2, and TGF-β3 that are highly conserved but differ in their binding affinity for the TGF-β receptors (Millan et al., 1991; Schmid et al., 1991). They have the characteristics of secreted polypeptides, carrying a hydrophobic signal sequence for translocation across the endoplasmic reticulum and are glycosylated. Each isoform is synthesized as part of a large precursor molecule and is encoded by a distinct gene that is expressed in both a tissue-specific and a developmentally regulated manner. While the expression of TGF-betas 2 and 3 is more developmentally and hormonally regulated, the type1 isoform is selectively induced in response to a variety of signals, including the products of immediate early genes and certain oncogenes (Roberts and Sporn, 1993). Also, unlike the other two isoforms, TGF-β1 is unique in its ability to induce its own expression through a process of auto induction (Kim et al., 1990). Despite a high degree of structural similarity, the isoforms are not functionally redundant. Isoform-specific activities are now more evident through models in which expression of each individual isoform has been disrupted. These knockouts demonstrate that the selective loss of each isoform *in vivo* yields a distinct, severely abnormal phenotype (Kaartinen et al., 1995; Kulkarni et al., 1993; Proetzel et al., 1995; Shull et al., 1992).

This part of the thesis describes work done on TGF-β1 within the realms of the immune system. Due to their multi-faceted roles, the background description on TGF-betas is vast. Therefore, to maintain brevity, the introduction is confined to TGF-β1 with emphasis on its role in immune regulation. Moreover, as this section describes the generation of a mouse model that will enable cell type specific deletion of TGF-β1, I will discuss the details of its role especially in B cells, T cells, monocytes, macrophages and dendritic cells.

A1 TGF-β1: Structure and signaling

TGF-β1, which was first isolated from human platelets (Assoian et al., 1983), is a disulfide-linked homodimer of two 112 amino acid chains. Each chain is synthesized as the C-terminal domain of a 390 amino acid precursor. The inactive precursor is cleaved from the amino-terminal glycopeptide at a tetrabasic cleavage site (Derynck et al., 1985). Although TGF-β1 is cleaved from the propeptide before the cell secretes the precursor, it remains attached to it by noncovalent bonds. It is secreted as a latent complex, consisting of a non-covalently associated 75-kDa glycoprotein, known as the latency associated protein (LAP), and a covalently bound 135-kDa binding protein (Diebold et al., 1995). There are four TGF-β1 latent binding proteins. These are encoded by different genes and are expressed in a tissue-specific fashion (Sinha et al., 1998). The attachment of TGF-β1 to the binding protein by disulfide bonds prevents it from binding to its receptors. Activation of this latent complex represents an important level of regulation of TGF-β1activity (Diebold et al., 1995). TGF-β1 is released from the complex by the matrix glycoprotein thrombospondin-1. This is accomplished by changing the conformation of the latent TGF-β1 binding protein (Crawford et al., 1998). TGF-β1 can also be activated by plasmin-mediated cleavage of the complex or in platelets from intracellular granules that are released on platelet activation (Borrelli et al., 2001). However not all mechanisms of TGF-β1 activation are known. Recently it has been demonstrated that the integrin $\alpha_v\beta_6$ binds to and activates latent extracellular complexes of TGF-β1 (Munger et al., 1999). This integrin-mediated activation is spatially restricted and requires direct presentation of activated TGF-β1 to receptors on adjacent cells. Although TGF-β1 LAP is a ligand for alphav beta6, the process of activation requires specific regions of the beta6-subunit cytoplasmic domain and an intact actin cytoskeleton, indicative of additional cellular mechanisms to regulate this process (Munger et al., 1999; Sheppard, 2001).

TGF-β1 regulates cellular processes by binding to three high-affinity TGF-β receptors known as types I, II, and III (Massague et al., 2000). The type III receptors are not involved in signaling themselves (Schlessinger et al., 1995), but facilitate it by binding TGF-β1 and then transferring it to its signaling receptors, the type I and II

Figure 1. Genomic organization of the TGF-β1 gene and biosynthesis of the mature peptide. (A) The coding region of the murine TGF-β1 gene is divided into 7 exons and 6 introns, is under the control of 2 promotors and is approximately 28 kb in size. **(B)** TGF-β1 is a disulfide-linked homodimer of two 112 amino acid chains. Each chain of the TGF-β1 homodimer is synthesized as the C-terminal domain of a 390 amino acid precursor. The inactive precursor is cleaved from the amino-terminal glycopeptide at a tetrabasic cleavage site. TGF-β1 is cleaved from the propeptide and it remains attached to it by noncovalent bonds. It is secreted as a latent complex, consisting of a non-covalently associated 75-kDa glycoprotein, known as the latency associated protein (LAP), and a covalently bound 135-kDa binding protein.

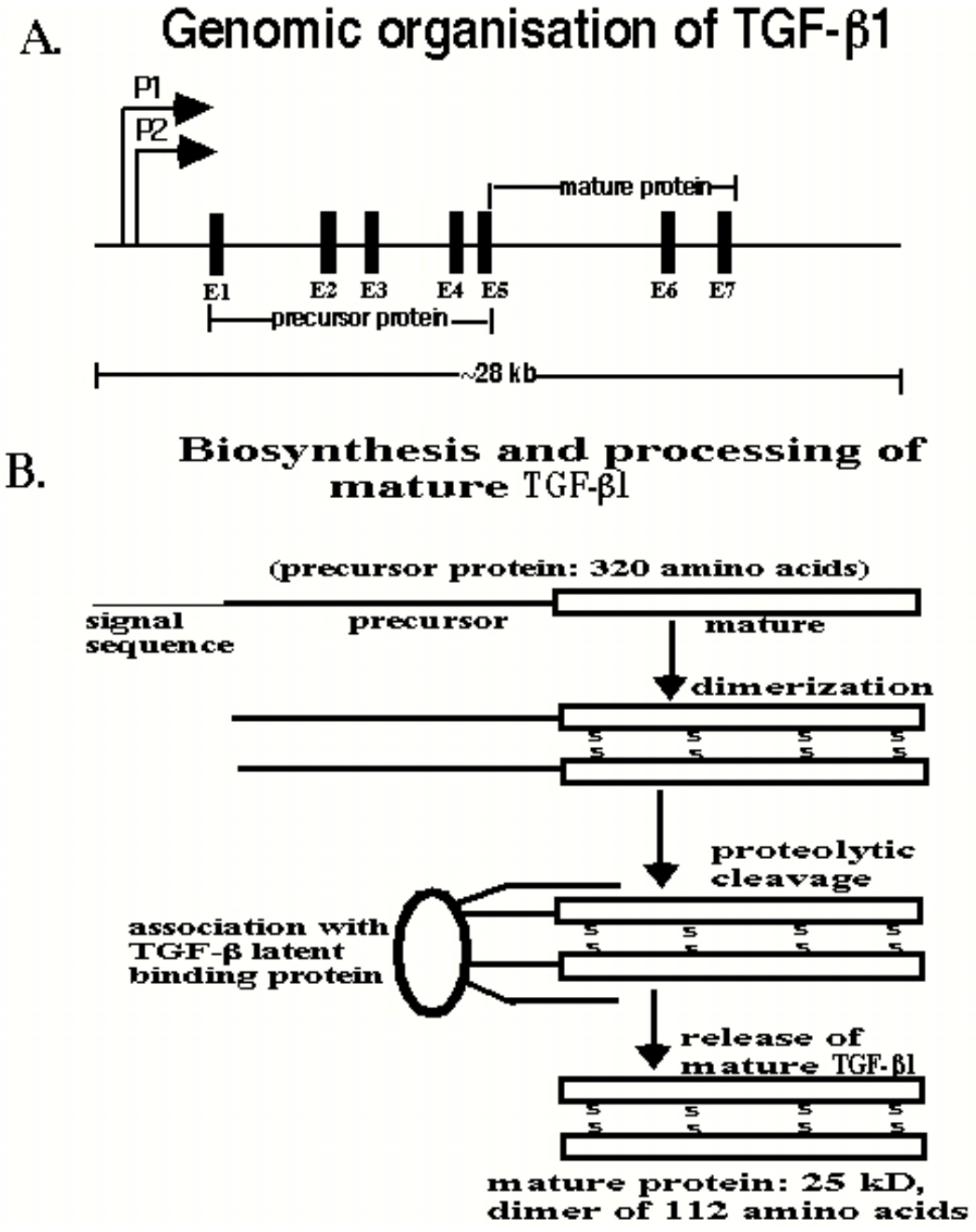
A.
Genomic organisation of TGF-β1
P1
P2
mature protein
E1
E2
E3
E4
E5
E6
E7
precursor protein
~28 kb
B.
Biosynthesis and processing of mature TGF-β1
(precursor protein: 320 amino acids)
signal sequence
precursor
mature
dimerization
proteolytic cleavage
association with TGF-β latent binding protein
release of mature TGF-β1
mature protein: 25 kD, dimer of 112 amino acids

Figure 1.

receptors (Lopez-Casillas et al., 1991; Lopez-Casillas et al., 1994; Wrana et al., 1994). Two other TGF-β receptors, endoglin and activin receptor-like kinase 1 (ALK-1) are specifically expressed on endothelial cells. ALK-1 is grouped with the type I TGF-β receptor family while endoglin contains a transmembrane region and a cytoplasmic tail homologous to the type III receptor (Blobe et al., 2000; Lux et al., 1999; McAllister et al., 1994). The type I and II receptors contain serine-threonine protein kinases in their intracellular domains that initiate intracellular signaling by phosphorylating transcription factors known as Smads (derived from the Sma and MAD gene homologues in *Caenorhabditis elegans* and *Drosophila melanogaster*). Smad genes are the intracellular mediators of TGF-β1 signalling (Weinstein et al., 2000). Ten Smad proteins have been described (Miyazono et al., 2000; Savage-Dunn, 2001). Smad2 and Smad3 are phosphorylated by activated type I TGF-β I receptors while Smad6 and Smad7 block the phosphorylation of Smad2 or Smad3, thus inhibiting TGF-β1 signaling (Liu et al., 2000; Miyazono et al., 2000). Smad4 is a common partner for all of the receptor-activated Smads. TGF-β1 also acts through the mitogen-activated and stress-activated protein kinase pathways (Piek et al., 1999). On activation by TGF-β1, type II receptors recruit, bind, and transphosphorylate type receptors, resulting in the activation of their protein kinases. The activated type I receptors phosphorylate Smad2 or Smad3, which then binds to Smad4. The resulting Smad complex then moves into the nucleus, where it interacts in a cell-specific manner with various transcription factors to regulate the transcription of TGF-β1 target genes (Massague, 1998; Miyazono et al., 2000; Nakao et al., 1997; Wrana et al., 1994).

A2 Multifunctional, context-dependent activities of TGF-β:

TGF-βs are produced by and act on a wide variety of cell lineages and play a role in vital processes like embryonic development, cell proliferation and differentiation, extra cellular matrix formation, hematopoiesis and immunoregulation (Massague et al., 1992; Roberts and Sporn, 1990; Wahl, 1994). TGF-βs influence designation of cell fates during development and inhibition of cell cycle progression (Zimmerman and Padgett, 2000). TGF-β1 messenger RNA (mRNA) is expressed in endothelial, hematopoietic, and connective tissue cells; TGF-β2 mRNA in epithelial and neuronal cells; and TGF-β3 mRNA primarily in mesenchymal cells. During development, TGF-β1 and TGF-β3 are expressed early in regions undergoing morphogenesis, and TGF-β2 is expressed subsequently in mature and differentiating epithelium (Schmid et al., 1991; Taipale and Keski-Oja, 1997). Starting from an eight-cell embryo to various stages of the postimplantation embryo, TGF-β1 is expressed in numerous cell types and modulates a variety of developmental processes (Akhurst et al., 1990). It is posited that TGF-β1 is involved in regulating hematopoietic stem cells (Heine et al., 1987), angiogenesis (Pepper, 1997) vasculogenesis (Dickson et al., 1995; Oshima et al., 1996) and the development of

enchondral bones (Millan et al., 1991). Developing tissues that have epithelial–mesenchymal interactions like atrio-ventricular cushion formation in the developing heart, show high levels of TGF-β1 in epithelial cells (Akhurst et al., 1990; Fitzpatrick et al., 1990; Lehnert and Akhurst, 1988; Pelton et al., 1991; Williams et al., 1991).

TGF-β1 influences the cell cycle by inhibiting proliferation and stimulating apoptosis in various cell types. It alters the level of RNA processing factors and arrests cells in G1 phase of the cell cycle. This is attained by stimulating production of the cyclin-dependent protein kinase inhibitor p15 and by inhibiting the function or production of essential cell-cycle regulators, especially the cyclin-dependent protein kinases 2 and 4 and cyclins A and E (Ravitz and Wenner, 1997; Seoane et al., 2001). Also, TGF-β1 plays an important role in mitochondrial metabolism, and the absence of TGF-β1 results in the disruption of mitochondrial membrane potential (Chen et al., 2001). A reduced mitochondrial potential may have an antagonistic effect on cellular metabolism, making cells more susceptible to apoptosis (Chen et al., 2001). TGF-β1 is one of the most robust regulators of extracellular matrix production (Massague, 1990). It achieves this by stimulating fibroblasts and other cells to produce collagen, fibronectin, and integrins and/or by decreasing the production of enzymes that degrade the extracellular matrix, including collagenase, heparinase, and stromelysin. Moreover, TGF-β1 augments the production of proteins that inhibit enzymes that degrade the extracellular matrix, including plasminogen-activator inhibitor type 1 and tissue inhibitor of metalloprotease (Maehra et al., 1999). It increases expression of diverse integrin receptors on monocytes including LFA-1, which binds ICAM-1 expressed on the surface of endothelial cells; VLA-3 (3β1), which binds collagen, fibronectin, and laminin; and VLA-5 (5β1), thereby increasing both cell-cell and cell-matrix interactions (Bauvois et al., 1992; Wahl et al., 1993). TGF-β1 inhibits the proliferation and promotes the differentiation and activation of leukocytes (Letterio and Roberts, 1998). Furthermore, it provides chemotactic stimuli for leukocyte migration and regulates their localization with the help of adhesion molecules.

A3 TGF-β1 and the immune system:

Diverse cytokine families are known to orchestrate the development and function of a variety of immunocompetent cells with unique and potent immunoregulatory properties (Paul and Seder, 1994). However, the preferential production of TGF-β1 by every leukocyte lineage, including lymphocytes, macrophages, and dendritic cells is indicative of it influencing the differentiation, proliferation, and state of activation of immune cells. TGF-β1 controls immune responses in a complex and often context-dependent manner. The primary level of control is not in the regulation of expression of TGF-β1 mRNAs, but in the regulation of both the secretion and activation of latent forms of TGF-β1. This is getting more evident via evaluation of transgenic and knockout mice with altered expression of TGF-β1 or their receptors (Amendt et al., 1998; Cazac and Roes, 2000; Gorelik and

Flavell, 2000; Kulkarni et al., 1993; Letterio and Bottinger, 1998; Shull et al., 1992). The phenotype of the TGF-β1 deficient mice has substantiated the importance of this isoform in the maintenance of immunological homeostasis. TGF-β1-deficient mice die from cardiac, pulmonary, and gastric inflammation, suggesting that TGF-β1 has a vital role in suppressing the activation and proliferation of inflammatory cells (Kulkarni et al., 1993; Shull et al., 1992). Although the TGF-β1 null mutation in the homozygous state causes some intrauterine lethality, more than one-third of the fetuses develop to term and appear clinically normal at birth. Evidence for maternal rescue of the targeted gene disruption in the fetus has been demonstrated (Letterio et al., 1994). Maternal sources of TGF-β1 contribute to the normal appearance and perinatal survival of TGF-$\beta1^{-/-}$ newborn mice. After two weeks the mice develop an acute wasting syndrome followed by death. No gross developmental abnormalities are detected however, prominent lesions due to multifocal, mixed inflammatory cell infiltration, similar to an autoimmune response is observed, that leads to premature death. The importance of macrophages, T and B lymphocytes in autoimmune disease caused by the lack of TGF-β1 has been demonstrated using TGF-$\beta1^{-/-}$ and SCID mice (Diebold et al., 1995).

Role of TGF-β1 B-cells:

TGF-β1 acts on every stage of B cell development. It is involved in lymphopoiesis and is essential for the development of plasma cells secreting secondary isotypes (Letterio and Roberts, 1998). TGF-β1 can induce apoptosis in immature B cells (Holder et al., 1992; Lomo et al., 1995) as well as in the fully differentiated plasma cells (Letterio and Roberts, 1998). It may be involved in germinal center development and regulation of B-cell proliferation at sites of high antigen load such as the gastrointestinal tract. The surface of the follicular dendritic cell (FDC) is the site of primary selection events in B cells that have undergone activation, clonal expansion, and somatic hypermutation of Ig v- region genes. Antigen trapped on the surface of FDC provides a survival signal for B cells with high-affinity surface membrane immunoglobulin (smIg), and TGF-β1 is the only factor known to interrupt this signal (Holder et al., 1992). Also, TGF-β1 is capable of inhibiting antigen-induced rescue of germinal center (GC) B cells and it works as a critical regulatory feedback loop to confine expansion of an activated population (Imai and Yamakawa, 1997).

In addition, B cell differentiation and immunoglobulin production are also regulated by TGF-β1. Synthesis and the secretion of IgG1, IgG2a, IgG3, and IgE are affected by TGF-β1 and low levels of autocrine TGF-β1 serve to enhance production and secretion of immunoglobulins under certain conditions (Snapper et al., 1993). Inhibition of IgM, IgD, IgA, CD23 (FcRII) and the transferrin receptor, and the induction of MHC class II expression on both pre-B and mature B cells is also influenced by TGF-β1 (Stavnezer, 1996). One of the best-characterized effects of TGF-β1 is its ability to stimulate isotype switching to IgA in both mouse and man. The ability of TGF-β1 to direct switch recombination in immunoglobulin isotypes IgA and IgG2b in mouse and IgA in human appears to be due to the induction of

transcription from the unrearranged I-C and I2b-C2b gene segments (Stavnezer, 1996). The influence of endogenous TGF-β1 is perhaps best elucidated by analyzing different classes of IgG production in mouse strains producing different levels of TGF-β1 (Lebman and Edmiston, 1999). In addition, active TGF-β1/IgG complexes secreted by B cells and plasma cells that interact with cellular receptors may modulate B cell responses, to mediate immunosuppressive effects on both T cells and neutrophils in a broad spectrum of diseases (Letterio and Roberts, 1997). Autocrine TGF-β1 is required for the secretion of IgG by B cells in response to LPS *in vitro*. Activation through antigen receptor by binding a monoclonal anti-IgM antibody to cell surface IgM induces expression of TGF-β1 mRNA in normal human peripheral blood B cells and stimulates murine B-lymphoma cell lines to secrete large amounts of active TGF-β1 (Warner et al., 1992). Recently the disparate roles of TGF-β1 in homeostasis and antigen responsiveness of B cell subpopulations have been confirmed *in vivo* via the generation of mice lacking the TGF-β1 receptor (TGF-βR) type II selectively in B cells (Cazac and Roes, 2000). Along with having an almost complete serum IgA deficiency, these mice have a reduced life span of conventional B cells, expansion of peritoneal B-1 cells, B cell hyperplasia in Peyer's patches and elevated serum immunoglobulin.

Role of TGF-β1 in T-cells:

TGF-β1 plays a pivotal role in multiple stages of T cell development, selection, activation and clearance (Wahl et al., 2000). Regulation of T-cell function by TGF-β1 is a function of their state of differentiation (Ludviksson et al., 2000; Schiott et al., 1996). The earliest studies of the effect of TGF-β1 on human lymphocyte function revealed that activated T cells themselves synthesize and secrete TGF-β1 and that exogenous TGF-β1 typically inhibits IL-2-dependent proliferation (Kehrl et al., 1986). Several studies have accentuated these inhibitory effects of TGF-β, especially its ability to interfere with events that occur subsequent to IL-2 production and receptor binding (Ahuja et al., 1993) such as the production of various cytokines (Espevik et al., 1987; Espevik et al., 1990; Fargeas et al., 1992) and cytolytic functions (Mule et al., 1988). On the other hand, TGF-β can also enhance the growth of T cells, predominantly of the naive phenotype (Cerwenka et al., 1994), induce T cell expression of specific cytokines and enhance their capacity to respond to subsequent stimulation, and even promote effector expansion through inhibition of T cell apoptosis (Cerwenka et al., 1996; Rich et al., 1996; Zhang et al., 1995).

Roles for both autocrine and paracrine TGF-β1 have been described during normal thymopoiesis (Letterio and Roberts, 1998; Suda and Zlotnik, 1991; Suda and Zlotnik, 1992). Data attained from fetal organ cultures further substantiates that TGF-β1 is required for murine thymocyte development (Plum et al., 1995). TGF-β1 synergizes with TNFα to advance the development of thymic precursor cells expressing CD8 (Suda and Zlotnik, 1992) and TGF-β1 induced expression of CD8 on $CD25^+$ triple negative ($CD4^-$,$CD8^-$,TCR^-) thymocytes represents a normal differentiation step (Suda and Zlotnik, 1991). The progression of $CD4^-CD8^-$

thymocytes to $CD4^{-}CD8^{lo}$, and to the subsequent $CD4^{+}CD8^{+}$ stages show that differentiation into the double positive state requires progression through at least one cell division which is effectively inhibited by thymic epithelial cells expressing TGF-β1 (Takahama et al., 1994). Moreover, TGF-β1 can enhance de novo CD8 expression also on the IL-2-dependent CTLL-2 cytotoxic T cell line which is contrary to the ability of TGF-β1 to inhibit IL-2-dependent increases in IL-2R, IL-2Rβ, and Granzyme B mRNA levels in these cells (Inge et al., 1992). That T cell homeostasis requires TGF-β1 signaling in T cells has been further corroborated by generating a mouse model in which signaling by TGF-β1 is blocked specifically in T cells by expressing a dominant-negative TGF-β1 receptor type II under a T cell-specific promoter (Gorelik and Flavell, 2000).

In the development of specific Th responses, contradictory data regarding the influence of TGF-β1 on Th differentiation exist. Initial work done on $CD4^{+}$ cells exposed to TGF-β1 during their priming phase showed an increase in INFγ producing cells and a decrease in IL-4 producing cells, indicating a move towards a Th1 phenotype (Swain et al., 1991). Further studies appear to have demonstrated TGF-β1 confers a Th2 phenotype by virtue of its reciprocal relationship with both IFNγ and IL-12 (Schmitt et al., 1994; Strober et al., 1997). Interaction between TGF-β1 and IL-12 has also been observed in human primary allogeneic proliferative and cytotoxic T cell responses. Effects of TGF-β1 are mediated through mechanisms associated with the abrogation of IL-12 production, a response which is irreversible even with the addition of exogenous IL-12 or IFNγ (Pardoux et al., 1997; Pardoux et al., 1999). Recent work has also revealed that TGF-β1 is an effective negative regulator of the $CD4^{+}$primary immune response. However, only Th1 cells are susceptible to such regulation after the memory stage of T cell differentiation has been attained (Ludviksson et al., 2000). Thus, TGF-β1 can inhibit the production of and response to cytokines associated with each subset, and the production of TGF-β1 by antigen-specific T cells may mark a unique subset already referred to as Th3 (Bridoux et al., 1997; Chen et al., 1994).

Role of TGF-β1 in monocytes / macrophages:

Both circulating monocytes and tissue macrophages secrete TGF-β1, predominantly as the type1isoform (Assoian et al., 1987; Grotendorst, 1997). In modulating macrophage/monocyte function, TGF-β1 plays a dual role as a 'macrophage-deactivating' agent and a monocyte-activator, depending on the other cytokines present and the state of differentiation or tissue origin of the cells (Ashcroft, 1999; Fan et al., 1992; Sato et al., 1996). An entire gamut of monocyte/macrophage activities like chemotaxis, host defense, phagocytic activity, activation and deactivation are regulated by TGF-β1 (Bogdan and Nathan, 1993; Wahl et al., 1992). During the initial stages of inflammation, TGF-β1 acts locally as a proinflammatory agent by recruiting and activating resting monocytes. As these cells differentiate specific immunosuppressive actions of TGF-β1 preponderate, leading to

resolution of the inflammatory response. (Ashcroft, 1999). Resting monocytes express high levels of TGF-β receptors type I and II, whereas receptor levels decline as cells mature and become activated. The difference in the expression pattern of receptors for TGF-β1 on these two populations of the mononuclear phagocyte system is considered the main cause for the different effects that TGF-β1 has on these two cell types.

Picomolar and even femtomolar concentrations of TGF-β1 can induce monocyte chemotaxis, growth factor production and angiogenetic activity (Munger et al., 1999; Wiseman et al., 1988). In contrast, TGF-β1's action on tissue macrophages is generally suppressive and contributes to the resolution of an inflammatory response. Suppressive effects of TGF-β1 on activated macrophages include its ability to modulate the profile of activating cytokines as by limiting production of IFN (Fargeas et al., 1992) or increasing expression of the IL-1 receptor antagonist (Turner et al., 1991). The result of this is similar to effects of IL-4, IL-10, and macrophage deactivating factor MDF; (Bogdan and Nathan, 1993). Furthermore, TGF-β1 is capable of reducing the production of cytotoxic reactive oxygen and nitrogen intermediates by activated macrophages. This has a crucial deactivating effect on macrophages (Bogdan and Nathan, 1993; Vodovotz et al., 1996). TGF-β1's role in the immunophagocytotic activity of monocytes/macrophages involves its ability to stimulate circulating monocytes to upregulate expression of cell surface FcRIII, which recognizes bound IgG (Wahl et al., 1992; Welch et al., 1990). Macrophage recognition of phosphatidyl serine is also TGF-β1–dependent. Phosphatidyl serine normally localized to the inner membrane, is transposed to the outer membrane leaflet of apoptotic cells, recognition of which can be enhanced by TGF-β1 (Rose et al., 1995).

Role of TGF-β1 in dendritic cells:

Dendritic cells (DC) are a distinct population of leukocytes that function as the primary antigen-presenting cells in the activation of T lymphocyte responses (Steinman, 1991). TGF-β1 influences the *in vitro* differentiation of these antigen presenting dendritic cells including highly specialized populations like Langerhans cells (LC) of the epidermis and the follicular dendritic cell (FDC) of lymph nodes (Borkowski et al., 1996; Riedl et al., 1997; Strobl et al., 1996). DC produce TGF-β1 ___ in the TGF-β1 knockout mouse the complete absence of the epidermal LC further stresses the importance of TGF-β1 in DC development (Borkowski et al., 1996). It is unlikely that inflammatory cytokines are effecting migration of LC from the TGF-β1 -null epidermis as even when the TGF-ß1 null mice are bred onto different immunodeficient backgrounds (SCID, athymic nude, RAG2-null) this absence of epidermal LC persists (Borkowski et al., 1996). Nonetheless, repopulation of recipient skin with donor-derived LC is observed in lethally irradiated recipients injected with TGF-$\beta1^{-/-}$marrow. This suggests that paracrine sources of TGF-β1 are adequate to support normal LC development and migration into the epidermis (Borkowski et al., 1997). Despite normal numbers of $CD11c^{+}$ DC, the

TGF-β1 null mice lack gp40+ lymph node DC, suggesting that the loss of TGF-β1 may affect other specialized DC populations participating in extrafollicular immune responses (Borkowski et al., 1997).

A4 ROLE OF TGF-β1 IN DISEASE

Various experimental models of disease have demonstrated that TGF-β1 regulates the cellular functions and interactions of the immune system in both humoral and cell-mediated immunity and disease pathogenesis. Activation of the latent forms of TGF-β1, which are blocked from receptor binding, is an important post-transcriptional control point in both physiological and pathological actions of TGF-β1 (Barcellos-Hoff and Dix, 1996; Flaumenhaft et al., 1993). Increased production and activation of latent TGF-β1 have been linked to immune defects associated with malignancy and autoimmune disorders, susceptibility to opportunistic infection, and to the fibrotic complications associated with chronic inflammatory conditions.

Many lines of evidence implicate TGF-β1 in the pathogenesis of autoimmune diseases (Kuruvilla et al., 1991; Miller et al., 1993; Miller et al., 1992; Racke et al., 1992; Weiner, 1997). Disruption of the tightly controlled cytokine network, as by the loss of TGF-β1, can potentially upset the normal regulation of self-reactivity and contribute to the development of systemic autoimmunity by disturbing the balance between Th1 and Th2 cells (Letterio and Roberts, 1997). When engineered to produce latent TGF-β1, antigen specific T cells down regulate Th1 cell-mediated autoimmune and Th2 cell-mediated allergic inflammatory processes (Thorbecke et al., 2000). Furthermore, mice null for the TGF-β1 gene confirm that it is a significant regulator of immune cell differentiation and function, and that loss of this gene is sufficient for development of an autoimmune-like phenotype. These mice show enhanced expression of MHC class I and II antigens, circulating SLE-like IgG antibodies to nuclear antigens, pathogenic glomerular IgG deposits. Also similar to human autoimmune syndromes such as Sjögren's disease, TGF-$\beta1^{-/-}$ mice have a progressive infiltration of lymphocytes into multiple organs. The inflammatory infiltrates compromise organ function and lead to the death of these mice between 2 and 3 weeks. Although tissue infiltration can be blocked by systemic administration of fibronectin peptides, which block adhesion of TGF-β1 null leukocytes to endothelium, this treatment does not block the primary autoimmune response (Hines et al., 1994). An aberrant expression of the MHC class I and II antigens clearly represents an important mechanism in development of this autoimmune phenotype because backcrosses of the TGF-β1 null mice onto either an MHC class I or class II deficient background develop neither circulating autoantibodies nor immune complex deposits (Letterio et al., 1996). TGF-β1 is known to suppress expression of MHC class II antigen, consistent with its overexpression in TGF-β1 deficiency. MHC class II molecules play a role in the selection and activation of $CD4^+$ T cells, which regulate both humoral and cell-mediated immune responses to antigens. The suppression of the autoimmune phenotype in TGF-$\beta1^{-/-}$/MHC-$II^{-/-}$ mice is perhaps

due to the absence of this $CD4^+$ T cell subset in the MHC-II-null background (Letterio et al., 1996). However, although the loss of endogenous TGF-β1 gene expression in the TGF-β1 null mouse results in the production of autoantibodies (predominantly IgG), it does not lead to a diffused polyclonal hypergammaglobulinemia or to a predominance of an Ig clonotype (Dang et al., 1995).

In case of malignancies, notwithstanding the existence of tumor-specific antigens and of tumor-specific immune cells, the majority of tumors manage to avoid immune-mediated eradication. One of the mechanisms for tumor evasion from immune response is presumed to be mediated by TGF-β1, an immunosuppressive cytokine found at the site of most tumors. Several murine and human B and T lymphoid malignancies have been identified that have become insensitive to the growth inhibitory effects of TGF-β1 and that express substantial amounts of an active form of the molecule (Berg and Lynch, 1991; DeCoteau et al., 1997; Kadin et al., 1994; Newcom et al., 1992). Furthermore, activation through antigen receptor stimulates murine B-lymphoma cell lines to secrete large amounts of active TGF-β1 (Warner et al., 1992). This autocrine inhibitory loop remains intact in some lymphoid malignancies and has been implicated in their reduced proliferative responses and slow rate of progression.

TGF-β1 is growth inhibitory for normal epithelial cells and melanocytes but can stimulate mesenchymal cells. Resistance to its inhibitory effects is characteristic of human melanoma. TGF-β1 produced by the melanoma cells activates the fibroblasts to produce matrix within and around the tumor mass. The tumors themselves show increased levels of collagen, fibronectin, tenascin, and α2 integrin. This is a positive feedback stimulation of tumor growth as remodeling of the surrounding stroma furnishes supporting scaffolding for tumor growth (Berking et al., 2001). Targeted deletion of the TGF-β1 gene results in rapid progression to squamous cell carcinoma (Glick et al., 1994). The role of TGF-β1 in controlling the balance of the immune response in tumor immunity has recently been further confirmed (Gorelik and Flavell, 2001). T-cell-specific blockade of TGF-β signaling leads to the enhancement of anti-tumor immunity the generation of an immune response capable of eradicating tumors in mice challenged with live tumor cells (Gorelik and Flavell, 2001). TGF-β1 contributes both to the formation of inflammatory foci by direct effects on chemotaxis and on adhesion. It also influences the resolution of acute inflammatory reactions and restoration of homeostasis by increasing the phagocytic activity of macrophages toward inflammatory cells and damaged parenchymal cells. Expression of active TGF-β1 by macrophages and other leukocytes plays a central role not only in inflammation, but also in accompanying fibrosis by paracrine stimulation of resident mesenchymal cells to produce excessive extracellular matrix. Thus, in bleomycin-induced pulmonary fibrosis in mice, nearly all of the active TGF-β1 implicated in pathologic matrix deposition is secreted by alveolar macrophages and is temporally concordant with the deposition of collagen in the lung (Khalil et al., 1996).

TGF-β1 is an important immunoregulator in parasite escape mechanism in all forms of human and murine leishmaniasis, Protozoan parasites such as Trypanosoma cruzi, and Leishmania (Barral-Netto et al., 1992). These parasites have evolved mechanisms to induce the infected host cell to secrete active TGF-β1, which then suppresses the killing activity of macrophages and enhances intracellular proliferation of the pathogen. TGF-β1 is now known to underlie control of the antimicrobial and tumoricidal pathways of macrophages and of immune responses in general (Bogdan and Nathan, 1993). Thus in general, TGF-β1 has a negative influence on host responses and a beneficial effect on the survival and growth of intracellular pathogens (Reed, 1999). This phenomenon has also been further substantiated by the generation of Smad3-deficient mice. These mice develop chronic mucosal infections due to impairment of T-cell activation and mucosal immunity, suggesting a key role for TGF-β1 in these processes (Yang et al., 1999).

Hence, *in vitro* and *in vivo* data demonstrate that TGF-β1 does indeed have a complex and context-dependent mode of controlling the immune system. In each case, TGF-β1 clearly provides regulatory signals that are shaped by ongoing cellular interactions, the cytokine context, and by the pertinent state of differentiation of the responsive cell. In lymphocytes, the primary level of control is not in the regulation of expression of TGF-β1 mRNAs, but in the regulation of both the secretion and activation of latent forms of TGF-β1. The phenotype of the TGF-β1-deficient mice has substantiated the importance of this isoform in the maintenance of immunological homeostasis. In these mice, prominent lesions due to multifocal, mixed inflammatory cell infiltration into many organs is observed, suggesting an uncontrolled inflammatory response that leads to premature death. The infiltrating cells in this inflammatory response have been shown to be primarily T cells and macrophages.

B OBJECTIVES OF THIS STUDY

The principal objective of this part of the thesis is to generate a conditional knockout mouse for the TGF-β1 gene, which would circumvent the lethal phenotype of the TGF-β1 total knockout mice (Kulkarni et al., 1993; Shull et al., 1992). With the help of the Cre/loxP system (Sauer and Henderson, 1988; Gu et al., 1994;) part of the TGF-β1 gene has been flanked by loxP sites (floxed) so as to get a TGF-$\beta1^{flox/flox}$ mouse. The TGF-$\beta1^{flox/flox}$ mice described here are viable and capable of deleting the TGF-β1 gene *in vivo* when bred to deleter-cre transgenics. The TGF-$\beta1^{\Delta/\Delta}$ mice generated by *in vivo* Cre-deletion have a phenotype comparable to the TGF-β1 complete knockouts (Kulkarni et al., 1993; Shull et al., 1992).

Using this conditional knockout, experiments are also underway to analyse autocrine TGF-β1 exclusively in the T cell compartment or in macrophages.
Therefore, TGF-$\beta1^{flox/flox}$ / $CD4Cre^{+}$ transgenics or TGF-$\beta1^{flox/flox}$ / $LysMCre^{+}$ transgenics have also been generated allowing T-cell specific (CD4Cre) and macrophage specific (LysMCre) ablation of the gene encoding TGF-β1 .

C EXPERIMENTAL PROCEDURES

C1 Molecular Biology Methods

C1.1 Cloning of the TGF-β1 targeting vector

The TGF-β1 genomic clone was received from Dr. Rajendra Raghow (University of Tennessee, Memphis) as a 28 kb fragment inserted into the Not1 sites of an 8.2 kb cosmid vector pWE15 (Sratagene). The coding region of the murine TGF-β1 gene is divided into 7 exons and 6 introns, is under the control of 2 promotors and is approximately 25 kb in size (Guron et al., 1995) (Figure 1A). The sequence for the promotor, 1kb of the 5' untranslated region, the 6 exons and the exon-intron boundaries as well as approximate intron sizes are published (Guron et al., 1995); (NCBI accession number L42456). I mapped the gene further to determine precise intron lengths as well as specific sites for cloning. The results of this mapping helped in designing the targeting strategy. The three fragments containing intron 5, exon 6, intron 6, exon 7 and 6 kb 3' of exon 7 were first subcloned into pSL1190 (Invitrogen) plasmid. The exons in the subclones were sequenced to confirm that the starting fragments were correct. As a result of this as well as subsequent subclonings into the multiple cloning site fragment of pSL1190, I was able to selectively incorporate specific restriction sites that were used for:

1. Final cloning of the three subclones into pEasy Flox which has a loxP flanked *neo*^{r} cassette, a third loxPsite and a HSV-1 thymidine kinase gene (tk) which would help select for homologous recombinants
2. Linearizing the vector for transfecting embryonic stem (ES) cells using a Sfi1 site
3. Screening targeted ES cell clones for homologous recombination and integration of the third loxP site using Sac1 and Xba1 sites
4. Screening partially deleted positive clones after *in vitro* cre mediated deletion of the loxP flanked *neo*^{r} cassette using Xba1 site.

Molecular cloning was performed according to standard protocols (Sambrook et al., 1989). The bacterial strain used in all cloning experiments was HB10 that were routinely transformed with plasmid DNA by heat shock. Plasmid DNA was prepared using either the Plasmid Miniprep or Maxiprep kits from Qiagen, Germany. Ligation of DNA fragments was achieved using the Takara Ligation Kit (Takara, Japan).

C1.2 DNA Sequencing

Exons and exon/intron boundaries in either the cosmid carrying the genomic clone or in the subclones were sequenced by Genterprise, University of Mainz. This was done using the dideoxy-chain-termination reaction protocols described earlier (Sanger et al., 1977) and using the non-radioactive flourescence-labeled dideoxynucleotides. (Taq-Dye-Deoxy-Terminator Cycle Sequencing Kit, Applied Biosystems). The samples were resolved and analyzed using an automated sequencer (ABI 373A, Applied Biosystems).

C1.3 Synthesis of oligonucleotides

Metabion Inc., Martinsried Germany, synthesized oligonucleotides used for DNA sequencing as well as for PCR. Standard desalted oligos were used for all procedures.

C1.4 Genomic DNA preparation from ES cells

DNA from ES cells was collected using the method described by Hogan et.al (Hogan et al., 1995). In brief ES cells were grown to confluence in 96 well plates that were coated with 0.1% gelatin. The plates were then washed three times with PBS. 100µl of lysis buffer (10mM Tris pH7.7, 10mM EDTA, 10mM NaCl, 0.5% w/v sarcosyl) was then added to each well and the plates were incubated at 56^{o}C overnight in a humidified container so as to prevent the plates from drying out. Following this incubation the plates were kept at room temperature for 30 min and 100µl of isopropanol was added to them and they were incubated at room temperature for another hour until strands of precipitated DNA became visible. The supernatant was then carefully drained off from the wells and the plates were washed twice with ice cold 70% ethanol. The wells were then air-dried after which the 35µl of the master mix having the respective restriction enzyme was added to each well for overnight digestion of the genomic DNA.

C1.5 Southern blot analysis of Genomic DNA

Southern blot analysis (Sambrook et al., 1989) was used for:

1. Identification of targeted ES cell clones
2. Screening mice to confirm germline transmission
3. Screening of mice carrying one copy of the floxed allele as well as the Deleter Cre, CD4-cre or the LysMcre transgene
4. Verify cre-mediated deletion of the TGF-β1$^{flox/flox}$ allele on breeding them to cre transgenic mice.

Between 5 and 10 µg of genomic DNA were digested overnight with 100 U of restriction enzyme. The digested DNA was resolved on a 0.8% agarose gel containing 0.5µg per ml ethidium bromide. For resolving closely running large fragments from Nco1 and Xba1 digests the gels were run overnight at a low voltage. To confirm digestion of the genomic DNA, each gel was exposed to UV-light and photographed. The gel was depurinated in 0.25M HCl solution for 20 to 30 min. The gel was then rinsed in distilled water and denatured by two 30 min incubations with gentle shaking in a solution of 1.5M NaCl and 0.5M NaOH. Finally the gel was neutralized by two incubations for 30 min. each in a solution of 1.5M NaCl and 0.5M Tris–HCl; pH 7.0. The gel was then rinsed in 10X SSC and blotted overnight using 20X SSC, so as to transfer the DNA onto a nitrocellulose membrane (Amersham). The nitrocellulose membrane was then dried and baked in an 80C oven for an hour.

The DNA was also crosslinked by exposing the membrane to UV light. The membranes were then pre-hybridised at 65^{o}C for one hour in hybridization solution. DNA probes (20-50ng) were radioactively labeled using the Random-Primer Labeling kit (Stratagene). The external probe B used for most of the Southern analyses was selected after screening several probes. Besides having the advantage of being able to screen for homologous recombination as well as integration of the third loxP site, this probe gave the lowest background hybridization while still giving clear specific endogenous bands. This probe is a 0.9 kb BamHI-HindIII fragment that is 3' of the short arm of homology and lies outside of the targeting vector. The labeled probes were purified over a Spin500 column (Amersham) and denatured by boiling for 5 min. The denatured probe was then added to the tubes incubating the membranes in prehybridisation buffer. Hybridization was carried out at 65^{o}C overnight. After this the membranes were washed (0.1X SSC and 0.1%SDS). The washed membranes were then sealed in plastic pouches and exposed to a film for 3 to 5 days or exposed to a Phosphoimager (Hewlet Packard) for 2 days.

C1.6 Genomic DNA preparation from mouse tails

Genomic DNA from mouse-tails was collected using previously published methods (Laird et al., 1991). In brief 0.5-1 cm of tail was digested overnight in lysis buffer. The digest was spun down at 15,000Xg for 10 min to get rid of any cell debris or hair. The supernatant was made to go through two rounds of phenol extraction and one round of chloroform extraction. The DNA in supernatant was then precipitated using NaAcetate and Isopropanol. A DNA pellet was collected by spinning down the supernatant at 15,000XG for 10 min. The pellet was washed three times in 70% ethanol and air dried. The dry pellet was resuspended in 100 µl of double distilled water.

C1.7 Polymerase chain reaction for screening transgenics

The FailSafe PCR kit (Biozym, Germany) was used to screen the deleter cre, CD4cre and LysMcre transgenic mice. This kit uses a premix of polymerase, dNTP's and buffers with varying concentrations of $MgCl_2$. All PCRs used for screening the below mentioned three transgenics used buffer D from the kit. The exact specifications for the buffer D are unknown as the company has a patent on it would not divulge the information.

C2 CELL CULTURE METHODS

C2.1 Embryonic Stem cell culture and generation of mutant mice

Once the targeting vector was cloned and sequenced, the 129/Sv x 129/Sv-CP derived ES cell line R1 (Nagy et al., 1993) was targeted using previously described protocols (Hogan et al., 1995). 25 μg of linearized targeting vector was mixed with $5x10^6$ R1 (passage 17) ES cells in a volume of 0.8ml of PBS. The ES cells were electroporated using a BioRad Gene Pulser at 240V, 500μF. The transfected cells were then plated out on irradiated neomycin resistant mouse embryonic primary fibroblasts at a density of $2.5x10^6$ cells per 10-cm dish and cultured in ES cell medium. 48 hours after electroporation G418 at 200 μg/ml and gancyclovir at 2μM was added to the ES cell medium for double selection of the targeted clones. Fresh selection media was added daily to the ES cells. Colonies of resistant ES cells developed between days 7-9 after starting selection. Undifferentiated, healthy looking, colonies were picked; trypsinised and transferred onto irradiated MEF coated 96 well plates for expansion and screening. For freezing down, 1 volume of ice cold 2X freezing medium (ES medium plus 10% DMSO) was added to confluent, trypsinised 96 well plates that had 1volume of trypsin in them. The wells were overlaid with sterile-filtered mineral oil (SIGMA), carefully wrapped in parafilm and gradually frozen down to -80^0C in styrofoam boxes.

To screen the cells for homologous recombination by Southern analysis, a replica plate was made of each plate having ES cell clones that was frozen. This replica plate was coated with 0.1%gelatin (SIGMA). These plates were grown to confluence and used to extract DNA to screen for targeted clones as described earlier. After identification by Southern Blot analysis, homologous recombinants, which also carried the third loxP site, were recovered from the frozen ES cell plates. Two of these positive clones were then expanded for another round of electroporation so as to undergo Cre-mediated deletion of the *neo*^{r} gene (Gu et al., 1994; Torres and Kuehn, 1997). The electroporation conditions were identical to the one described above except that 25 μg of pPGK-cre vector was used (cloned by Kurt Fellenberg, University of Cologne). Two days after transfection, ES cells were picked and grown in duplicate 48-well plates. 48 hours later, one of these plates was frozen down as described earlier and the other one was placed under G418 selection. The G418 sensitive clones were then thawed from the frozen stocks, expanded and screened by Southern analysis for candidates that had deleted only the neomycin resistance gene but retained the floxed exons 6 and 7 of the TGF-β1 gene.

C3 MICE

C57BL/6 female mice used as blastocyst donors were between 8 to 12 weeks old. Blastocysts were collected from either naturally mated or superovulated females. Mice used to study the progeny of TGF-β1 $^{flox/+}$ bred to TGF$^{flox/+}$ x deleter-cre double-transgenics were between 2 to 3 weeks old. This was due to the fact that the TGF-

β1 null mice resulting from this breeding did not survive any longer. Their littermates that were used as controls were also 2 to 3 weeks old.

C3.1 Generation of TGF-β1$^{flox/+}$, TGF-β1$^{flox/flox}$ and TGF-β1 $^{\Delta/\Delta}$ mice

Targeted ES cell clones were used to generate chimeras by injecting them into blastocysts obtained from C57BL/6 females (Hogan et al., 1995). Injected blastocysts were then implanted into the uterine horns of time-matched pseudo-pregnant females (C57BL/6 x C3H). Chimeras were identified by coat color and backcrossed to C57BL/6 mice to achieve germline transmission. Germline transmission was confirmed by Southern blot analysis. Heterozygous mice were then either intercrossed to get homozygous floxed TGF$^{flox/flox}$ mice or were crossed to the deleter-Cre to get TGF-β1 $^{\Delta/\Delta}$ mice. TGF$^{flox/+}$ mice were also crossed to CD4-Cre, LysM-cre transgenics.

Other transgenic mice used in experiments:

C3.2 LysMcre mice

Two homozygous breeding pairs (6 to 8 week old) of the LysMcre mice were received from Prof. Irmgard Foerster at the Technical University of Munich, Munich, Germany. These mice express Cre in myeloid cells due to the targeted insertion of the cre cDNA into their endogenous M lysozyme locus (Clausen et al., 1999). They were on a C57BL/6 background and were transferred from the SPF facility to the University of Mainz animal facility.

PCR for screening LysMcre mice

1μl of genomic DNA prepared from tail snips was used for each PCR reaction. The conditions for the PCR, which were provided by Prof. Irmgard Foerster, were 95^{O}C for 3 min followed by 35 cycles of 94^{O}C for 45 seconds, 63^{O}C for 45 seconds and 72^{O}C for 1 min. These conditions were used to get the following PCR products:
1. A 350 bp wild type band with the primer pair Mlys1 and Mlys2
2. A 700bp LysMcre transgene band with the primer pair Mlys1 and Cre 8.
3. A 1.7 kb WT/Cre band with the primer pair Mlys1 and Mlys2. Here the primers bind to the endogenous WT locus and the PCR product shows the WT LysM locus with Cre knocked-in

The sequence information on the primers and where they bind is given below.

Oligo	Sequence	Specificity
Cre 8	5'CCC AGA AAT GCC AGA TTA CG-3'	495 bp upstream of Cre cDNA ATG, anti-sense
Mlys 1	5'-CTT GGG CTG CCA GAA TTT CTC-3'	5'of Mlysozyme-ATG start
Mlys 2	5'-TTA CAG TCG GCC AGG CTGAC-3'	3' Mlysozyme gene exon 1

C3.3 CD4Cre mice

Two 12 weeks old CD4-cre male mice, on a mixed 129/Sv x C57BL/6 background (originally from Dr. Christopher Wilson, University of Washington, Seattle) were transferred from University of Cologne to University of Mainz, where they were kept in quarantine. These transgenic mice have Cre under the control of the CD4 promotor (without the silencer) and therefore should express Cre in $CD4^+$ and $CD8^+$ T cells (Lee et al., 2001; Sawada et al., 1994). These mice were mated to superovulated TGF-β1 $^{flox/+}$ females or C57BL/6 females, which were sacrificed at day 1.5 post coitum to collect fertilized embryos, which were transferred to pseudo-pregnant female mice in the Specific Pathogen Free facility at the University of Mainz.

PCR for screening CD4cre mice

To screen for these mice I designed six primers that were within the 90 bp region of the 3' end of intron 1 and the 5' end of exon 2 of the CD4 gene. All these primers were individually used in combination with a primer designed around 495 bp upstream of the region of cre cDNA (AG-Cre6). All six primer pairs gave the expected PCR product. Finally for all the screenings, AG-CD4L6 and AG-Cre6 primer pair were used which amplified a 600bp CD4cre band. The primer AG-CD4L6 binds to base pairs 140 to 157 of the published CD4 gene sequence (Genbank Accession number NM013488). 1μl of genomic DNA prepared from tail snips was used for each PCR reaction. The conditions for the PCR were 95^OC for 3 min followed by 35 cycles of 94^OC for 45 seconds, 58^OC for 45 seconds and 72^OC for 1 min.

The sequence information on the primers and where they bind is given below.

Oligo	Sequence	Specificity
AG-CD4L6	5'-CCC AAC CAA CAA GAG CTC -3'	3'intron1 to 5'exon2 of CD4 gene (nt140-157)
AG-Cre6	5'CCC AGA AAT GCC AGA TTA CG-3'	495 bp upstream of Cre cDNA ATG, anti-sense

C3.4 Deleter mice

An eight-week-old deleter-cre transgenic male mouse (Schwenk et al., 1995) on a C57BL/6 background was received from Dr. A. Bockamp (University of Mainz). The deleter-cre transgenic mice can ubiquitously delete loxP-flanked regions of DNA *in vivo*. The male deleter-cre was bred to a $TGF^{flox/+}$ female mouse. As the Cre gene in these transgenics is X-linked the female progeny were screened for the presence of Cre and the TGF-β1 floxed allele. Positive females were backcrossed to a TGF-$\beta 1^{flox/+}$ male mouse so as to generate $Cre^{+}/TGF^{\Delta/\Delta}$ mice.

PCR for screening deleter-Cre mice

Conditions for screening the deleter cre transgenics were $95^{O}C$ for 3 min followed by 35 cycles of $94^{O}C$ for 45 seconds, $58^{O}C$ for 45 seconds and $72^{O}C$ for 1 min. A 150 bp kb amplicon was attained with these conditions. 1μl of genomic DNA prepared from tail snips was used for each PCR reaction.
The sequence information on the primers and where they bind is given below. (Bacteriophage P1 Cre Genbank Accession number X03453)

Oligo	Sequence	Specificity
Cre-P1	5'-CCG GTT ATT CAA CTT GCA CC-3'	Cre cDNA, sense
Cre-P2	5'-CTG CAT TAC CGG TCG ATG CAA C-3'	Cre cDNA, anti-sense

D Histology

D1 Preparation of paraffin sections

For preparing tissue that would be used to make paraffin sections, organs collected from mice that were sacrificed or found dead were immediately rinsed in PBS and fixed in formalin (4% paraformaldehyde). After this the organs were sequentially treated at room temperature in 50%, 80%, 96% ethanol for three hours each. This was followed by two washes each of 100% ethanol and of Xylol for four hours. The organs were then incubated at $56^{o}C$ in a Xylol/paraffin (1:1) solution. They were then incubated in paraffin for 4 hours at $56^{o}C$; this step was repeated two times with fresh batches of paraffin. The organs were then embedded into cassettes at room temperature and the resulting solid molds used for sectioning. The 5-7 μM sections were carefully spread out onto slides and left at $40^{o}C$ for 6 hours. The sections were then stained with Hematoxylin / eosin (H/E) as described bellow.

D2 Staining

Hematoxylin / eosin (H/E) staining was performed according to standard protocols using hematoxylin Gill's solution#3 and eosinY (SIGMA). Five to seven micron paraffin sections on slides that were H/E stained were treated as follows. They had three 10-min incubations in Xylol followed by two 5-min washes in 100% ethanol. 5-min washes in 95%, 70%, 50%, 30% ethanol, and then a two-min wash in distilled water followed this. The slides were stained with Hematoxilin for 30-60 seconds and washed in normal, running tap water for 10 mins to allow differentiation. The slides were washed in 0.1% $NaHCO_3$ for 1-2 mins and stained with eosin for 15 min, washed 2 times for 1 min each in 70% ethanol and in 100% ethanol for 2mins. Finally the sections were washed three times for 5- min. in Xylol following which they were mounted using entalan.

TABLE OF VECTORS USED

Plasmids/ Vectors	**Description**	**Source**
pWE15-TGF	pWE15 cosmid vector from Stratagene carrying the 28 kb genomic TGF-β1 clone	Dr. Rajendra Raghow, University of Tennesse, Memphis
pSL1190	cloning vector with multiple cloning sites	Pharmacia Biotech
pBSIIKS	cloning vector with multiple cloning sites	Stratagene
pEasy Flox	loxP flanked *neo*^{r} cassette and a third loxPsite	M.Alimzhanov, University of Cologne
pPGKcre-bpa	Cre vector for eukaryotic expression	Kurt Fellenberg, University of Cologne

TABLE OF PRIMERS USED FOR SEQUENCING TGF-β1 GENE

Oligo	**Sequence**	**Specificity**
AG-C1	5'-GGT ATC GCG CTC CGA TTC GCA CG-3'	5' exon 6, TGF-β1 gene
AG-C2	5'-CCA GCC TGT CCC ATG AGC CGA GCC-3'	3' exon 6, TGF-β1 gene
AG-E1	5'-GAG ATC CTG GTG GGG AGA GAG ATA GG-3'	5' exon 7, TGF-β1 gene
AG-E2	5'-GGC GGC CAA TGA CAC GGA GAC CAC C-3'	3' exon 7, TGF–β1 gene

E RESULTS:

E1 Generation of TGF-β1$^{flox/flox}$ mice

E1.1 Targeting of ES cell clones

The active form of mammalian TGF-β1 consists of a disulfide-linked homodimer, with each monomer representing the 112 carboxy-terminal amino acids of a large, inactive precursor that is cleaved from the amino-terminal glycopeptide at a tetrabasic cleavage site (Derynck et al., 1985) (Figure 1A and B). The targeting strategy was designed to effectively disrupt the mature peptide only, while leaving the precursor pro-region intact. To achieve this, I decided to delete exons 6 and 7 which code for the mature peptide only, leaving behind exons 1 to 5 which code for the pro-region that forms the latency associated protein (LAP).

As depicted in the targeting strategy (Figure 2A), loxP sites (floxed) flanked exons 6 and 7. This was achieved by inserting the floxed neomycin resistance gene 5' into an Sph1 site 1 kb upstream of exon 6. The third loxP site was introduced 1.2 kb downstream of exon 7 into a Sph1 site. The long arm of homology was 3.8 kb upstream of the neo gene. The short arm was 1.2 kb downstream of the third loxP site, which was followed by a HSV-1 thymidine kinase gene (tk) which would help select for homologous recombinants. The loxP flanked fragment having exons 6 and 7 is 3.6 kb in length. 25 μg of the targeting vector was linearized using the Sfi1 site at the 5'end of the long arm of homology and transfected into R1 ES cells. Transfectants were selected with G418 and gancyclovir and a total of 1000 colonies were picked and expanded for freezing down as well as preparing genomic DNA. DNA from 600 individual double resistant clones was digested with Sac1 and used to screen for homologous recombinants as well as integration of the third lox-P by Southern blot analysis using a 3'external probe B (Fig. 2A). In targeted clones, homologous recombination as well as integration of the third loxP site introduces a Sac1 restriction site. Sac1 digested genomic DNA from targeted clones yields restriction fragments of 5.7 kb (wild type) and 3.8 kb (targeted) when hybridized with an external probe B (Figure 2B). 11 out of the 600 double resistant clones were positive and all 11 were thawed, expanded and screened again by digesting their genomic DNA with Sac1. Also the insertion of the floxed neomycin resistance cassette introduces an Nco1 restriction site downstream of the endogenous Nco1 site. Further verification of the positive clones was done by digesting the genomic DNA with either Nco1 (Figure 2C) or Apa1 (data not shown) and screening by Southern analysis, using Probe B. The Nco1 digest results in a 8kb fragment for the targeted allele as opposed to a 10.4 kb fragment for the wild type allele.
To enable *in vitro* deletion of the *neo*^{r} gene, clones AG4E6 and AG5F10 were transiently transfected with Cre expressing vector pPGK-cre. Following expansion as described earlier, 500 colonies were picked and tested for G418 sensitivity.

Of the 76 G418-sensitive colonies only 5 clones had partial *neo*^{r} gene deletion while the majority of them carried the totally deleted allele or were unidentifiable by Southern analysis. This was determined by digesting the ES cell DNA with Xba1 and screening by Southern analysis, using Probe B. This yields an 11.2 kb wild type band, 9 kb partially deleted band and a 7 kb totally deleted band using the external probe B (Figure 3A and B). Four of these clones, AG4E6.1, AG4E6.6, AG5F10.1 and AG5F10.4 were checked once again (using Xba1 digest) by Southern blot analysis for homologous integration (Figure 3B).

I injected each of these four clones into 150-175 blastocysts i.e. over a period of about 12 weeks a total of around 500 blastocysts were injected and transplanted into 25 pseudopregnant foster mothers. Of these 25 fosters, 5 females died within two days of transfer. From the surviving 20 females a total of 94 pups were born. Of these 14 were chimeras with varying degrees of chimerism (20% to 100%). Another 16 pups, which included 3 chimeras, were lost within the first two weeks after birth. The surviving 9 male and 2 female chimeras were backcrossed to C57BL/6 mice and germline transmission as observed by coat color was obtained only from male chimeras that were derived from AG4E6.1 and AG4E6.6. Both of these were derived from the parental clone AG4E6. Genomic DNA prepared from tail snips got from progeny of chimeras was digested by Sac1 and used to confirm germline transmission by Southern blot analysis using Probe B (Figure 4A). Heterozygous mice were then either intercrossed to get homozygous floxed mice or were crossed to Deleter-Cre or CD4-Cre or LysM-cre transgenics.

Figure 2. **Targeting strategy for the generation of TGF-β1 conditional allele.**
(A) Construction of targeting vector and screening of homologous ES-cell recombinants. The targeting strategy was designed to effectively disrupt the TGF-β1 mature peptide only by deleting exons 6 and 7 and leaving behind exons 1 to 5. This was achieved by inserting the loxP flanked neomycin resistance gene into an Sph1 site 1 kb upstream of exon 6. The third loxP site (along with a Sac1 site) was introduced 1.2 kb downstream of exon 7 into an Sph1 site. The arms of homology were 3.8 kb upstream of the neo gene and 1.2 kb downstream of the third loxP site. The floxed fragment having exons 6 and 7 is 3.6 kb in length.
Southern blot analysis of homologous ES-cell recombinants. (B) In targeted clones, homologous recombination as well as integration of the third loxP site introduces a Sac1 restriction site. Sac1 digested genomic DNA from targeted clones yields restriction fragments of 5.7 kb (wild type) and 3.8 kb (targeted) when hybridized with an external probe B. **(C)** To target the genomic TGF-β1 locus, the loxP flanked neomycin resistance gene was cloned into a Sph1 site 1 kb upstream of exon 6. This helped introduce an Nco1 site into the floxed fragment having exons 6 and 7. Nco1 digested genomic DNA from targeted clones yields restriction fragments of 10.4 kb (wild type) and 8 kb (targeted) when hybridized with an external probe B. This was used for confirming homologous recombination and integration of third loxP site in clones AG2B4, AG4E6 and AG5F10

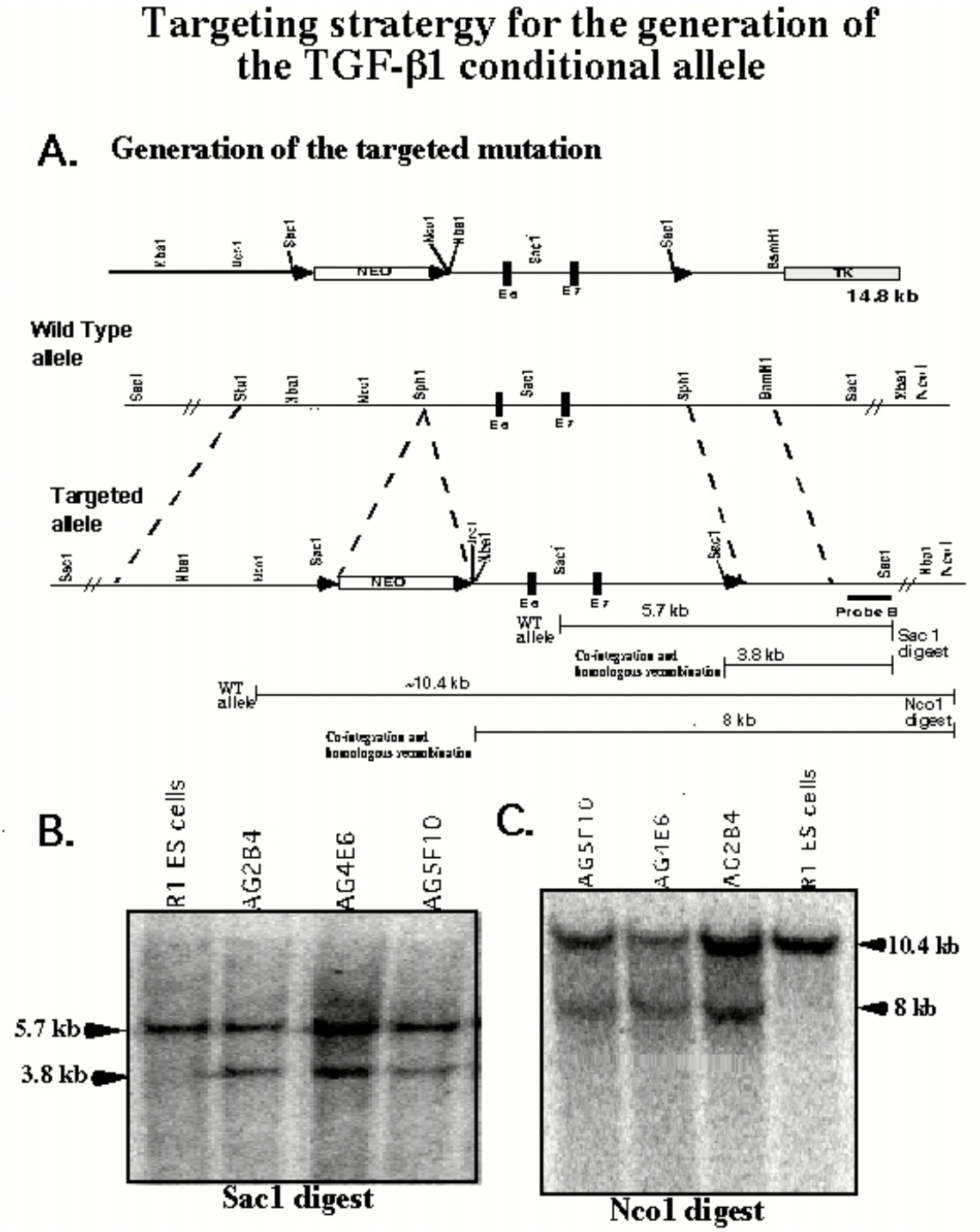
Targeting stratergy for the generation of the TGF-β1 conditional allele
A. Generation of the targeted mutation
Wild Type allele
Targeted allele
NEO
TK
14.8 kb
Probe B
5.7 kb
WT allele
Sac 1 digest
3.8 kb
~10.4 kb
Nco1 digest
8 kb
B.
R1 ES cells
AG2B4
AG4E6
AG5F10
5.7 kb
3.8 kb
Sac1 digest
C.
AG5F10
AG4E6
AG2B4
R1 ES cells
10.4 kb
8 kb
Nco1 digest

Figure 2

Summary of ES cell transfections

# clones picked	# clones screened	# Targeted with third loxP site	# clones transfected with pGKCre for *neo* deletion	# clones picked after *neo* deletion	# clones with partial deletion
1000	600	11	2 (AG4E6, AG5F10)	500	5 (AG4E6.1, AG4e6.6, AG5F10.1, AG5F10.4)

Summary of blastocyst injections and germline transmission:

Clone Injected	Male chimeras	Germline transmission	Female chimeras	Germline transmission
AG4E6.1	3	Yes, 1 out of 3 transmitted	0	NA
AG4E6.6	2	Yes, 1 out of 2 transmitted	0	NA
AG5F10.1	3	None after 10 litters	0	NA
AG5F10.4	1	None after 12 litters	2	No

Figure 3. Cre mediated in vitro deletion of the neomycin cassette.
(A) GenomicTGF-β1 locus and targeted allele showing inserted Xba1 site. To target the genomic TGF-β1 locus the loxP flanked neomycin resistance gene was cloned into an Sph1 site 1 kb upstream of exon 6. In this processes an Xba1 site was introduced into the floxed fragment having exons 6 and 7 that is 3.6 kb in length. **(B)** Southern blot analysis of partially and totally deleted homologous ES-cell recombinants. When hybridized with an external probe B, Xba1 digested genomic DNA from targeted clones yields restriction fragments of 11.2 kb for wild type, 9 kb for partially deleted and 7 kb for totally deleted alleles. This was used for screening partially and totally deleted clones got after the Cre mediated *in vitro* deletion of the neomycin resistance cassette from clones AG4E6 and AG510.

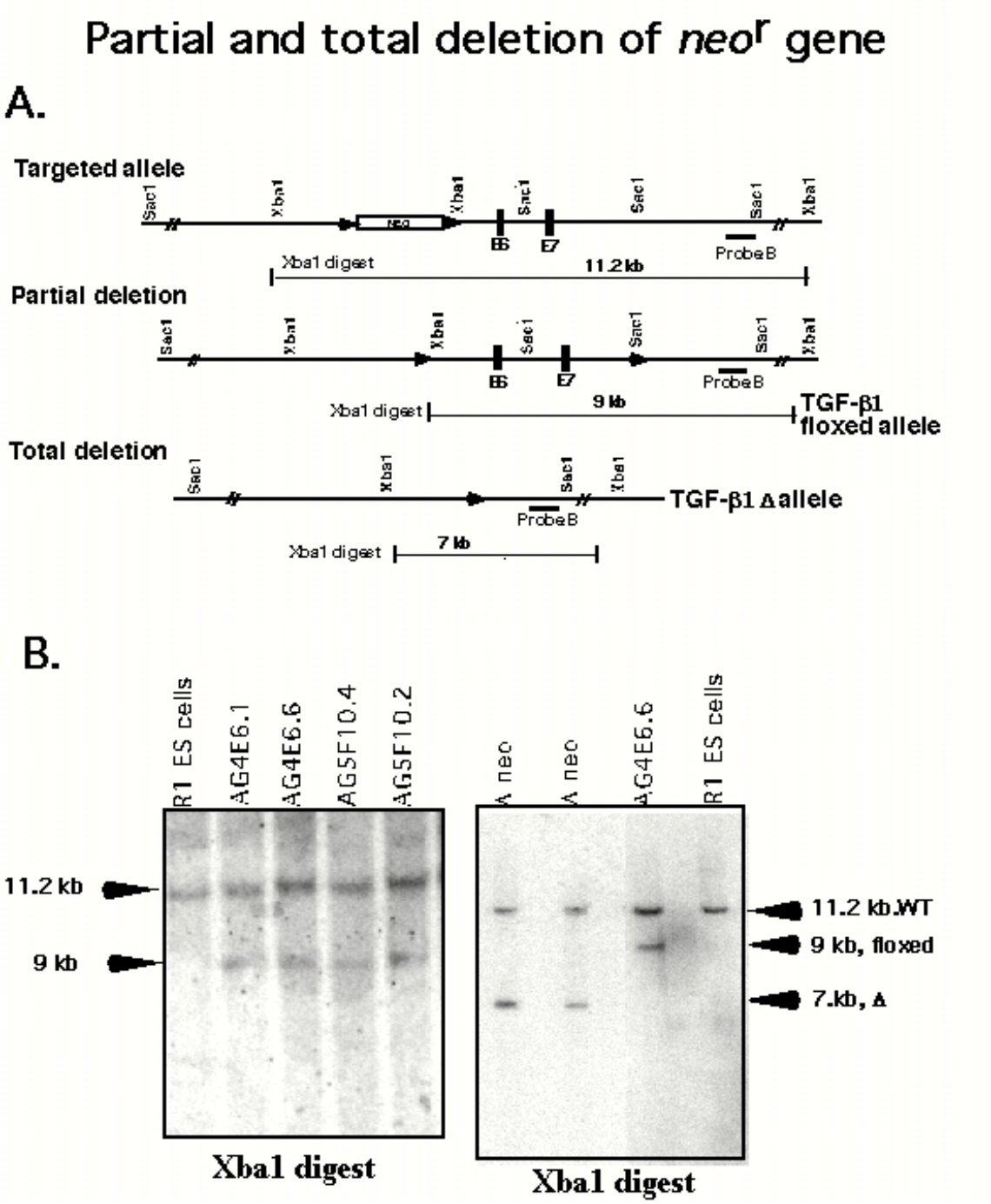
Partial and total deletion of neor gene
A.
Targeted allele
Sac1
Xba1
neo
E6
E7
ProbeB
Xba1 digest
11.2 kb
Partial deletion
9 kb
TGF-β1 floxed allele
Total deletion
TGF-β1 Δ allele
7 kb
B.
R1 ES cells
AG4E6.1
AG4E6.6
AG5F10.4
AG5F10.2
Δ neo
11.2 kb
9 kb
11.2 kb.WT
9 kb, floxed
7.kb, Δ
Xba1 digest

Figure 3.

E2 Homozygous TGF-β1$^{flox/flox}$ mice are viable and have no obvious phenotype

In contrast to the complete knockout of TGF-β1, the heterozygous TGF-β1 null mice are viable and do not show any phenotypic differences when compared to wild type mice. Hence, healthy heterozygous TGF-β1$^{flox/+}$ mice would not indicate if the *in vivo* manipulations of the TGF-β1 locus were in any event deleterious to the gene or its product. Therefore it was imperative to confirm that the homozygous TGF-β1$^{flox/flox}$ mice were viable and capable of breeding. This was shown by intercrossing the TGF-β1$^{flox/+}$ mice and checking for the presence of the two floxed alleles in the progeny by screening genomic DNA isolated from tail snips by Southern analysis using Probe B (Figure 4B). A normal Mendelian distribution was seen in the viable TGF-β1$^{flox/flox}$ mice that were born from an interbreeding of TGF-β1$^{flox/+}$ mice. These mice showed no developmental abnormalities and were healthy 4-5 weeks after weaning, at which point they were used for further breeding.

E3 Generation of TGF-β1$^{flox/flox}$ / Cre^{+} transgenic mice and *in vivo* deletion of TGF-β1

To ascertain that in the TGF-β1$^{flox/flox}$ mice, the floxed allele could indeed be deleted, a deleter-cre transgenic male mouse, on a C57BL/6 background was bred to a TGF-β1$^{flox/+}$ female mouse. As the Cre transgene is X-linked (Schwenk et al., 1995) the female progeny were screened for the presence of Cre and the TGF-β1 floxed allele. Two positive females were then backcrossed to a TGF-β1$^{flox/+}$ male mice so as to obtain TGF-β1$^{flox/flox}$ / Cre double transgenic mice. Over a period of about 12 weeks, six litters were born from these crosses yielding 40 pups, of which a total of 9 newborns died or were cannibalized by the mothers. Pieces of the dead bodies were collected and also screened. The surviving pups were observed daily at 6-8 hour intervals. At day 18 one of the male pups suddenly showed signs of respiratory distress that was very rapidly followed by a severe swelling of its abdominal cavity (in about 6 hours) although the rest of the mouse looked like it was gradually wasting away. This mouse was sacrificed early on day 19 as it looked too unhealthy to

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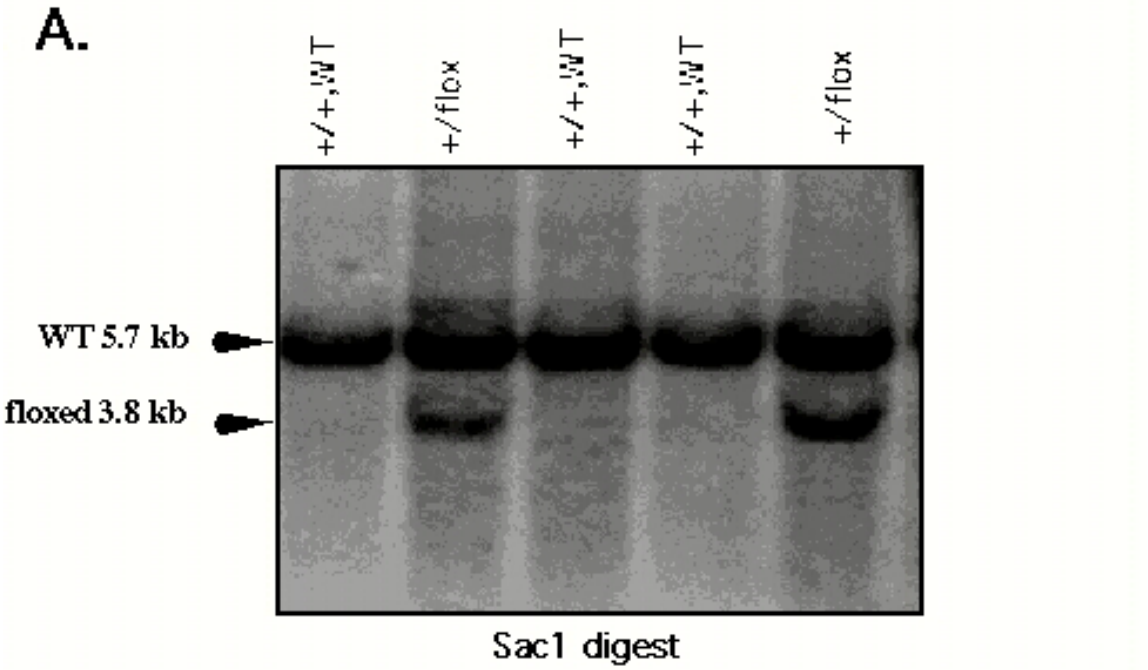
Figure 4. Germline transmission of the TGF-β1$^{flox/+}$ allele. (A) Genomic DNA from progeny of chimeras generated using TGF-β1$^{flox/+}$ targeted ES cells (AG4E6.1) was digested with Sac1 and screened for presence of the targeted allele by Southern blot analysis. Using external probe B, heterozygous TGF-β1$^{flox/flox}$ mice show a 5.7 kb wild type band and a 3.8 kb targeted band. **(B)** Genomic DNA from the progeny got as a result of breeding heterozygous TGF-β1$^{flox/+}$mice was digested by Sac1 and using probeB, screened by Southern blot analysis. The presence of only one targeted 3.8 kb band as opposed to a 5.7 kb wild type and a 3.8 kb targeted band in heterozygous mice indicates the generation of homozygous TGF-β1$^{flox/flox}$ mice.

Germline transmission of the TGF-β1 loxP flanked allele



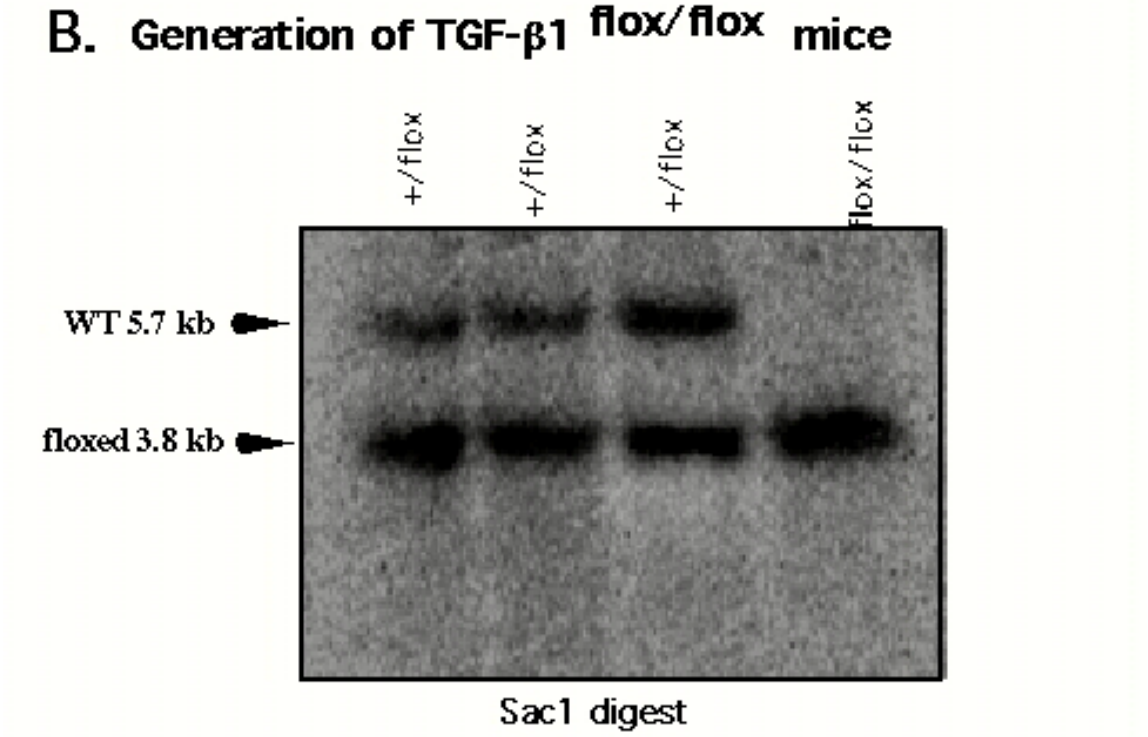


Figure 4

survive any longer. Immediately after sacrificing the mouse its heart, spleen, small and large intestine, stomach, kidneys, liver, lungs and pieces of skin were fixed in 4% formalin. Part of its tail was digested overnight at 56^0C in lysis buffer to collect genomic DNA for Southern blot analysis. The surviving mice were weaned at day 23 after birth. On day 25 another male mouse started showing signs of wasting and by late day 27 it was also sacrificed and the same procedure as mentioned above was used to collect its various organs. Although the genotype of the mice at this point was unknown, two healthy looking male littermates of this sick mouse were also sacrificed as controls.

The thirty-one pups mentioned above, including the two that succumbed were screened for the presence of Cre and the TGF-β1 floxed allele(s). Genomic DNA collected from tail snips of the pups was digested overnight by Xba1 and screened for the presence of the TGF-β1 floxed allele(s) by Southern blot analysis using 3'external probe B (Figure 5A and B). The endogenous TGF-β1 band is about 11.2 kb, the targeted allele gives a 9 kb band while the total deletion of the TGF-β1 gene gave a band around 4 kb. The genomic DNA collected from tail snips was also screened by PCR for the presence of Cre. A total of eleven out of the surviving thirty-one pups were heterozygous for the floxed/deleted allele. Two out of the thirty-one pups born were TGF-$\beta1^{\Delta/\Delta}$ / Cre^{+} double transgenic mice. These were the two that had died prematurely on day19 and day 27. Both of these showed physical signs of distress and waisting before they died. The two healthy male littermates that were sacrificed along with the sick pups were both heterozygous for the floxed allele and negative for Cre (Figure 5B). Therefore, this breeding resulted in only around 6.45% (2 out of 31 surviving pups) instead of the normal expected 25% TGF-$\beta1^{\Delta/\Delta}$ / Cre+ double transgenic mice, indicating intrauterine lethality. DNA that was collected from the pieces of dead pups was too degraded for screening by Southern Blot analysis (data not shown), however three out of the nine did come up positive for the presence of the Cre transgene by PCR.

Figure 5. **Cre mediated *in vivo* deletion of TGF-β1 gene. (A)** To ascertain that in the TGF-$\beta1^{flox/flox}$ mice, the floxed allele could be deleted *in vivo*, a deleter-cre transgenic male mouse, on a C57BL/6 background was bred to a TGF-$\beta1^{flox/+}$ female mouse. Two females positive for Cre and the floxed allele were then backcrossed to a TGF-$\beta1^{flox/+}$ male mouse. Genomic DNA from a pup that died at day nineteen, as well as two healthy littermates was digested with Xba1 and screened by Southern blot analysis. Using probe B, the healthy littermates gave two bands: an 11.2 kb wild type and a 9 kb targeted band. The pup that died prematurely gave only one 7 kb band for total deletion of both alleles indicating it was a TGF-$\beta1^{\Delta/\Delta}$ / Cre transgenic mouse.

Cre mediated *in vivo* deletion of the TGF-β1 loxP flanked alleles

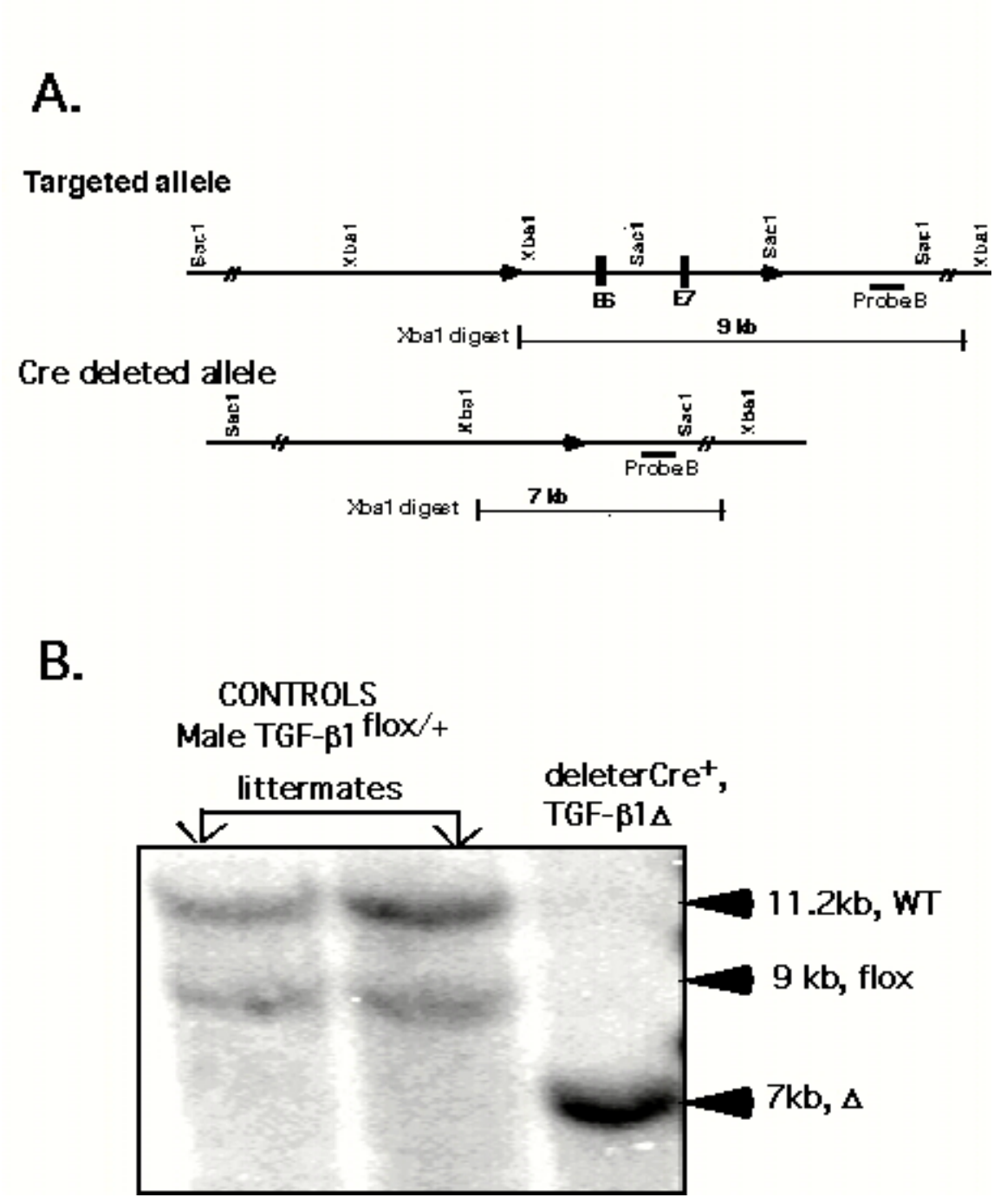


Figure 5.

E4 Histology

By Southern analysis and PCR I confirmed that the two pups that had died prematurely out of surviving twenty-five pups were TGF-$\beta1^{flox/flox}$ / Cre^{+} double transgenic mice. External physical examination of these two dead pups showed normal skin. Internally in both cases, an extremely deformed and necrotic looking heart was observed. The mouse that had developed a severe abdominal swelling showed an extremely swollen and bloody stomach, which was difficult to even manipulate for fixing.The other mouse showed massive swelling and blood in regions of its small intestine. The spleen, liver, kidneys, thymus, lungs, tongue, most of the connective tissue and brain looked normal. All organs were fixed in 4% paraformaldehyde and then embedded in paraffin. Also organs from the two healthy male littermates that had been sacrificed at the same time point as the death of the sick pups were fixed. The healthy, normal hearts from control mice and the necrotic hearts were then cut to get 5-7 μM sections which were then stained with Hematoxylin / eosin (Fig. 6). Microscopic examination of the necrotic hearts from TGF-$\beta1^{\Delta/\Delta}$/ Cre double transgenic mice showed a loss in the structural integrity of the heart. Dense infiltrates of cells (Figure 6B) were observed in the sections made from TGF-$\beta1^{\Delta/\Delta}$/ Cre double transgenic mice hearts when compared to the stained sections made from healthy hearts. The infiltrates were made up predominantly of mononuclear cells. The myocytes in the TGF-$\beta1^{\Delta/\Delta}$/ Cre double transgenic mice hearts looked abnormally packed close together.

E5 Generation of TGF-$\beta1^{flox/flox}$/CD4-Cre, TGF-$\beta1^{flox/flox}$ /LysM-Cre double transgenic mice

In addition to breeding to the deleter-cre transgenics, the TGF-$\beta1^{flox/+}$ mice were also bred with either CD4-Cre transgenics or with LysMCre transgenic mice. This was done to achieve T-cell specific or macrophage specific deletion of TGF-β1. Genomic DNA collected from tail snips of the progeny was screened by Southern analysis and PCR for the floxed allele and the specific cre transgenes. The TGF-$\beta1^{flox/+}$ / CD4-Cre^{+} transgenics born were healthy and bred to a TGF-$\beta1^{flox/+}$ so as to get homozygous TGF-$\beta1^{flox/flox}$ / CD4-Cre^{+}mice (Figure 7A and B). In a similar manner, TGF-$\beta1^{flox/+}$ / LysMCre^{+} transgenics were bred to a TGF-$\beta1^{flox/+}$ so as to generate TGF-$\beta1^{flox/flox}$ / LysMCre^{+} transgenics (Figure 8 A and B). The small pools of mice generated showed no phenotypic abnormalities and have developed normally upto around 5 weeks of age.

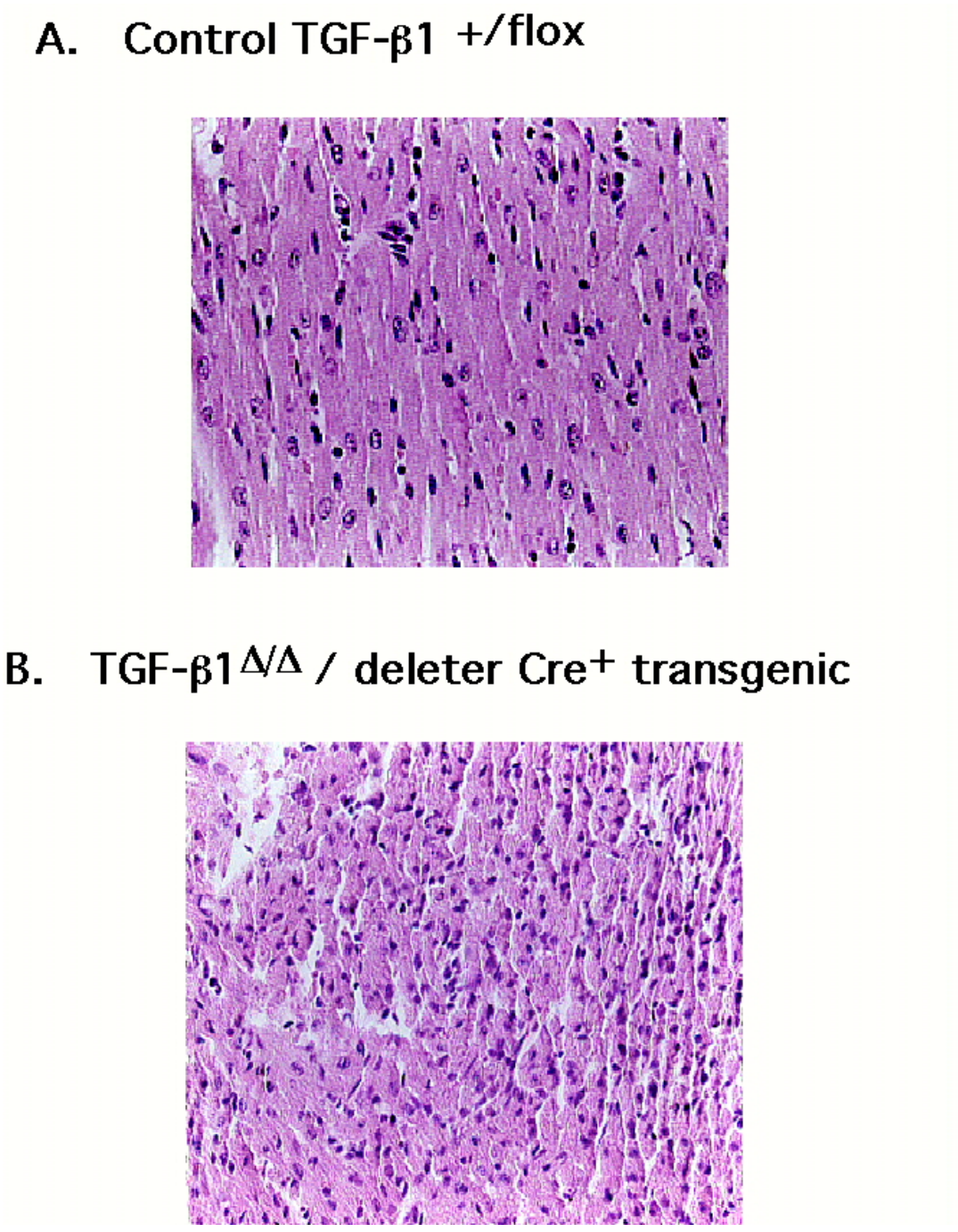


Figure 6. **Histological examination of control (A)** TGF-$\beta1^{flox/+}$ and **(B)** TGF-$\beta1^{\Delta/\Delta}$ / deleterCre double transgenic mice. Healthy, normal hearts from control mice and the necrotic hearts collected from TGF-$\beta1^{\Delta/\Delta}$ / Cre^{+} were fixed in 4% paraformaldehyde and then embedded in paraffin. These were then cut to get 5-7 μM sections which were stained with Hematoxylin / eosin (H/E). Compared to the TGF-$\beta1^{flox/+}$ mouse, the TGF-$\beta1^{\Delta/\Delta}$ /Cre^{+} mouse shows a very high number of mononuclear cells and a distortion of the normal cellular structures (40X magnification, 1cm approx. =100μm).

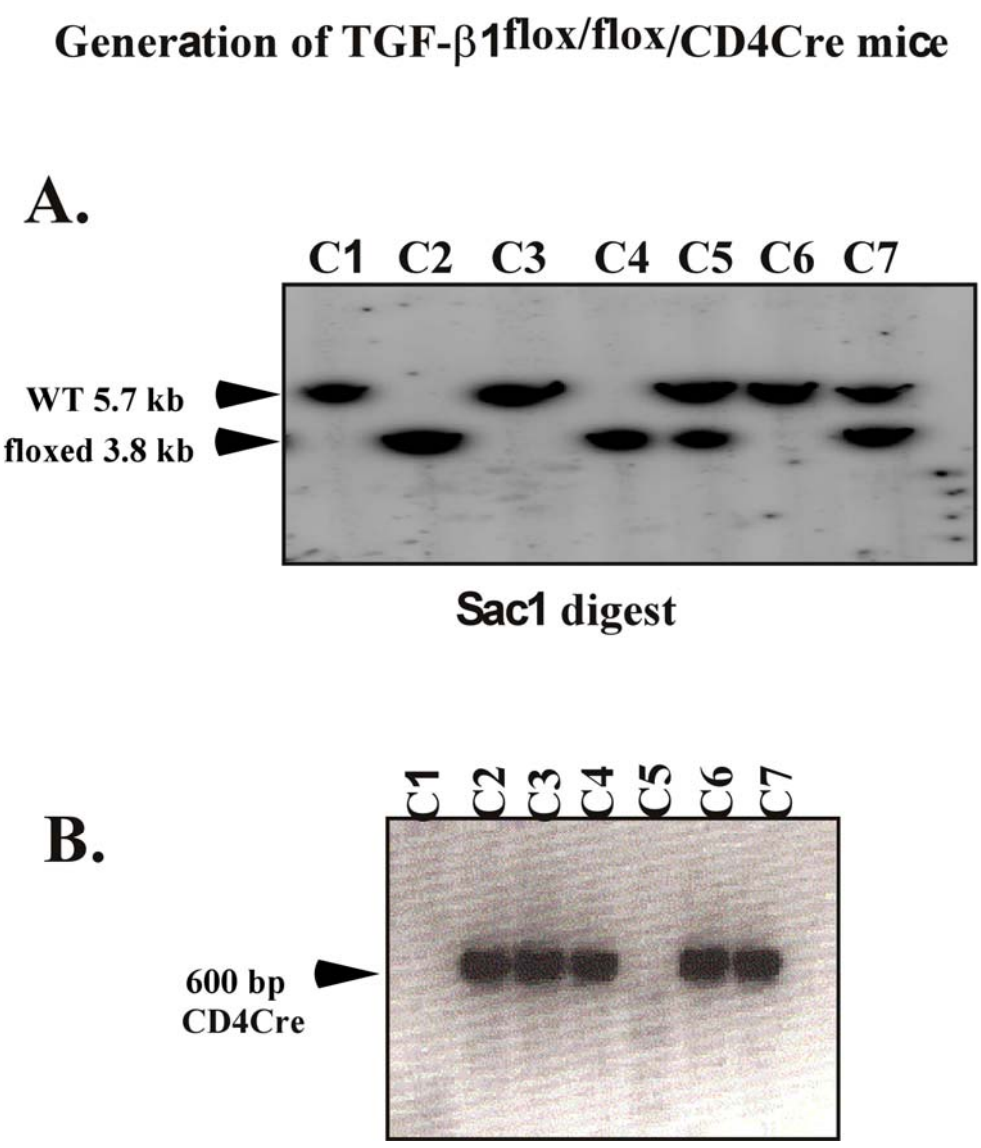


Figure 7. Generation of TGF-β1$^{flox/+}$ / CD4Cre mice. (A) Genomic DNA from progeny born to TGF-β1$^{flox/+}$ / CD4Cre female bred to a TGF-β1$^{flox/f+}$ male mouse was digested with Sac1 and screened for presence of the targeted allele by Southern blot analysis. Using external probe B, heterozygous mice (numbers C5 and C7) show a 5.7 kb wild type band and a 3.8 kb targeted band while numbers C1, C3 and C6 are wild type. Mouse numbers C2 and C4 5 are TGF-β1$^{flox/flox}$ **(B)** PCR screening of TGF-β1$^{flox/f+}$ / CD4Cre mice. 1 μl of genomic DNA from the same mice as above were screened by PCR for the CD4Cre transgene. Primer pairs AG-CD4L6 and AG-Cre6 were used for the screening, which amplified a 600bp CD4cre band. C1, C2, C3,C5 and C6 were CD4Cre positive while C4 and C7 were negative. Based on the PCR and Southern data, mouse number C2 was the only TGF-β1$^{flox/flox}$ / CD4Cre+ mouse

Figure 7

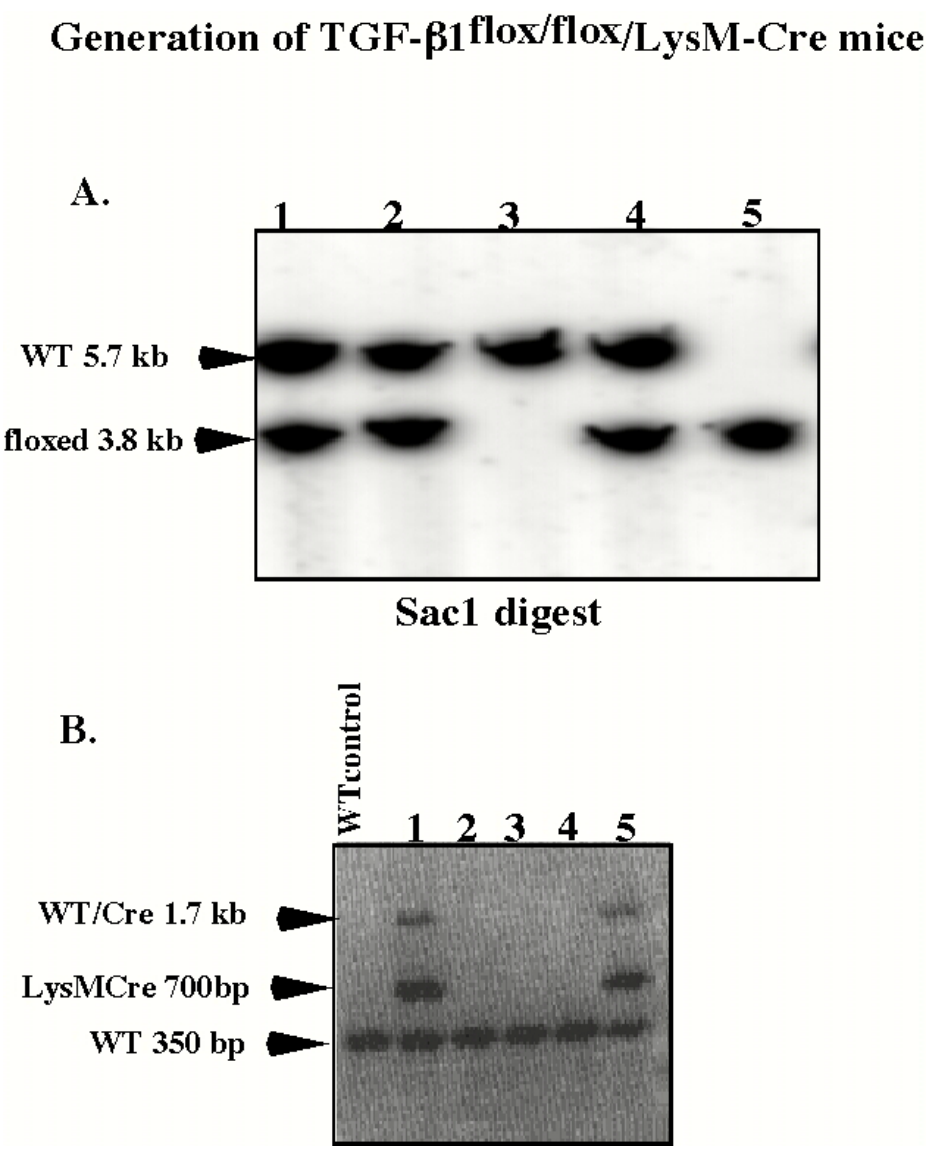


Figure 8 . Generation of TGF-β1$^{flox/+}$ / LysMCre mice. (A) Genomic DNA from progeny born to a TGF-β1$^{flox/+}$ female bred to a TGF-β1$^{flox/f+}$ / LysMCre male mouse was digested with Sac1 and screened for presence of the targeted allele by Southern blot analysis. Using external probe B, heterozygous mice (numbers 1, 2 and 4) show a 5.7 kb wild type band and a 3.8 kb targeted band. Mouse number 5 is TGF-β1$^{flox/flox}$ **(B)** PCR screening of TGF-β1$^{flox/flox}$ / LysMCre mice. The mice described above were also screened for the presence of the LysMCre transgene by PCR . Using primers Cre8, Mlys1 and Mlys2, mouse numbers 2, 3 and 4 gave only a wild type band of 350 bp while mouse numbers 1 and 5 gave a wild type band as well as a 700bp LysMcre transgene band and a 1.7 kb WT/Cre band with the primer pair Mlys1 and Mlys2. Based on these data, mouse number 5 was a TGF-β1$^{flox/flox}$ / LysMCre+ mouse.

Figure 8

F DISCUSSION

Transgenic and knockout mice that have an altered expression of TGF-β1 or its receptors have been generated to elucidate the highly pleiotropic properties of TGF-β1 and its biologic role in the regulation of immune homeostasis. The widespread expression of TGF-β1 and its receptors possess a major impediment in discerning its role immune regulation. In this endeavor, the knowledge gained via the generation of the following three models of mice has been especially valuable. I would like to describe these existing models and then discuss the importance of generating the TGF-$\beta1^{flox/flox}$ mice.

TGF-β1 null mice

The targeted disruption of the murine gene encoding TGF-β1 results in fifty to one hundred per cent embryonic lethality (Kallapur et al., 1999) demonstrating that TGF-β1 is vital for embryonic development. Surviving TGF-$\beta1^{-/-}$ pups do not show any gross developmental abnormalities at birth, but within 3-4 weeks they succumb to a wasting syndrome accompanied by a multifocal, mixed inflammatory cell response and tissue necrosis, leading to organ failure and death (Kulkarni et al., 1993; Shull et al., 1992). TGF-$\beta1^{-/-}$ mice show an excessive inflammatory response with massive infiltration of lymphocytes and macrophages in many organs, but primarily in heart and lungs. The infiltrates are primarily made up of T-cells and macrophages, further substantiating a role of TGF-β1 in these cell lineages. This tissue inflammation in TGF-$\beta1^{-/-}$ mice can be partially inhibited by immune suppressants, but the severe autoimmune phenotype is not reversed by systemically injected TGF-β1, suggesting that immune cells rely on autocrine and/or intracrine TGF-β1 (Kulkarni and Letterio, 1998). This hypothesis is further corroborated by bone marrow transplantation studies in which the reconstitution of lethally irradiated wild-type mice with marrow of TGF-$\beta1^{-/-}$ donors leads to an autoimmune phenotype resembling that of a TGF-$\beta1^{-/-}$ mouse. This transfer of phenotype, despite the presence of TGF-β1 produced by non marrow-derived cells of the wild-type recipient alludes to the important role of TGF-β1 producing immune cells. Also, there is a noteworthy slowdown in the development of autoimmunity in breeding studies that place the TGF-$\beta1^{-/-}$ genotype onto background strains carrying immune system defects such as the SCID mouse, RAG1 or RAG2 knockout mice, nude mice, MHC class I and class II knockout mice (Letterio and Roberts, 1998).

T-cell specific dominant-negative TGF-β1 receptor type II transgenic mice

In this model, signaling by TGF-β is blocked specifically in T cells by expressing a dominant-negative TGF-β1 receptor type II under a T cell-specific promoter (Gorelik and Flavell, 2000). In this study, TGF-β signaling in CD4-dnTGFRII mice is affected only in T cells and is normal in all other cell types. Hence these mice lack most of the problems associated with the use of TGF-$\beta1^{-/-}$ mice as a model for dissecting the role of TGF-β signaling in T cells. They show that T cell–specific blockade of TGF-β signaling leads to autoimmune disease characterized by multifocal inflammatory infiltration and autoantibody secretion

leading the authors to conclude that disregulation of TGF-β signaling in T cells is sufficient to disrupt their homeostasis and result in their activation. Their data substantiates the fact that TGF-β is an important anti-differentiation factor for T cells under physiological conditions in vivo. In addition, CD4-dnTGFRII mice provide a good model to study the regulation of various immune responses in the absence of TGF signaling. Also, notwithstanding the fact that the nature of the stimuli activating T cells in the absence of TGF-β signaling in CD4-dnTGFRII mice is not determined, the presence of autoimmune IgG antibodies in these mice implies that maybe part of the T cell response is directed against self-antigens.

TGF-β1 receptor type II conditional knockout mouse

These mice lack the TGF-β receptor (TGF-β R) type II selectively in B cells (Cazac and Roes, 2000). The generation of these mice involved using the Cre/loxP system (Gu et al., 1994; Sauer and Henderson, 1988). Exon 3 of the gene coding for the TGF-β receptor was flanked by lox/P sites, so as to enable deletion of the receptor when bred to a B-cell specific cre transgenic mouse. These mice have a reduced life span of conventional B cells, expansion of peritoneal B-1 cells, B cell hyperplasia in Peyer's patches, an almost complete serum IgA deficiency and elevated serum immunoglobulin. In this study they have shown that TGF-β receptor may mediate a higher level of control by setting B cell responsiveness independently of antigen and antigen receptors unlike antigen receptor–associated regulatory molecules such as the CD19/CD21 complex. They propose that TGF-β1 production by local tissues, leukocyte subsets, or B cells themselves may provide a mode to alter B cell responsiveness to the antigenic stimuli and thereby counterbalance "bystander" effects of stimulatory cytokines in a proinflammatory environment.

Rationale for generating TGF-β1$^{flox/flox}$ mice

As discussed above, the complete knockout for TGF-β1 has helped establish the significance of this growth factor. However, it has provided us with an inadequate and ambiguous model for delineating the role TGF-β1 plays in discrete cell lineages, tissues and systems. Also, in the CD4-dnTGFRII mice, there are a few points that could hinder the unraveling the TGF-β1 enigma in T cells. One being that the TGF-β1 $^{-/-}$ mice develop a much more severe autoimmune phenotype and at a much faster rate than CD4-dnTGFRII mice. The relative severity of the total knockout could be due to disregulated homeostasis of multiple tissues and an abnormal upregulation of MHC class I and II molecules in all tissues of the TGF-β1 $^{-/-}$ mice, resulting in presentation of self-antigens that are ordinarily not presented. Hence, the lack of TGF-β1 control of T cells along with the aberrant display of self-antigen to T cells could bring about the accelerated and more severe autoimmune response relative to CD4-dnTGFRII mice, where TGF-β1 signaling is blocked only in T cells. Alternatively, inherent to a transgenic system that does not eliminate the endogenous TGF-βRII, in the CD4-dnTGFRII mice complete blockade of TGF-β1 signaling in T cells is not achieved. So if there is a "leakage" of dominant-negative

TGF-β1RII, this enables residual TGF-β1 signaling, encumbering spontaneous activation of self-reactive T cells, accordingly decreasing the rate at which autoimmune disease manifests itself in these mice. Furthermore, TGF-β1 alone does not signal through the TGF-β1RII; TGF-β2 and TGF-β3 use it too. In addition, TGF-β1 is required in the development of the hematopoietic system and disregulation of hematopoiesis in adult TGF-$\beta1^{-/-}$ mice makes it extremely difficult to study the role of TGF-β1 in regulation of the immune system.

Hence, the establishment of a model system in which the expression of TGF-β1 can be regulated in a cell-type specific manner only, would provide us with an opportunity to precisely discern its activities in various systems. As mentioned above, an elegant method for cell-type specific deletion of the TGF-β1RII has already been generated using the Cre/loxP system. The utilization of a similar system would be ideal for the generation of a conditional knockout mouse for the TGF-β1 gene that will overcome the problem of the lethal phenotype of the TGF-β1 total knockout mice (Kulkarni et al., 1993; Shull et al., 1992).

TGF-$\beta1^{flox/flox}$ mice

To achieve this conditional deletion of TGF-β1, I generated a mouse wherein loxP sites flank a crucial part of the TGF-β1 gene is flanked (TGF$^{flox/-flox}$ mice). Heterozygous TGF-β1 null mice are viable and do not show any phenotypic differences when compared to wild type mice (Kulkarni et al., 1993; Shull et al., 1992). This is a critical observation when disscussed in view of a conditional knockout which entails the *in vivo* manipulations of the TGF-β1 locus involving the insertion of loxP sites within the introns of the gene. To confirm that these manipulations were not deleterious to the gene or its product, the TGF-$\beta1^{flox/+}$mice were interbred to generate TGF-$\beta1^{flox/flox}$ mice. In the resulting progeny, a normal Mendelian distribution was observed of viable TGF-$\beta1^{flox/flox}$ mice.

The conditional knockout was made with the intention of having the capability of deleting TGF-β1 in a spatially, temporally and perhaps even inducibly controlled manner. This idea is based on the premise that Cre mediated deletion of the TGF-β1 gene would occur without any hindrance *in vivo* . But, before venturing on such an intricate investigation of the autocrine loops of TGF-β1 in specific cells it was absolutely crucial to demonstrate the feasibility of the system by achieving Cre mediated deletion of the floxed allele *in vivo* in all cells. Ideally, such a deletion of TGF-β1 would result in a phenotype identicle to that of the complete knockout. To achieve this TGF-$\beta1^{\Delta/\Delta}$ /Cre+ transgenic mice were generated by crossing a deleter-cre transgenic male mouse (Schwenk et al., 1995), on a C57BL/6 background to a TGF-$\beta1^{flox/+}$ female mouse. Female mice, positive for Cre and the TGF-β1 floxed allele were backcrossed to a TGF-$\beta1^{flox/+}$ male mice so as to obtain TGF-$\beta1^{\Delta/\Delta}$ / Cre transgenic mice. This breeding resulted in an extremely low number of TGF-$\beta1^{\Delta/\Delta}$ /Cre mice indicating intrauterine lethality. In the total knockout of the same phenomenon is observed (Kallapur et al., 1999) . The TGF-$\beta1^{\Delta/\Delta}$ / Cre+ transgenic pups developed a wasting syndrome to which they succumbed prematurely comparable to the complete knockout mice. Internal examination showed an

extremely deformed and necrotic looking heart in both cases. One mouse showed a severely swollen and bloody stomach while the other mouse showed massive swelling and blood in regions of its small intestine. The spleen, liver, kidneys, thymus, lungs, tongue, most of the connective tissue and brain looked normal. Microscopic examination of the necrotic hearts from TGF-β1$^{\Delta/\Delta}$ / Cre double transgenic mice showed a loss in the structural integrity of the heart and densely packed infiltrates. The infiltrates were made up predominantly of mononuclear cells. It is important to note that the TGF-β1$^{-/-}$ mice also show an excessive inflammatory response with massive infiltration of lymphocytes and macrophages in many organs, but primarily in heart and lungs (Kulkarni et al., 1993; Shull et al., 1992). Specifically, necrotic hearts have been observed in the total knockout of TGF-β1. The phenotype of the TGF-β1$^{\Delta/\Delta}$ / Cre confirmed that the *in vivo* deletion of TGF-β1 gene in all cells was able to recapitulate the phenotype that is characteristic of the TGF-β1 complete knockout (Kulkarni et al., 1993; Shull et al., 1992). In the complete knockout, mice die between the ages 2 to 4 weeks similar to the time window within which the TGF-β1$^{\Delta/\Delta}$ /Cre mice died. Hence the phenotype of the TGF-β1$^{\Delta/\Delta}$ /Cre mice was indeed reaffirmation of the fact that Cre mediated ablation of the gene encoding TGF-β1.

Hence, the generation of TGF-β1$^{flox/flox}$ mice has provided us with a model that will help in delineating the precise role of TGF-β1 in in various other cell lineages by using cell-type specific cre transgenics

G FUTURE PERSPECTIVES

Work in our laboratory has focused on gaining a better understanding of various aspects of the immune system and the TGF-β1$^{flox/flox}$ mice described here can indeed serve as valuable tools in this process.

Currently, there is an insufficient comprehension of the underlying mechanism resulting in the activation of T cells and their acquisition of an effector phenotype. Does TGF-β1 itself in a paracrine or autocrine manner govern the fate of T cells or are its effects on non-lymphoid cells like the thymic epithelial cells or lymphoid cells like antigen presenting cells in the end effecting T cells? This tissue inflammation in TGF-β1$^{-/-}$ mice can be partially inhibited by immune suppressants, but the severe autoimmune phenotype is not reversed by systemically injected TGF-β1, suggesting that immune cells rely on autocrine and/or intracrine TGF-β1 (Kulkarni and Letterio, 1998). This hypothesis is further corroborated by bone marrow transplantation studies in which the reconstitution of lethally irradiated wild-type mice with marrow of TGF-β1$^{-/-}$ donors leads to an autoimmune phenotype resembling that of a TGF-β1 $^{-/-}$ mouse. This transfer of phenotype, despite the presence of TGF-β1 produced by non marrow-derived cells of the wild-type recipient alludes to the important role of TGF-β1 producing immune cells. Also, there is a noteworthy slowdown in the development of autoimmunity in breeding studies that

place the TGF-$\beta1^{-/-}$ genotype onto background strains carrying immune system defects such as the SCID mouse, RAG1 or RAG2 knockout mice, nude mice, MHC class I and class II knockout mice (Letterio and Roberts, 1998). Furthermore, anti-CD4 treatment of TGF-β1 null mice also results in decreased inflammation and improvement in longevity (Kulkarni and Letterio, 1998; Letterio and Roberts, 1998). On close scrutiny of the underlying aberrant regulation of the immune response mechanisms in the TGF-β1 homozygous null mice, a role for TGF-β1 in controlling apoptosis and T cell selection patterns has emerged (Chen et al., 2001). As a result of increased levels of apoptosis and TCR mediated cell death normal negative and positive T cell selection in the thymus are disrupted. Furthermore, TGF-β1-dependent regulation of viability is independent from the TGF-β1 membrane receptor-Smad3 signaling pathway, but linked with a mitochondrial antiapoptotic protein Bcl-XL (Chen et al., 2001). So, TGF-β1 may protect T cells at multiple sites in the death pathway, particularly by maintaining the essential integrity of mitochondria. These data demonstrate a pivotal role for TGF-β1 in multiple stages of T cell apoptosis, selection, activation and clearance**.** Also, increased apoptosis of T cells occurs along with or perhaps is followed by massive inflammation. Apoptosis and removal of apoptotic cells are non-inflammatory events, involving immunoregulatory cytokines such as TGF-β1. Macrophages usually generate TGF-β1 during clearance of apoptotic cells which contributes to dampening of inflammation and its subsequent steps associated with phagocytosis. However, in the absence of requisite TGF-β1 release during phagocytosis, the balance of the immune response shifts toward the production of proinflammatory cytokines such as TNFα by phagocytically activated macrophages (Yang et al., 1999). Increasing our understanding of the bi-directional regulation of macrophage function will facilitate prediction of the ultimate outcome of modulating TGF-β1 levels in vivo.

Hence, the TGF-$\beta1^{flox/flox}$ mice have been bred to either CD4-Cre transgenics or to LysMCre transgenic mice. The TGF-$\beta1^{flox/+}$ / CD4-Cre^{+} transgenics or TGF-$\beta1^{flox/+}$ / LysMCre^{+} transgenics born from these breeding so far are healthy and have been interbred so as to get homozygous floxed TGF-β1 cre positive transgenics which delete specifically in T cells or macrophages respectively. The small pools of TGF-$\beta1^{flox/flox}$ / CD4-Cre^{+} transgenics or TGF-$\beta1^{flox/flox}$ / LysMCre^{+} transgenics mice generated have shown no phenotypic abnormalities and have developed normally till around 5 weeks of age. In the absence of a severe phenotype, and taking into consideration that the cre transgenics used are known to be specific and functional (Lee et al., 2001 cells) (Clausen et al., 1999), we can speculate that the loss of autocrine TGF-β1 in T-cells and macrphages can perhaps be compensated for. However, cell type-specific deletion of TGF-β1 demonstrated in these mice in order to validate this speculation.

Hopefully in the near future, the deletion of this gene exclusively in the T cell compartment or in macrophages will help elucidate further the role of this complex cytokine in immune regulation. In addition to this, the TGF-$\beta1^{flox/flox}$ mouse, when bred to various cell-type specific Cre transgenics should offer an opportunity to explicate *in vivo* the cell-type specific function of TGF-β1 producing cells in diverse developmental, physiological and disease states.

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CHAPTER 2

Generation of T-cell Specific Cre Transgenic Mice

A INTRODUCTION

Gene ablation and transgenic mice expressing constitutively active or dominant-negative molecules have provided valuable insights into the physiological roles of numerous genes. However, interpretations of the phenotype are often limited due to embryonic lethality, which impedes attempts to study gene function in older animals. Furthermore, analysis of these mice can be difficult due to compensation for the hypo- or hyper- activity of expressed genes or inappropriate expression of genes that can be cytotoxic or cytostatic. Hence to overcome these intrinsically confounding problems, the development of conditionally active proteins that can be spatially and temporally regulated is essential. Initial attempts were made to regulate the activity of transgenes by using mammalian control elements such as metallothionein promotor, heat shock promotors or steroid response elements (Yarranton, 1992). Although these systems were able to regulate gene expression, the inducing agents were at times toxic to mammalian cells, had pleiotropic effects and influenced the expression of endogenous genes. Also, in the absence of the inducers, often these systems had a high background expression of their target genes. In recent times, new approaches have developed to achieve regulated target gene expression in mammalian systems. As the field evolved, the use of recombinases to ablate or express specific genes at later stages of development or adulthood and hence evade embryonic lethality took root. Lakso et al. (Lakso et al., 1992) were the first to describe a method for controlled modulation of a transgene *in vivo* using site-directed recombination. In transgenic mice, using the Cre-loxP system they mediated activation of an SV40 T antigen under the control of the αA-crystallin promotor, resulting in the tumor production in the lens only.

A1 Cre/loxP System

Bacteriophage P1 recombinase, Cre is a member of the integrase family of site-specific recombinases that catalyze recombination between loxP DNA elements as a part of the normal viral life cycle (Sternberg and Hamilton, 1981; Sternberg et al., 1981). In a similar manner the Flp integrase of *Saccharomyces cerevisiae* mediates recombination between FRT (FLP recombination target) sites within yeast plasmids (Kilby et al., 1993). All members of the integrase family share a similar mechanism of action (Argos et al., 1986). In each case, the only requirements for DNA rearrangement are the integrase and the recombination sites. No additional cellular factors are necessary. The loxP site is a 34 bp DNA sequence comprising two 13 bp palindromes separated by an asymmetric 8 bp core. (Hoess et al., 1986; Hoess et al., 1982). The recombinase catalyses DNA strand exchange between two aligned recombination sites, resulting in deletion, duplication, integration, inversion or translocation of sequences, according to the orientation of the recombination sites and the number of molecules involved. During cleavage, Cre Tyr324 becomes co-valently linked to the 3'-phosphate, forming a DNA-phosphotyrosine intermediate (Gopaul et al., 1998). Interaction with a second loxP-bound Cre dimer allows nucleophilic attack of the 3'-phosphotyrosine in one loxP site by the free 5'-hydroxyl

of the other. This ligation reaction induces a structural rearrangement of the tetrameric complex, allowing the same reaction to occur on the second strand of each loxP site, thus completing the strand exchange reaction (Gopaul et al., 1998; Sauer and Henderson, 1988; Wierzbicki et al., 1987).

Recent structural models of site-specific recombinases from the integrase family have shed light on the three-dimensional nature of the recombination pathway (Van Duyne, 2001). Members of this family exchange strands between DNA substrates in a stepwise process wherein one pair of strands is exchanged to form a Holliday junction intermediate, and the second pair of strands is exchanged during resolution of the junction to products. Crystal structures of reaction intermediates in the Cre-loxP site-specific recombination system, together with biochemical studies support a strand swapping model for recombination (Van Duyne, 2001). It does not require branch migration of the Holliday junction intermediate in order to test homology between recombining sites in Cre-mediated recombination, individual loxP sites are bound by Cre homodimers (Mack et al., 1992). Primarily, only small-scale DNA rearrangements affecting single loci have been achieved by using the Cre-loxP system. However, chromosomal rearrangements like the deletion of large region can also be attained by this system. Deletions up to at least 60 centimorgan (cM) in ES cells *in vitro* and up to 2 cM *in vivo* have been achieved (Ramirez-Sollis et al., 1995; Zheng et al., 2000). Furthermore by generating acentric/dicentric chromosomes exchanges, whole chromosome loss can also be engineered (Lewandoski and Martin, 1997).

While studying a gene, therefore, one would desire to control not only the site of its expression or deletion but also the timing. In the Cre/loxP system, this requires the incorporation of two recombination sites within a gene of interest, and then cell type specific expression of Cre. Site or tissue specificity is determined by the choice of promoter driving Cre recombinase expression while temporal and quantitative control can be achieved by the use of inducible forms of Cre. This technology relies on mice derived from targeted embryonic stem cells(Thomas et al., 1987), with loxP sites flanking the region of interest that can be bred to homozygosity for the floxed allele, and crossed to Cre transgenics wherein Cre is under the control of a specific promoter. In this way, the activity of the gene can be modified in a limited range of cells at a particular developmental stage, leaving the genetic makeup of the rest of the animal unaltered. Breeding to distinct Cre transgenic mice would thereby enable the generation of a different temporal and spatial pattern of recombination, and hence perhaps different phenotypes (Buchholz et al., 2000; Collins et al., 2000; Su et al., 2000).

A2 Temporal Control of Cre

A requisite for temporal control of Cre-mediated recombination is generating inducible Cre recombinase (Logie and Stewart, 1995). To attain this, inducible fusion proteins containing the original Cre recombinase and a modified ligand-

binding domain have been generated. To avoid activation of the engineered Cre recombinases by endogenous ligands, such as circulating glucocorticoids, estrogen, or progesterone, mutated hormone ligand-binding domains have been used. In such cases, synthetic ligands bind the modified Cre recombinases but endogenous ligands cannot. Ligand binding produces conformational changes of the fusion protein and/or changes in the intracellular localization resulting in the targeting of Cre to the nucleus (Metzger and Feil, 1999; Sauer, 1998). An example of this is the fusion of the ligand-binding domain (LBD) of a mutated estrogen receptor to Cre This hybrid binds synthetic antagonists such as tamoxifen or its derivative 4-hydroxy-tamoxifen but not endogenous circulating estrogens (Metzger et al., 1995). The LBD allows sequestration of hybrid Cre in the cytosol by heat-shock proteins. Tamoxifen releases Cre, allowing recombination to proceed at a specified time in only tissues expressing Cre. Transgenic mice with ligand-activated Cre have demonstrated no detectable recombination in the absence of tamoxifen (Feil et al., 1996) and almost 100% recombination in Cre-expressing cells after 3 days of exposure (Brocard et al., 1997). However, one major limitation of using tamoxifen *in vivo* is its toxicity. Comparable hybrids have also been developed using modified progesterone and glucocorticoid receptor LBDs, responsive to synthetic steroid RU486 (a glucocorticoid receptor antagonist) and dexamethasone respectively but not endogenous progesterone or glucocorticoids (Brocard et al., 1997; Kellendonk et al., 1996). However, in some cases incomplete and ligand-independent leaky recombination have been observed (Kellendonk et al., 1999; Schwenk et al., 1998) resulting in constitutive rather than inducible activation. To obtain spatial and temporal restriction of the recombination process, the activity of the engineered Cre recombinase should be strictly dependent on the presence of the exogenous ligand. To minimize these problems and obtain a more stringent control of the recombination process new forms of tamoxifen and RU486-inducible Cre are now available with enhanced ligand sensitivity and low background activity, (Danielian et al., 1998; Imai et al., 2001; Indra et al., 1999; Kuhbandner et al., 2000; Wunderlich et al., 2001; Zhang et al., 1996).

A3 Inducible promotors

An alternative strategy to control the functional activity of the Cre recombinase is the use of an inducible promoter that directs expression of Cre. In an example of this approach, Cre is placed under the control of the Mx1 promoter, whose activity is inducible by interferonα/β as well as by pI-pC (an interferon inducer) *in vivo* administration of which results in Cre recombination (Kuhn et al., 1995). This has been used successfully to delete the low-density lipoprotein receptor-related protein in hepatocytes (Rohlmann et al., 1997). However, although deletion of the floxed gene was almost complete in liver and spleen, DNA recombination reached only 20 to 70% in other tissues, reflecting either poor bioavailability of the inducer in these organs or restricted tissue-dependent activity of the Mx1 promoter (Kuhn et al., 1995).

A4 Regulatory systems for gene expression

These are inducible systems that combine efficient spatial and temporal control of Cre recombinase expression. The two essential elements in these systems are a chimeric transactivator producing a transcription factor, which can be regulated and Cre under the control of a minimal promoter whose activity is dependent on the presence of the chimeric transcription factor (Saam and Gordon, 1999; St Onge et al., 1996; Utomo et al., 1999). The chimeric transcription factor contains three important components: (I) A domain able to bind a specific DNA-sequence, (II) a domain that regulates the ability of the receptor to bind DNA upon administration of an exogenous chemical (ligand) and (III) a domain with the potential to activate or repress transcription. To be able to regulate target genes *in vivo*, ideally the DNA-binding domain should not bind to DNA elements in the nucleus and alter endogenous gene expression. Also, the ligand-binding domain (LBD) should be stimulated by a nontoxic drug that retains biological activity at low concentrations without the interference of endogenous compounds. Also, to prevent "leakiness" in the absence of the inducer, the regulation mediated by the LBD should only occur in the presence of the ligand. However, it should be noted that this approach requires the *in vivo* combination of several independent transgenes or mutated loci. They include the transactivator construct, the acceptor construct (Cre recombinase in this case), and the transgene or locus to be modified (one in the case of an expression construct or two in the case of conditional gene inactivation). Examples of systems that facilitate transcriptional regulation of target genes are discussed below.

A5 Tetracyclin based regulatory systems

The tetracyclin (tet)-inducible system comprises of the tet transactivator protein (tTA), a chimeric protein containing the HSV VP16 activation domain fused to the tet repressor of Escherichia coli. In the absence of tetracyclin, tTA exhibits a high binding affinity to the tet resistance operator (tetO). In the presence of tet, a conformational change in the tet repressor prevents tTA from binding to tetO. When multiple copies of tetO are fused in front of a minimal promotor linked to a reporter gene, expression of the reporter gene is suppressed in presence tet. Conversely, constitutive expression of tTA causes gene activation in the absence of tet. The tetracyclin inducible system has been used in a variety of different studies such as stable mammalian cell lines, transgenic mice, tissue specific gene expression and adenoviral or retroviral regulated gene delivery (Fruh, 1995; Shockett et al., 1995; Shockett and Schatz, 1996). However, a disadvantage using this regulatory system is that high doses of antibiotics over a long time period may cause major side effects and that the slow clearance of tetracyclin in the bone and liver of transgenic animals is likely to cause problems in these animals. Also**,** the continuous expression of the tTA protein is toxic to eukaryotic cells (Gossen et al., 1993). To overcome these problems of toxicity a modified system contains a reverse transactivator (rtTA) that only binds tetO in the presence of doxycyclin, a derivative of tetracyclin. In this case to administration of doxycyclin induces expression of target genes (Gossen and

Bujard, 1992; Gossen et al., 1995). This inducible system has been used to control target gene expression in a mammalian system *in vivo.*

A6 Estrogen-based regulatory system

The main component of this inducible system is a chimeric transcriptional activator (GLVP) consisting of an N-terminal VP16 transcriptional activation domain fused to a yeast GAL4 DNA binding domain and a mutated human progesterone receptor (hPR) ligand binding domain (LBD) This chimeric regulator binds to a target gene containing the 17-mer GAL4 upstream activation sequence (UAS) in the presence of anti-progesterone, RU486 (Braselmann et al., 1993; Wang et al., 1997b). The combination of two different types of domains (VP16 and poly-glutamine stretch) into one chimeric molecule results in a further increase in transcriptional activation potency. Through mutational analysis, the original GLVP was modified to generate a more potent version of the RU486 inducible regulator GL914 VPc with a 19 amino acid deletion of the hPR-LBD (delta C19) and a C-terminally located VP16 activation domain. This new chimeric regulator can effectively activate target gene expression at a much lower concentration of RU486. The concept of RU486 regulated gene expression is not limited to gene activation. By replacing the VP16 activation domain with a KRAB transcriptional repression domain, we are able to achieve inducible repression of target gene expression. Individual functional domains within a chimeric protein can modulate each other's function depending on their relative positions within the molecule. This inducible system can be used to control target gene expression in a mammalian system *in vivo* (Wang et al., 1997a; Wang et al., 1997b).

A7 Ecdysone based regulatory system

This regulatory system consists of a truncated ecdysone receptor (EcR) attached to the VP16 activation domain (No et al., 1996) and uses the insect steroid hormone ecdysone as an inducer to activate target gene expression. The chimeric EcR must heterodimerise with its partner ultra spirical (USP) so as to be able to bind to its response element. Ultra spirical (USP) is the insect homologue of the mammalien retinoid X receptor (RXR). A hybrid response element containing a glucocorticoid response element and a type II nuclear receptor element (like RXR) enables the modified regulator to bind DNA (No et al., 1996). This artificial element called E/GRE is not able to bind any receptors other than the modified ecdysone inducible regulator which, by itself, does not bind any other response elements. Using this inducible system, ecdysone dependent gene expression has been demonstrated in stable cell lines and transgenic mice (No et al., 1996). However, a major disadvantage of this system is severe pleiotropic effects in mammalian cells, as the activation potency of this regulatory system depends on interaction with the retinoid receptor.

A8 IPTG based regulatory systems

This inducible system is based on the bacterial lactose repressor protein (lacR) (Gossen et al., 1993). The lacR binds in the presence of isopropyl beta-D-thiogalactoside (IPTG) to a specific DNA element, the lac operator (lacO) (Baim et al., 1991; Deuschle, 1989). Inserting the lac operon upstream to a TATA box modifies the promoter and allows it to regulate eukaryotic genes. The lac repressor is modified by excision of its repressor function and fusion to the activation domain of the herpes simplex virus protein (VP16) creating a lacI activator protein (LAP) that can drive the expression of lacO-bearing genes. IPTG causes the chimeric regulator to bind lacO and activate the TATA box coupled reporter gene. Murine cell lines containing both the LAP gene and a LAP-inducible chloramphenicol acetyltransferase (CAT) reporter gene have shown a 1200-fold induction of CAT activity within 24 h after addition of IPTG (Baim et al., 1991). Using this IPTG based regulatory system it is possible to achieve high induction of target genes, however, a disadvantage is that IPTG is toxic to mammalian cells, thereby severely limiting the scope of its application.

A9 CID based regulatory system

This regulatory system is based on "chemical inducers of dimerization" (CIDs) and their dimerization activity (Crabtree and Schreiber, 1996). The system is based on the ability of rapamycin to induce heterodimerization of two cellular proteins FKBP12 and FRAP. A DNA binding domain (ZFHD1) that binds to a novel DNA response element and is not recognized by any endogenous transcription factors was fused to FKBP12. To be able to activate target gene expression, the activation domain of p65 is added to the second partner, FRAP. (Rivera, 1996) and gives this inducible system the potency for various applications. However, the main disadvantage of this system is that rapamycin is involved in many signaling transduction pathways which therefore induce other side effects in eukaryotic cells.

Along with the systems discussed above, adenoviral delivery of Cre can also be used to determine the timing of recombination in certain tissues: after systemic delivery most recombination occurs within the liver, none in the central nervous system, and intermediate levels in other tissues (Akagi et al., 1997; Wang et al., 1996) However, immune responses to adenovirus limit long term Cre expression (Akagi et al., 1997).

B Objectives of this study

The aim of this part of the thesis was to establish two T-cell specific systems for conditional gene targeting using the Cre/loxP system (Gu et al., 1994; Kuhn et al., 1995; Rajewsky et al., 1996). Each one of these was designed to enable, in a T-cell specific manner, either the constitutive or the inducible expression of Cre. This can be a crucial tool in investigating the role of T-cell specific genes, complete inactivation of which may lead to embryonic lethality. Furthermore, it would facilitate the analysis of genes whose production cannot be tolerated constitutively or at certain stages of development. The CD3δ promotor was used to get T-cell specificity (Dave et al., 1997; Georgopoulos et al., 1990). For constitutive expression of Cre in T cells, a transgene was designed wherein Cre was put under the control of the CD3δ promotor. To enable the temporal and quantitative control of Cre expression I generated a transgene that utilized the CD3δ promotor as well as a binary transactivation system that controls the inducible expression of Cre at a transcriptional level. The main component of this steroid hormone receptor based method is a chimeric transcriptional activator (GLVP) consisting of an N-terminal Herpes Simplex Virus VP16 transcriptional activation domain fused to a yeast GAL4 DNA binding domain and a mutated human progesterone receptor (hPR) ligand binding domain (LBD). The mutated progesterone receptor is unable to bind progesterone, but can bind its antagonist mifepristone (RU486). This chimeric regulator binds to a target gene containing the 17-mer GAL4 upstream activation sequence (UAS) in the presence of anti-progesterone, RU486 (Braselmann et al., 1993; Wang et al., 1997b). The 17x4 multimer is placed upstream of a Thymidine kinase (TK) minimal promotor and Cre, with a nuclear localization signal was placed under the control of 17x4TK, Therefore, Cre expression was dependent on the presence of the specific chimeric transcriptional activator. It is important to note that in the case of binary system I placed both parts of the system on one transgene so as to generate a GLVP-CD3δ-Cre transgene. This would generate mice that carry the regulatory system in its entirety, thereby avoiding crossing between strains of mice, which carry one element each of the regulatory system.

C EXPERIMENTAL PROCEDURES

C1 T-cell specific promotor:

pNEz, 4.9 kb plasmid carrying the CD3δ enhancer and promotor was received from Dr. Vibhuti Dave, Fox Chase Cancer Center, Philadelphia, USA. From this the 1.1 kb CD3δ enhancer and promotor were excised as a Pst1/Kpn1 fragment (Genbank Accession number M18222).

C2 Transgenes:

C2.1 Constitutive expression of Cre: CD3δ-Cre transgene

For the constitutive expression of Cre, a CD3δ-Cre transgene was generated. In this transgene, Cre with a 5'splice substrate, a nuclear localization signal and bovine polyA was placed under the control of the CD3δ promotor. A maxiprep of the plasmid carrying the transgene was purified using a Qiagen kit. The DNA was checked by restriction digests and Cre was sequenced again. The final transgene was 2.6 kb in length and was excised from the pBSK vector backbone by first double digesting the plasmid with Kpn1 and Sac1, precipitating the DNA (sodium acetate/ethanol) and then digesting it again with Sca1. This way the 2.9 kb vector backbone was further cut into a 1 kb and 1.9 kb fragments, enabling precise gel purification of the 2.6 kb transgene.

C2.3 Inducible expression of Cre GLVP- CD3δ-Cre transgene

For transcriptional regulation I generated a vector using the GLVP binary transactivation system. This steroid hormone receptor based method combines a truncated form of the progesterone receptor hormone-binding domain with a yeast GAL4 DNA-binding moiety, and the transactivation domain of the VP16 protein. The mutated progesterone receptor is unable to bind progesterone, but can bind its antagonist mifepristone (RU486). In the presence of RU486 the fusion protein (GLVP) activates transcription through a multimer of the GAL4 DNA-binding site (called 17X4) that is placed upstream of a Thymidine kinase promotor (TK). It is important to note that in the case of binary systems I placed both parts of the system on one transgene. This would generate mice that carry the regulatory system in its entirety thereby avoiding crossing between strains of mice which carry one element each of the regulatory system.

Construct for the GLVP-CD3δ-Cre system:

As shown in Fig. 2 a transgene for regulating Cre using the GLVP system was designed as follows:

1. The modified transactivator GLVP was placed under the control of the CD3δ promotor so as to get T-cell specific expression.
2. Cre was placed under the control of 17x4TK that is a GAL4 DNA-binding site (17x4) sequence placed upstream of a TK minimal promotor.

The above-mentioned two parts of the binary system were placed on one transgene in opposite orientation. Following this, two copies of the chicken beta-globin insulator (Chung et al., 1997) were placed 5' and 3' of the Cre transgene. Finally a Westphal Stop Site (Lakso et al., 1992) was placed between the CD3δ promotor and the 17x4TK promotor. A maxiprep of the plasmid carrying the transgene was purified using a Qiagen kit. This DNA was checked by restriction digests and Cre was sequenced again. The final transgene was 11.8 kb in length and was excised from the vector backbone by digesting the plasmid with Asc1.

The two parts of the GLVP system were received from Dr. Bert O'Malley, Baylor College, Houston, USA. The Cre DNA that was cloned into the transgene had a nuclear localization signal and a splice substrate placed upstream to prevent aberrant splicing. It also had a bovine polyA (Pfarr et al., 1986). The Westphal Stop Site was excised out of the RAGE vector (pBS302) from GibcoBRL. This is a 1.5 kb stretch of DNA made up of a 550 bp C-terminal sequence of yeast His3 gene, 825 bp of the simian virus 40 poladenylation signal region, and a synthetic oligonucleotide where ATG is a false translation initiation signal and GTAAGT is a 5' splice donor site (Lakso et al., 1992). The sequence for this synthetic oligonucneotide is 5'GATCTGACAATGGTAAGTAAG-CTT3'. The insulator used is a DNA sequence at the 5' end of the chicken beta-globin locus, which in a specific orientation is capable of shielding a gene from the activating effects of a nearby locus control region. Most of the insulating activity lies in a 250-bp CpG island (core element), which contains the constitutive DNase I-hypersensitive site (5'HS4). It was received from Dr. Gary Felsenfeld, NIH, USA. The insulating activity of the core element is multiplied when tandem copies are used (Chung et al., 1997). Hence, I placed two copies of the insulators in the same orientation (5" to 3') on each side of the 17x4TK-Cre part of the transgene.

C3 T-cell GFP reporter line:

The pWSS-GFP plasmid was transfected using a liposome mixture, DMRIEC (GibcoBRL) into BW58, a murine thymoma line. The pWSS-GFP plasmid carries a neomycin selection cassette; hence the cells were selected using G418 (200 μg/ml). Stable transfectants were got after two weeks of selection. These cells were then tested by FACS analysis to check for the absence of any GFP expression and confirm that GFP expression was indeed blocked by the presence of the WSS. This line called BW58-WSS-GFP was then used to test the efficacy of the CD3δ-Cre plasmid.

Construct for GFP repoter:

The 1.5 kb loxP flanked Westphal Stop Site (WSS) was excised out of the pBS302 vector (GIBCO) and cloned into the pGFP-C1 plasmid (CLONETECH). This was done so as to place the WSS between the CMV promotor and the GFP gene in the in pGFP-C1. The resulting plasmid was called pWSS-GFP.

C3.1 Transfections:

The BW58-WSS-GFP line was used to check to check the CD3δ-Cre transgene. All transfections were done using the liposome-based reagent DMRIEC from GibcoBRL. The transfection protocol was as follows: 2 μl of DMRIEC and 1 μg of the appropriate DNA were first incubated for 30 min at room temperature. This DMRIEC and DNA mix was then added to a well containg $2x10^6$ cells in 0.5 ml of OPTIMEM (GibcoBRL), a serum free media. The cells were then incubated for 4 hours at 37^O after which another 1 ml of OPTIMEM + 15% FCS was added to each well. Cells were then incubated for another 24 to 48 hours and then either put under G418 selection to get the stable reporter line or collected and analysed using FACS ananlysis.

Molecular cloning was performed according to standard protocols (Sambrook et al., 1989). The bacterial strain used in all cloning experiments was DH5α that were routinely transformed with plasmid DNA by heat shock. Plasmid DNA was prepared using either the Plasmid Miniprep or Maxiprep kits from Qiagen, Germany. Standard restriction digest enzymes were used and ligation of DNA fragments was achieved using the Takara Ligation Kit (Takara, Japan).

C4 Cytofluorometric analysis:

FACS analysis was performed as following: $1\text{-}5x10^6$ cells were washed with PBS/1%BSA/0.01%sodium azide (PBS/BSA/azide) and surface stained for 20 min at 4^OC with the anti-hCD14 antibody in 20μl of PBS/BSA/azide. After a final washing step the cells were resuspended in PBS/BSA/azide and then analysed on a FACScan (Becton Dickinson). Cells that were analyzed for GFP expression (after Cre-mediated deletion of the Westphal Stop Site) were treated the same as described above, except that they were not stained with an antibody. For two color staining (GFP and CD14-PE), non-viable cells were excluded by adding propidium iodide (PI) (0.2μg/ml) during the analysis.

C5 Transgenic mice:

Using these transgenes, Dr. Kurt Reifenberg at the University of Ulm, Germany generated transgenic mice via pronuclear injections.

TABLE OF VECTORS USED:

Plasmids/ Vectors	**Description**	**Source**
pNN265	Plasmid from which Cre^{NLS}-bpA was excised.	Ralf Kuhn, University of Cologne, GEMANY
PNEz	4.9 kb plasmid from which the CD3δ enhancer and promotor were excised as a Kpn1/Pst1 fragment	Vibhuti Dave, Fox Chase Cancer Center, Philadelphia, USA
PAPCMV-GL1914c'VP-SV	Plasmid from which a 1.6 kb fragment was excised to get the chimeric transactivator GLVP.	Bert O'Malley, Baylor College, Houston, USA
17x4-TK-CAT	Plasmid from which a 1.6 kb fragment was excised to get GAL4 DNA-binding site (17x4) sequence placed upstream of a TK minimal promotor.	Bert O'Malley, BaylorCollege, Houston, USA
PJC13-1	Plasmid from which two copies of the insulator were excised as one contiguous 2.4 kb fragment (BamH1/Sal1 fragment)	Gary Felsenfeld, NIH, USA
RAGE vector (pBS302)	Plasmid carrying the 1.57 kb Westphal Stop Site flanked byloxP sites	GibcoBRL
pSL1190	Cloning vector with multiple cloning sites	Pharmacia Biotech
pSL1190	Cloning vector with multiple cloning sites	Pharmacia Biotech
pGFP-C1	Plasmid carrying GFP which can be used for fusing heterologous proteins to the C-terminus of GFP	CLONETECH

D RESULTS

D1 CD3δ-Cre transgene and GLVP-CD3δ-Cre:

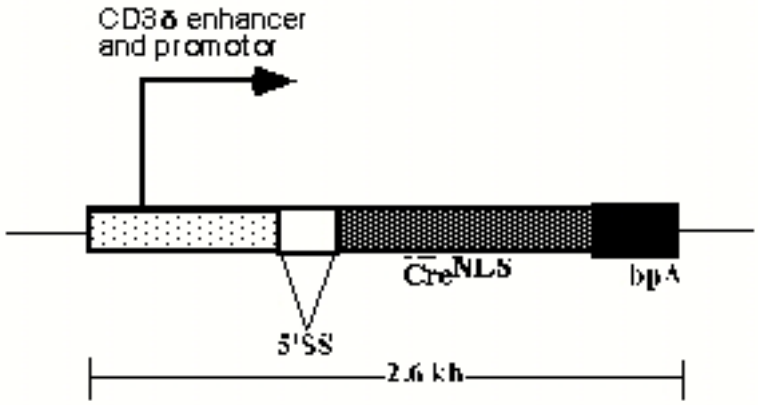
The CD3δ-Cre transgene (Fig.1) and the GLVP-CD3δ-Cre transgenes were constructed (Fig. 2) and checked by restriction digests for the correct orientation of each element and Cre were sequenced. A large-scale preparation of the transgenes was purified, verified by restriction digests and Cre was sequenced once again. The Qiagen Maxi-prep purified plasmids were then mailed to Dr. Kurt Reifenberg for pronuclear injections to generate transgenic mice.

D2 In vitro CD3δ-Cre mediated loxP deletion:

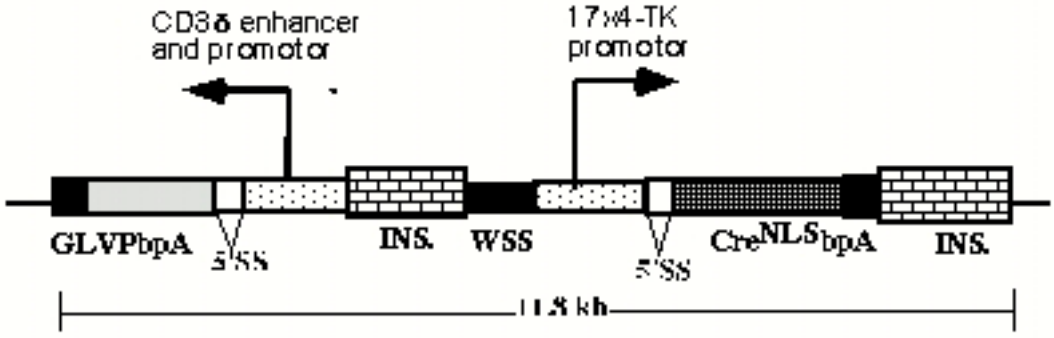
To test the CD3δ-Cre transgene *in vitro*, prior to making transgenic mice, I generated a T-cell reporter line using BW58 murine thymoma cells. This line carries a CMV driven GFP gene, expression of which is blocked by the presence of a loxP flanked Westphal Stop Site (WSS). BW58-WSS-GFP cells were transiently trasnfected with CD3δ-Cre. The cells were also transiently co-transfected with a plasmid carrying CMV driven human CD14 gene as a control. Cells were stained with an anti-hCD14 antibody conjugated to Phycoerytherin (PE). As shown in Figure 4 in the presence of an "empty vector" (the plasmid backbone without the CD3δ-Cre transgene) and hCD14, no expression of GFP was detectable although CD14 was (left hand upper panel). When the cells were transfected with CD3δ-Cre and CD14, both GFP and CD14-PE were detected (right hand upper panel), demonstrating the deletion of the loxP site flanked Westphal Stop Site and hence the transcription of the GFP gene by the CMV promotor. The low numbers of transfected cells is probably due to the fact that. getting a high transfection efficiency T-cell is rather difficult. Furthermore, these were transient transfections wherein cells were analysed within 48 hours of transfection. However, the data were able to clearly indicate that the CD3δ-Cre transgene was able to delete a loxP flanked DNA fragment.

FIG.1. Schematic depiction of transgenes for T-cell specific expression of Cre. For the constitutive expression of Cre (1A), a CD3δ-Cre transgene was generated. In this transgene, Cre with a 5'splice substrate, a nuclear localization signal and bovine polyA was placed under the control of the CD3δ promotor. The transgene was 2.6 kb in length. For the inducible expression of Cre, a GLVP-CD3δ-Cre transgene was generated (1B.) In this transgene a chimeric transcriptional activator (GLVP) was placed under the control of the CD3δ promotor. This chimeric regulator binds to the 17x4 multimer that was placed upstream of a Thymidine kinase (TK) minimal promotor. Cre, with a 5'splice substrate, a nuclear localization signal and bovine polyA was then placed under the control of 17x4TK. Both parts of this binary system were placed on one transgene, in opposite orientations. Cre, under the control of 17x4TK, was flanked by two copies each of the chicken beta-globin insulator. A Westphal Stopo Site was also placed in between the the two parts of the binary system to prevent any constitutive expression of Cre. The transgene was 11.8 kb in length.

A. CD3δ-Cre Transgene



B. GLVP-CD3δ-Cre Transgene



5'SS = 5' splice substrate
INS. = Insulators
NLS = nuclear localization sequence
WSS = Westphal stop site
bpA = bovine polyA

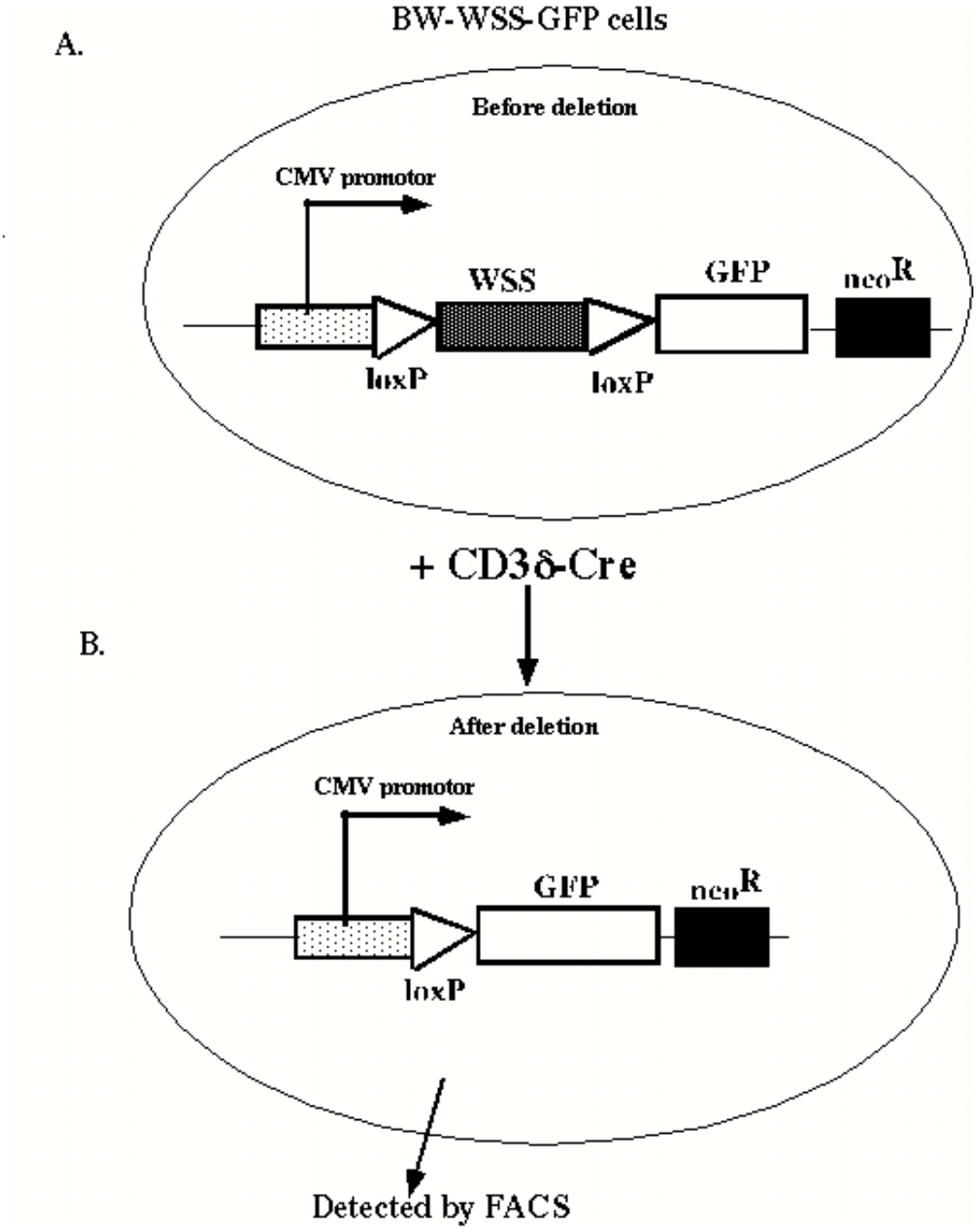


FIG.2. Schematic depiction of T-cell reporter line (BW-WSS-GFP) the GFP gene, expression of which is blocked by the presence of a loxP flanked Westphal Stop Site (WSS). In the presence of the Cre transgene the WSS is deleted leading to GFP expression that can be detected by FACS analysis.

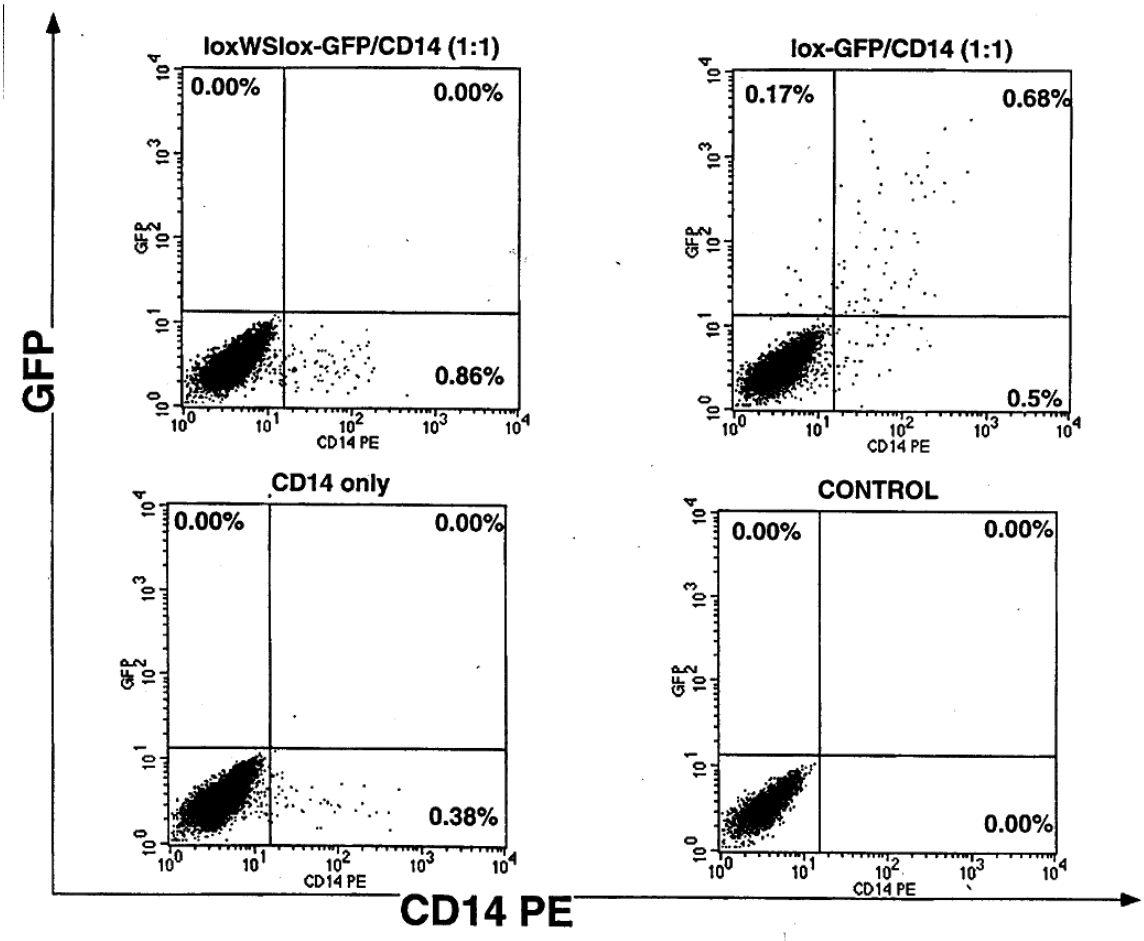


FIG.3. Cytoflourometric analysis of the T-cell reporter line (BW-WSS-GFP). To test for deletion of the WSS, the BW-WSS-GFP line was transiently transfected with the CD3δ-Cre transgene. The left hand upper panel shows cells transfected with (a.) only the control backbone vector not carrying the CD3δ-Cre transgene and (b) h-CD14 which is stained for with anti-CD14PE. The left hand lower panel shows the cells transfected with only hCD14, which is stained with anti-CD14PE. The right hand upper panel shows cells transfected with (a.) CD3δ-Cre transgene, and (b) h-CD14 that is stained for with anti-CD14PE. The right hand lower panel shows control cells that were treated in an identical manner as cells undergoing transient transfections, except no plasmid DNA was added to the cells.

D3 Generation of CD3δ-Cre transgenic mice:

Pronuclear injections of the CD3δ-Cre transgene were carried out by Dr. Kurt Reifenberg at the University of Ulm. A total of seventy-four offspring were born as a result of these injections. The genomic DNA got from tails of these mice was screened for the presence of Cre by Southern blot analysis. Christian Vosshenrich at the University of Cologne did this. He got a total of twenty-six Cre positive mice out of seventy-four mice that he screened. Bojan Polic at the University of Cologne, then bled these mice and tested by FACS analysis their Peripheral Blood Lymphocytes (PBL) for expression of Cre in T-cells only. He found a total of fifteen (six females and nine males) that looked promising for further analysis. These mice were then bred to mice homozygous for the IL-2 receptor gamma chain allele flanked by loxP sites. The offspring from this cross were analysed by Christian Vosshenrich, Bojan Polic and Hendrik Wildner for T-cell specific deletion. First, from the resulting offspring, 8 litters (70 pups) were screened for the presence of Cre. Twenty-five of these pups were positive for Cre and these were further checked for deletion of the gamma chain allele in a T-cell specific manner. The final results from these analyses have demonstrated that none of the CD3δ-Cre transgenic mice were T-cell specific. The details of these results are presented in Hendrik Wildner's "Diplom" thesis.

D4 Generation of GLVP-CD3δ-Cre transgenic mice:

Pronuclear injections of the GLVP- CD3δ-Cre transgene were also done by Dr. Kurt Reifenberg at the University of Ulm. A total of eighty-four offspring were born as a result of these injections. The genomic DNA got from tails of these mice was screened for the presence of Cre by Southern blot analysis. This was done by Christian Vosshenrich at the University of Cologne. He got a total of twenty-six Cre positive mice out of eighty-four mice that he screened. Bojan Polic at the University of Cologne, then carried out the further analysis of these mice to determine, inducible, T-cell specific, deletion of floxed alleles. He communicated to me that none of the mice he had tested demonstrated RU486 induced, T-cell specific, deletion of floxed alleles.

E DISCUSSION:

The ability to switch gene expression 'on' or 'off' in restricted tissues at specific times allows unique flexibility for studying individual gene function in a spatio-temporal manner. This part of the thesis describes an attempt at establishing two T-cell specific systems for conditional gene targeting using the Cre/loxP system by enabling either the constitutive or the inducible expression of Cre. The use of these systems in mice would result in the deletion of genetic material in T-cells only and at a specific time. In this way, one can avoid the complications of embryonic lethality and developmental compensatory changes. The CD3δ promotor was used to get T-cell specificity (Dave et al., 1997; Georgopoulos et al., 1990). For constitutive expression of Cre in T cells, a CD3δ-Cre transgene was designed. To enable the temporal and quantitative control of Cre expression I generated transgenes that used the CD3δ promotor as well as a binary transactivation system that controls the inducible expression of Cre at a transcriptional level. The main component of this steroid hormone receptor based method is a chimeric transcriptional activator (GLVP) consisting of an N-terminal VP16 transcriptional activation domain fused to a yeast GAL4 DNA binding domain and a mutated human progesterone receptor (hPR) ligand binding domain (LBD). The mutated progesterone receptor is unable to bind progesterone, but can bind its antagonist mifepristone (RU486). This chimeric regulator binds to a target gene containing the 17-mer GAL4 upstream activation sequence (UAS) in the presence of anti-progesterone, RU486 (Braselmann et al., 1993; Wang et al., 1997b). The 17x4 multimer was placed upstream of a Thymidine kinase (TK) minimal promotor. Cre, with a nuclear localization signal was placed under the control of 17x4TK whose activity is dependent on the presence of a specific chimeric regulator. In this binary system I placed both parts of the system on one transgene so as to generate a GLVP-CD3δ-Cre transgene. This would generate mice that carry the regulatory system in its entirety, thereby avoiding crossing between strains of mice, which carry one element each of the regulatory system.

To test for the efficacy of the CD3δ promotor to be able to drive Cre expression, the BW58-WSS-GFP reporter T-cell line was generated. This carries a CMV driven GFP gene, expression of which is blocked by the presence of a loxP flanked Westphal Stop Site (WSS). Transient transfection of these cells with CD3δ-Cre resulted in GFP expression, demonstrating the deletion of the loxP site flanked Westphal Stop Site and hence the transcription of the GFP gene by the CMV promotor. The CD3δ-Cre and GLVP-CD3δ-Cre transgenes were then injected into pronuclei of fertilized embryos. After screening by Southern blot analysis twenty-six transgenic founders were generated for each of the transgenes. The twenty-six CD3δ-Cre founders were tested for expression of T-cell specific expression of Cre in PBL's by FACS analysis as well as for T-cell specific deletion of a floxed allele. None, of the CD3δ-Cre founders were found to show T-cell specific Cre expression. Also, twenty-six of the GLVP-CD3δ-cre founders were tested. None of these were determined to be ideal candidates for inducible, T-cell specific deletion. The main problems faced according to Christian Vosshenrich Hendrik Wildner and Bojan Polic

who screened and analysed CD3δ-Cre mice were either no expression of cre at all or the lack of T-cell specificity. The CD3δ-cre transgenics behaved as "deleter" mice, showing expression of cre and hence deletion of a floxed allele in all tissues.

The problem of not attaining T-cell specific expression of cre may have to do with the CD3δ promotor that was used to generate these transgenic mice. However, the promotor was selected based on available information regarding its specificity as well as an already existing T-cell specific transgenic mouse model that had used the same promotor (Dave et al., 1997). The rationale behind specificity was as follows. A requisite towards T cell maturation is expression of the CD3 gene products which occurs very early during thymic differentiation and may even precede migration to the thymus. The CD3 complex is expressed in immature and mature T cells throughout T cell development.. The expression of the CD3δ gene in the T-cell receptor (TCR) complex is regulated by a T-cell-specific enhancer. A highly conserved 40-bp motif (element delta A) within the CD3δ enhancer is responsible for mediating its activity and specificity (Clevers et al., 1989). A 400 bp region 3' of the CD3δ gene functions as a transcriptional enhancer with strong specificity for T cells (Georgopoulos et al., 1990). Two elements in the CD3δ enhancer which mediate its T cell restricted function are element delta A and element delta B. Element delta A can function as an independent enhancer while element delta B has no independent function but augments the activity of element delta A. Together, delta A and delta B are sufficient to reconstitute the activity of the CD3δ enhancer (Georgopoulos et al., 1990; Georgopoulos et al., 1992). The promotor that was used to generate the transgenics described here had this enhancer essential for endowing it with T-cell specificity.

Another issue especially in the case of the GLVP-CD3δ-Cre transgenics may have to do with the insulators used. The core element in these has CpG islands. Although CpG islands are often associated with promoters of housekeeping genes, there is little evidence based on existing work that the core element in the insulator is a promoter. Furthermore, the insulator differs from a promoter in its ability to block the locus control region effect directionally (Chung et al., 1997). If indeed the core element does impart to the insulator properties of a promotor, in the case of the GLVP-CD3δ-Cre transgene this would result in constitutive expression of Cre in all tissues. Furthermore, the genetic background on which transgenics are produced is of great importance in conditional transgenic experiments as it may influence not only the primary pathology but also the efficiency of the conditional system itself. Evidence from previously carried out studies suggests that the regulation of the rtTA system differs between mouse strains (Zhu et al., 2001). In a similar manner, the efficiency of GLVP-CD3_-Cre recombination maybe strain dependent, although this has not been formally investigated.

In summary, despite having fifty-two founders for the constitutive and inducible systems, not a single T-cell specific transgenic mouse was obtained. Furthermore, the inducibility of the GLVP system was not well regulated leading to constitutive expression of Cre. It is always difficult to envisage *a priori* the

functionality of a transgene *in vivo* after it has integrated into a previously unpredictable region of the genome. The negative outcome of this part of the thesis reflects the rather complex nature of achieving the spatial and temporal control of Cre-mediated DNA recombination. That the strategy of placing both the parts of the GLVP system, the transactivator and the acceptor constructs within the same transgene as annunciated in this study is tenable has been recently demonstrated (Utomo et al., 1999). Perhaps the use of the next generation of chimeric transactivators from the O'Mailley laboratory will overcome the issue of leakiness and therefore result in a tightly regulated inducible system.

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G THESIS SUMMARY

The objective of this thesis was the conditional ablation of the murine transforming growth factor β1 gene as well as the generation of two T-cell specific systems for conditional gene targeting using the Cre/loxP system. The details of this work are described in two separate chapters of the thesis, a summary of which is as follows:

1. Generation of transforming growth factor β1 conditional knockouts

A murine system in which the expression of TGF-β1 can be regulated in a cell-type specific manner only, will _circumvent the lethal phenotype of the TGF-β1 total knockout mice as well as provide us with an opportunity to elucidate its specific activities in various systems. For the generation of the TGF-β1 $^{flox/flox}$ mouse the Cre/loxP system was used to flank part of the TGF-β_ gene by loxP sites (floxed) so as to get a TGF-β1 $^{flox/flox}$ mouse. TGF-β1 $^{flox/flox}$ mice were generated from targeted embryonic stem cells wherein via homologous recombination the exons coding for the mature peptide were floxed. The TGF-β_$^{flox/flox}$ mice are viable and capable of deleting the TGF-β1 gene *in vivo* when bred to deleter-cre transgenics. These TGF-β1 mice generated by *in vivo* Cre-deletion recapitulate the phenotype seen in the TGF-β1 total knockout. In order to look at the role of autocrine TGF-β_ in Tcells and macrophages, TGF-β1 $^{flox/flox}$ / CD4-Cre^{+} and TGF-β1 $^{flox/flox}$ / LysMCre^{+} transgenics mice have also been generated.

2. Generation of T-cell specific Cre transgenic mice.

The second aim of the thesis was to establish two T-cell specific systems for conditional gene targeting using the Cre/loxP system. Each one of these was designed to enable, in a T-cell specific manner, either the constitutive or the inducible expression of Cre. The CD3δ promotor was used to get T-cell specificity. For constitutive expression of Cre in T cells, a transgene was designed wherein Cre was put under the control of the CD3_ promotor. To enable the temporal and quantitative control of Cre expression a transgene was generated that utilized the CD3_ promotor as well as a steroid hormone receptor based binary transactivation GLVP system that controls the inducible expression of Cre at a transcriptional level. The main component of this steroid hormone receptor based method is a chimeric transcriptional activator (GLVP) consisting of an N-terminal Herpes Simplex Virus VP16 transcriptional activation domain fused to a yeast GAL4 DNA binding domain and a mutated human progesterone receptor (hPR) ligand binding domain (LBD). The mutated progesterone receptor is unable to bind progesterone, but can bind its antagonist mifepristone (RU486). In this system, Cre expression was dependent on the presence of the specific chimeric transcriptional activator. Both parts of the system were place on one transgene so as to generate a GLVP-CD3δ-Cre transgene. This would generate mice that carry the regulatory system in its entirety, thereby avoiding crossing between strains of mice, which carry one element each of the regulatory system.

Mice generated from both the above mentioned transgenes did not result in a T-cell specific and/inducible Cre transgenics and hence could not be used for attaining the T-cell specific ablation of TGF-β1 . However, concurrently as the CD3Cre mice were being tested in our lab, CD4Cre transgenic mice were generated in Dr. Christopher Wilson's laboratory at the University of Washington, Seattle. These were shown to be T-cell specific and therefore were used instead to generate TGF-$\beta1^{flox/flox}$ / $CD4Cre^{+}$ transgenics. Furthermore, to study the role of TGF-β1 in macrophages, TGF-$\beta1^{flox/flox}$ mice have also been bred to $LysMCre^{+}$ mice and TGF-$\beta1^{flox/flox}$ / $LysMCre^{+}$ transgenics have also been generated.

G1 ZUSAMMENFASSUNG

Das Ziel der vorliegenden Arbeit war die Generierung einer konditionalen knock out-Maus für Transforming Growth Factor-β1 (TGF-β1) und die Generierung von zwei transgenen Mauslinien, die eine konditionale T-Zell-spezifische Inaktivierung von Genen mit Hilfe des Cre/loxP-Systems erlauben.

1. Generierung einer konditionalen knock out-Maus für Transforming Growth Factor β1.

Eine zelltypspezifische Inaktivierung der TGF-β1 Expression kann zum einen die Entstehung des letalen Phänotyps der TGF-β1 knock-out Mäuseverhindern und ermöglicht zum anderen die Untersuchung der spezifischen TGF-β1 Funktionen in verschieden Zellarten. Für die Generierung der TGF-$\beta1^{flox/flox}$ Maus wurde das Cre/loxP System verwendet. Mittels homologer Rekombination in embryonalen Stammzellen wurden loxP-Sequenzen auf beiden Seiten des kodierenden DNA-Abschnitts für die reife Region von TGF-β1 eingeführt. Die TGF-$\beta1^{flox/flox}$ Mäuse sind lebensfähig und verlieren den von loxP-Sequenzen umgebenen DNA-Abschnitt, wenn sie mit transgenen Mäusen gekreuzt werden, die das Cre-Enzym in der Keimbahn exprimieren. Die aus dieser Kreuzung entstehenden TGF-$\beta1^{D/D}$ Mäuse rekapitulieren erwartungsgemäß den Phänotyp der TGF-β1 knock-out Maus.

2. Generierung von transgenen Mäusen, die T-Zell-spezifisch Cre exprimieren.

Das zweite Ziel der Arbeit war die Etablierung von zwei transgenen Mauslinien, die T-zell-spezifisch Cre exprimieren. Diese transgenen Linien können für die Generierung von konditionalen knock-out Mäusen verwendet werden. Dazu wurden zwei T-Zell-spezifische Expressionsvektoren entwickelt, die entweder eine konstitutive oder eine induzierbare Expression von Cre ermöglichen. Der CD3δ Promotor wurde verwendet, um eine T-Zell-spezifische Expression zu erhalten. Für die Generierung eines Cre-Expressionsvektors mit konstitutiver Expression in T-Zellen wurde das Cre-Gen unter die Kontrolle des CD3δ Promotors gebracht. Für eine zeitlich induzierbare und quantitative kontrollierbare Cre-Expression wurde der CD3δ Promotor und ein auf dem Steroid Rezeptor basierendes binäres Transaktivierungs-GVLP-System verwendet. In diesem System ist die Cre-Expression abhängig von der Anwesenheit eines spezifischen chimären Transkriptionsaktivators. Sowohl der CD3δ Promotor als auch das binäre Transaktivierungs-GVLP-System wurden in einen Vektor kloniert. Dieser GVLP-CD3δ-Cre Expressionsvektor, der das vollständige Regulationssystem als eine Einheit beinhaltet, vermeidet die Notwendigkeit der Kreuzung von transgenen Linien, die nur Einzelkomponenten enthalten.

Mit den oben genannten Vektoren konnte jedoch keine konstitutive oder induzierbare T-Zell-spezifische Expression von Cre erzielt werden, so dass für die T-Zell-spezifische TGF-β1 Inaktivierung CD4Cre Mäuse verwendet wurden. Diese

Mäuse zeigen eine T-Zell-spezifische Cre Expression und wurden kürzlich im Labor von Dr. Christopher Wilson an der Universität von Washington, Seattle generiert. Um auch die Rolle von TGF-β1 in Makrophagen untersuchen zu können, wurden zusätzlich Makrophagen-spezifische TGF-β1 knock out-Mäuse generiert (TGF-$\beta 1^{flox/flox}$/LysMCre+). Dazu wurden TGF-$\beta 1^{flox/flox}$ Mäuse mit LysMCre Mäusen gekreuzt, die Makrophage- spezifische Cre exprimieren.

H ACKNOWLEDGEMENTS

The work described in this thesis was carried out between December 1996 to December 1998 in Prof. Klaus Rajewsky's laboratory at the Institute for Genetics, University of Cologne and between March1999 and March 2002 in Prof. Manfred Blessing's laboratory at the University of Mainz. I thank both Klaus and Manfred for their advice and support. A Deutsche Forschungsgemeinschaft (DFG) Fellowship Graduate Program funded the work. I thank Prof. Leptin in Cologne and Prof. Streeck in Mainz for spearheading this program. I also very much appreciate the guidance and encouragement I received from Prof. Irmgard Foerster at the University of Munich and Dr. Ashok Kulkarni at the NIH. I would also like to thank Dr. Werner Mueller for his critical reading of this thesis and helpful suggestions.

I would like to take this opportunity to thank past and present members of the Rajewsky lab in Cologne. These include S.Casola, B. Clausen, G. Esposito, Y. Fukita, B. Hampel, R. Kuehn, I. Lieberman, B. Polic, G. Schmall, C. Schmedt, C. Uthoff-Hachenberg, C. Vosshenrich and A. Waisman. I very much appreciate the support I received from Kai Breuhahn, Borut Klopcic, Briggite Kulen, Thorsten Maas, Karin Nicole, Arnd Wilhelmi and Marina Snetkova. My sincere thanks to Dr. Kurt Reifenberg and the staff of the animal facility, specially Christiane Steiger for the responsible care of my mice. I am also grateful to Martina Protschka for helping with the breedings and excellent record keeping of the mice and to Amrit Mann for the long hours she spent helping me with histology. I would also like to convey my heartfelt gratitude to Christiane Amendt, whose professional help, encouragement and personal friendship made my stay in Mainz especially enjoyable and memorable.

I would also like to acknowledge Professor David Scott, my Master's thesis advisor, whose wonderful example as a mentor and continuing support helped me through this phase of my graduate work too. Most impotantly, I am deeply grateful to Amma and Didi in Delhi, my father and Leah and Jim Watson in Rochester, Julie and Al Hofmann in Mainz and Mukul. Their love and good wishes instilled the strength in me to make this possible.

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