SIGNAL TRANSDUCTION OF TRANSFORMING GROWTH FACTOR-BETA IN CYTOTOXIC T CELLS

Dissertation zur Erlangung des
naturwissenschaftlichen Doktorgrades
der Bayerischen Julius-Maximilians-Universität

vorgelegt von

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aus

Würzburg

Würzburg 2002

Eingereicht am:

Mitglieder der Promotionskommission:

Vorsitzender:
Gutachter: Prof. Dr. Walter Sebald
Gutachter: Prof. Dr. Georg Krohne

Tag des Promotionskolloquiums:

Doktorurkunde ausgehändigt am:

Contents

I Introduction

Cytokines are a highly variable type of protein mediators that act as growth factors, differentiation factors or activators of immune responses. In most cases they are produced by more than one cell type and may have effects on many cell types. Their actions are sometimes redundant, and some of them are able to influence each other. Cytokines may act in an autocrine or paracrine fashion by binding to high affinity receptors on the target cell surface, and certain types are reported to be produced in sufficient quantity to circulate and exert endocrine actions.

Thus, cytokines serve as important factors in embryonal development, the regulation of cellular growth and the immune system. Dysregulation of their production may lead to developmental defects, impairment of immunological defence mechanisms and tumor progression. On the contrary, cytokine application or inhibition in an ongoing disease may provide a strategy for the therapy of complex diseases such as cancer or autoimmune responses. Important mediators hereby are the T lymphocytes of the immune system, especially those that have the ability to kill other cells directly: The cytotoxic T cells. This stresses the importance of understanding the exact mechanisms by which cytokines such as TGF-β exert specific and defined actions on various cell types. The subject of this work is to understand TGF-β action on cytotoxic T cells by investigating TGF-β1 signal transduction pathways in these cells.

1 TGF-β and its superfamily

1.1 Overview

The TGF-β superfamiliy received its name from the member discovered first: Transforming Growth Factor-β (TGF-β) was discovered by virtue of its capacity to induce anchorage-independent growth of normal rat kidney cells and fibroblast cell lines, i.e. to induce transformation [Moses HL #216; Roberts AB #262]. It soon became apparent that the biological activity of TGF-β was not restricted to this single effect on cell growth, but rather exerts a variety of effects on many kinds of cells [Roberts AB #263; Saltis J #271; Dunker N #74]. According to sequence similarities, TGF-β family proteins can be grouped into four subfamilies: The TGF-β family itself, the BMP (Bone morphogenetic proteins)-family (often referred to as DVR-group: decapentaplegic (Dpp) and vegetal-1 (Vg1) related group), the

Activin family and further members such as the GDNFs (Glial-cell derived neurotrophic factors), MIS (Muellerian-inhibiting substance), Inhibin-α, Ebaf (endometrial bleeding-factor, othologue to lefty in mice), and several others identified predominantely in *Caenorhabditis elegans*.

1.1.1 TGF-β family

Five isoforms of TGF-β have been isolated so far: TGF-β1, -β2 and -β3 are expressed in mammalian cells [Derynck R #64; Derynck R #65], whereas TGF-β4 and -β5 are thought to represent the chick and *Xenopus* homologues of TGF-β1 [Burt DW #26; Burt DW #25]. The functions of TGF-β isoforms are pleiotropic and include cell cycle control, ECM (Extracellular matrix) production, chemotaxis, apoptosis and suppression of the immune system. TGF-βs are expressed in numerous tissues such as mesenchyme, connective tissues, endothelium, platelets, immune and bone cells [Sporn MB #291]. Effects of distinct isoforms are dependent on type and differentiation state of the target cells

1.1.2 BMP family

The BMP family is the largest family within the TGF-β superfamily. This family consists of more than 30 members that were cloned from primitive organisms like sea urchins up to mammals. Like all members of the TGF-β superfamily, BMPs are highly pleiotropic proteins. They induce bone and cartilage and regulate the ontogenetic development of many organs, including the nervous system, lung, kidney, gonads, and skin [Hogan BL;# 118]. In addition to several mammalian GDFs, the BMP group includes Drosophila 60A, Dpp and Screw, as well as several proteins isolated from *Xenopus*. BMP family members exert important functions in early development and morphogenesis. Dpp organizes dorsoventral patterning in *Drosophila* [Ferguson EL #81]. Vg1 is encoded by maternal mRNA and is localized to vegetal blastomeres in *Xenopus* embryos [Weeks DL #334]. Subsequently, local posttranscriptional activation of the Vg1 precursor protein leads to formation of axial mesoderm [Thomsen GH #309]. In mice, nodal is important for mesoderm formation and development of axial structures [Zhou X #368].

1.1.3 Activin/Inhibin family

Activins consist of two inhibin-β subunits forming homo-or heterooligomers. By association of one β-subunit with an α-subunit, the inhibins are formed. The terms “activins” and “inhibins” initially referred to the antagonistic activities observed in several biological

systems. Whereas activins were thought to stimulate synthesis of follicle-stimulating hormone, inhibins blocked follicle-stimulating hormone production [Sairam MR #269; Bottner M #18]. Activins and inhibins also regulate steroid hormone synthesis by granulosa cells, production of progesterone, gonadotropin releasing hormone and choronic gonadotropin in cultures of placental cells [Petraglia F #245]. Activins also play important roles in in erythroid differentiation [Eto Y #79] and induction of mesoderm in embryonic *Xenopus* explants [Smith JC #288].

1.1.4 GDNF family

GDNF was first isolated from a glial cell (B49) conditioned medium as a survival factor for cultured midbrain dopaminergic neurons [Lin LF #183]. In the presence of TGF-β, the protein is a potent trophic factor for CNS neurons [Unsicker K, Suter-Crazzola C., and Krieglstein K.;# 318]. In addition to GDNF, the family contains neurturin, persephin, and artemin/neublastin [Bottner M #18]. These proteins represent the most divergent subgroup within the TGF-β subfamily, as their C-terminal domains share only 20% sequence similarity with other members. In contrast to other TGF-β superfamily members, factors of the GDNF group signal through a receptor complex consisting of the tyrosine kinase c-Ret and a GPI-anchored α-receptor [Unsicker K, Suter-Crazzola C., and Krieglstein K.;# 318].

1.2 TGF-β signal transduction

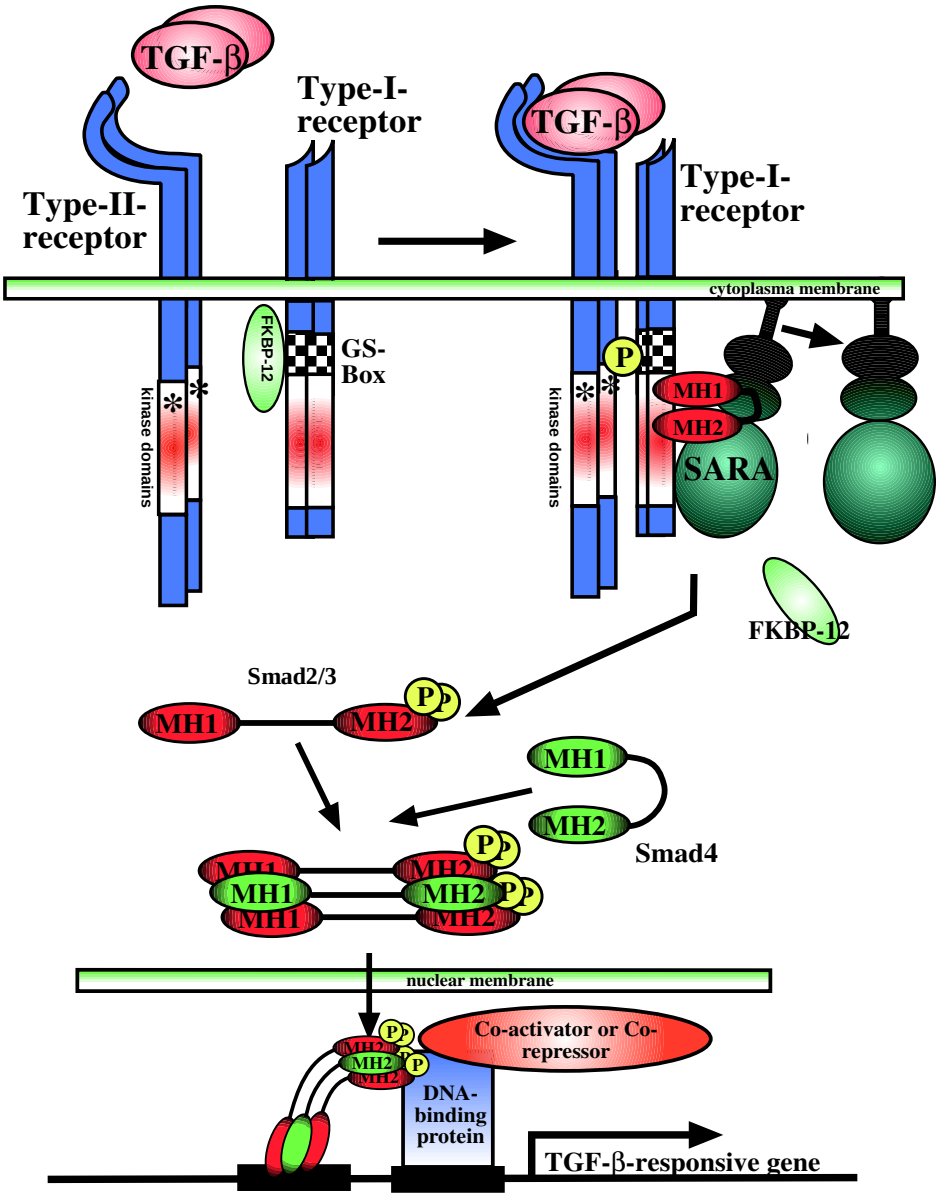


Figure 1: Signal transduction of TGF-β. The binding of TGF-β follows the sequential binding mode. TGF-β first binds to the type II receptor. The type I receptor still has FKBP-12 bound to protect the GS box from spontaneous phosphorylation. The type II receptor bears a constitutive active kinase domain (noted with an *) that phosphorylates the GS-box after the type I receptor has come into proximity of the type II receptor in the course of ligand binding. Then, FKBP-12 is released. The R-Smad (Smad2) is brought into contact with the now activated type I receptor by SARA, and is phosphorylated by the type I receptor at the SSXS motif of the MH2 domain. This leads to a conformational change in the Smad2 molecule that allows the release from SARA and from the receptor. Together with the common mediator Smad4, Smad2 forms heterooligomers. These oligomers then translocate into the nucleus, and drive gene expression together with coactivators/corepressors and DNA binding proteins.

1.2.1 The TGF-β molecule

TGF-βs are synthesized as prepro-proteins. A pro-domain is involved in correct folding, dimerization of subunits and regulation of activity [Kingsley DM;# 154]. Cleavage of the pro-domain from the mature part of the protein occurs at the RXXR-motif cleavage site in the intracellular compartment. The pro-domain, however, which is called latency-associated peptide (LAP), remains non-covalently bound to the bioactive C-terminal domain (110-140aa). An amino-terminal signal peptide targets the precursor molecule to the secretory pathway and the molecules are secreted as a non-covalent complex together with the LAP. Crystallography studies and NMR analysis of the bioactive domains of TGF-β2 and TGF-β1 [Daopin S #57; Schlunegger MP #276; Archer SJ #7; Hinck AP #115] have resolved the common structural motif in the TGF-βs.

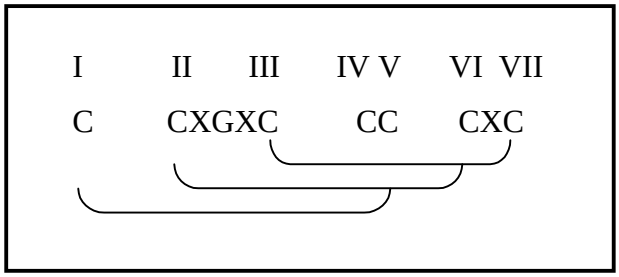


Figure 2: Seven characteristic cysteine residues within the mature domains of the monomeric subunits are highly conserved among the TGF-β family members. Six of these cyteines form three intramolecular disulfide bonds, which are in close neighborhood to form a structure named "cystine knot": For this structure, two of these disulfide bridges form a ring structure, which is crossed by the third cystine bond. The residual seventh cysteine forms the intermolecular cystine bridge that links the two monomers to a biologically functional dimer [Archer SJ #7; Hinck AP #115].

1.2.2 The TGF-β receptors

1.2.2.1 *Structure*

Cross-linking studies have shown that TGF-β binds to three different receptors. According to their size, they are named TβRI (53kD), TβRII (70-100kD) and TβRIII (200-400kD) [Massague J #202]. The varying molecular weights depend on the extent of receptor glycosylation. The type I and type II receptors, as illustrated in Figure 1 are responsible for signal transduction, while the type III receptor (betaglycan) might modulate ligand access to the signaling receptors [Cheifetz S #38; Lopez-Casillas F #189]. The type II receptor exists in two splice variants. The TβRII binds TGF-β1 and -β3 with high affinity while TβRII-B, containing a specific insert, is able to bind TGF-β1, -β2 and -β3 [Rotzer D #267]. Type I and

type II receptors belong to the serine/threonine class of transmembrane protein kinase receptors. Serine/threonine kinase receptors are glycoproteins composed of a short extracellular ligand binding domain, a single transmembrane domain, and an intracellular domain with serine/threonine kinase activity.

1.2.2.2 *Binding of the ligand*

In 1992, Wrana *et al*. initially proposed the model of sequential binding of TGF-β ligands to the receptor complexes [Wrana JL #343]. As illustrated in Figure 1, TGF-β first binds to the type II receptor and subsequently the type I receptor is recruited. In contrast to the TGF-β ligands, the BMPs appear to be more flexible with respect to the ligand binding mode. Upon binding of BMP-2 to complexes of BRI and BRII, the BRI kinase is activated [Gilboa L #92], and subsequently the R-Smads (Smad1, Smad5 or Smad8) are phosphorylated and associate with Smad4. Alternatively, another pathway exists where activated BMP-receptor complexes are able to induce signaling via the p38 MAP kinase pathway, independent of the Smad pathway [Miyazono K #213]. Both pathways are thought to be initiated by distinct BMP receptor complexes. Binding of BMP-2 to preformed receptor complexes (PFC) activates the Smad pathway, whereas binding to BMP-induced signaling complexes (BISC) induces the p38 MAP kinase pathway [Nohe A #228]. Here, BMP-2 could initially mediate homodimerization of BRI, the high affinity receptor. Then, it is thought to recruit the BRII. Consequently, the proposed ratio of BRI:BRII is suggested to be 1:1 in the preformed complexes and, in contrast, 2:1 in the BISCs [Nohe A #228].

1.2.2.3 *Receptor activation*

The crucial event in ligand-mediated receptor activation is the phosphorylation of serine and threonine residues in the sequence of the receptor known as GS-box, marked with black and white squares in Figure 1. This GS-box is a highly conserved region in the juxtamembrane part of the TGFβ–RI with a high frequency of glycine and serine residues. This phosphorylation event is catalyzed by the kinase activity of the type II receptor [Franzen P #85; Wieser R #339]. Upon phosphorylation of the GS-box, the type I receptor is activated and phosphorylates the signal transducer proteins, the Smads.

1.2.3 Receptor-associated proteins

FKBP-12 (view Figure 1) is an immunophilin that interacts with the type I receptor through an FK506-like Leu-Pro motif preceding the kinase domain. FKBP-12 inhibits phosphorylation of the type I receptor by the type II receptor, possibly by sterical hindrance, and may function to prevent leaky signaling in the basal cell state. FKBP-12 dissociates from TβRI after ligand-induced phosphorylation of TβRI by TβRII [Chen YG #46] [Wang T #331]. FKBP-12 is also proposed to act as a negative regulator of TGF-β receptor internalization [Yao D #362]. **Caveolin-1** interacts with the type I-receptor and mediates the localization of the receptor complexes to caveolae, thus interfering with Smad-mediated signal transduction [Razani B #258]. Proteins of the **SNX**-family (sorting nexin) of vesicle and receptor-trafficking adaptors also interact with TGF-β receptor complexes [Parks WT #239]. **GIPC** (GAIP-interacting protein, C-terminus) is a scaffolding protein for Gα subunits that associates with clathrin vesicles and interacts with the TβRIII, also enhancing Smad2 signaling [Blobe GC #15]. GIPC is a PDZ-domain protein, and these types of proteins are in general able to act as multiprotein-complex organizing centres [Harris BZ #106]. **SARA, Dab-2**, **Axin** and **TRAP1** are discussed in the Smad-interacting proteins-section. All receptor-associated proteins are reviewed in detail by Lutz *et al.* [Lutz M #193].

1.2.4 Smad proteins

1.2.4.1 *Gene organization*

Smads were first identified as the products of the *Drosophila Mad* and *Caenorhabditis elegans Sma* genes. The human genome encodes 8 Smad family members (Mad-homologues; MADH). The 8 human Smads have been mapped to four chromosomes. *MADH2, -4,* and *-7* are closely clustered at 18q21.1, a region that is frequently deleted in human cancers. Three are found on chromosome 15 (*MADH3, -5,*and *-6*), while *MADH1* and *MADH8* are located on chromosomes 4 and 13, respectively [Moustakas A #218]. Related proteins are known in the rat, mouse, *Xenopus*, zebrafish and the helmith *Schistosoma mansoni*. Smads are ubiquitously expressed throughout development and in all adult tissues [Flanders KC #83; Luukko K #194]. Smad2, -3, -5, and -8 are produced from alternatively spliced mRNAs. Functionally, Smads fall into three subfamilies: Receptor-activated Smads (R-Smads: Smad1, -2, -3, -5, -8) which become phosphorylated by the type I receptors; common mediator Smads (Co-Smads: Smad4) which oligomerize with activated R-Smads; the third group are the inhibitory Smads

(I-Smads: Smad6 and -7) whose expression is induced by TGF-β family members. The I-Smads exert a negative feedback mechanism by competing with the R-Smads for receptor interaction and by marking the receptors for degradation.

1.2.4.2 *Protein structure*

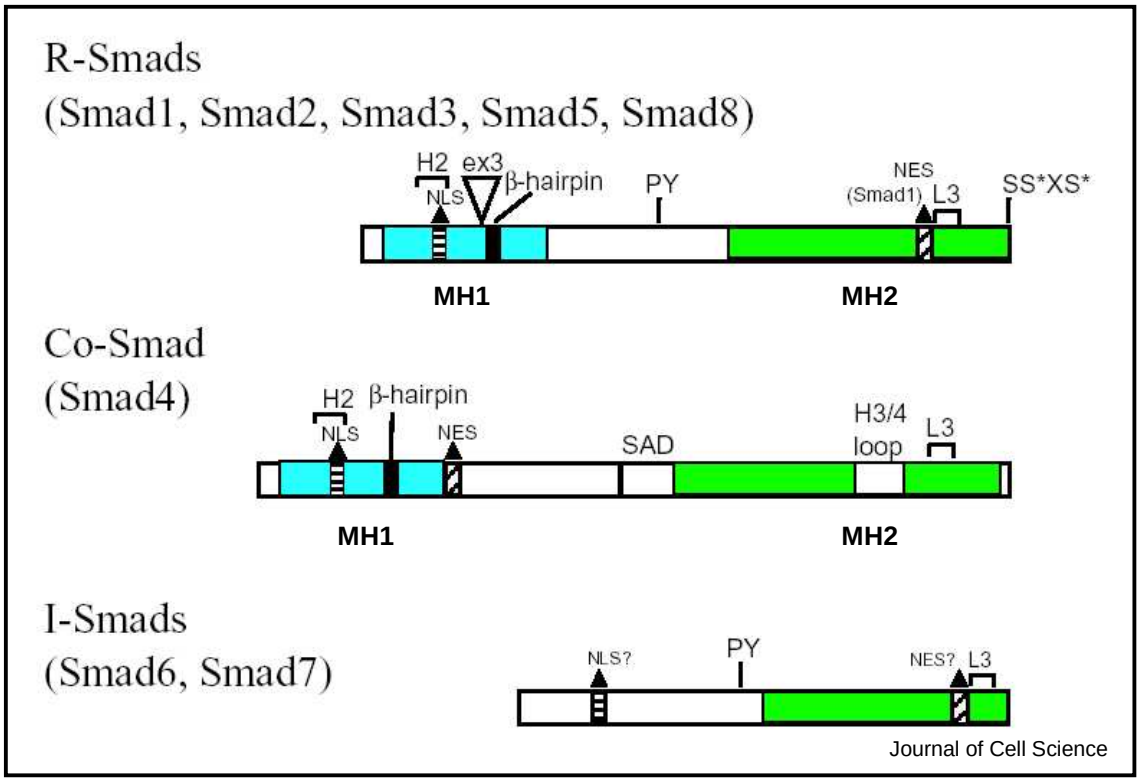


Figure 3: The Smad family. Scematic representation of the three subfamilies of Smads. The MH1 domain is coloured in blue and the MH2 domain in green. Selected domains and sequence motifs are indicated as follows: α-helix H2, L3 and H3/4 loops, β-hairpin, the unique exon 3 of Smad2 (ex3), NLS and NES motifs, the proline-tyrosine (PY) motif of the linker that is recognized by the Hect domain of Smurfs, the unique SAD domain of Smad4 and the SSXS motif of R-Smads with asterisks indicating the phosphorylated serine residues [Moustakas A #218].

As depicted in Figure 3, Smads have two conserved domains, the N-terminal Mad-homology domain (MH1) and the C-terminal MH2 domain, separated by a proline-rich linker region. In the I-Smads, the MH1 domain is replaced by divergent amino-termini. The MH1 domain regulates nuclear import and transcription by binding to DNA and interacting with nuclear proteins, and is highly conserved between R-Smads and Co-Smads. Sequence and structural analysis indicate that the MH1 domain is homlogous to the His-Me (histidine-metal-ion) finger family of endonucleases, and it might have evolved from an ancient enzymatic domain that lost its catalytic activity, but retained its DNA binding property [Grishin NV;# 100]. Certain R- and Co-Smads possess DNA-binding activity, although this interaction is of relatively low specificity. The crystal structure of the MH1 domain of Smad3 bound to an 8

base pair SBE (Smad-binding element) GTCTGTCT demonstrates that the MH1 domain forms a compact globular fold that uses a highly conserved 11-residue β-hairpin to contact the DNA in the major groove [Shi Y #280; Shi Y;# 278]. In Smad2, a 30aa insertion encoded by exon3 displaces the β-hairpin loop, providing the explanation for Smad2 lacking DNA binding activity.

The MH2 domain is highly conserved among all Smads, as indicated in Figure 3. Its structure contains several α-helices and loops surrounding a β-sandwich [Shi Y;# 278] and resembles the forkhead-associated domain (FHA), a phosphopeptide-binding domain common in transcription and signaling factors [Li J #178]. The first crystal structure of a Smad MH2 domain to be solved was that of Smad4 [Shi Y #279; Shi Y;# 278]. This study revealed that the MH2 domain is composed of 5 α-helices (H1-H5) and three loops (L1-L3) that enclose the β-sandwich. Figure 4 shows a model for the interaction of Smad2 with the TβRI, the GS region binding to the highly basic surface patch to provide affinity, and the L45 loop recognizing the L3 loop. Analysis of the trimeric Smad4 crystal showed that the loop-helix-region (L1, L2, L3 and H1) of one subunit makes extensive contacts with the three-helix bundle of another subunit, and that many of the conserved residues are located within the trimer interface. The MH2 domain regulates Smad oligomerization, recognition by type I receptors and interacts with several cytoplasmic adaptors and transcription factors.

The linker region located between MH1 and MH2 (Figure 3) is the target for several Smad-interacting proteins, and its main function is Smad ubiquitination. It thus contains a number of important peptide motifs. These include potential phosphorylation sites for MAP-Kinases and a nuclear export signal encoded by exon 3 of Smad4 [Pierreux CE #247; Watanabe M #333; Kurisaki A #162]. R-Smads and I-Smads also contain a conserved proline-tyrosine (PY) motif that mediates interaction with the WW domains in the Smad-interacting proteins Smurf1/2. The linker region of Smad4 also contains a Smad Activation Domain (SAD) that is required for transcriptional activation (Figure 3). Crystal structure of Smad4 fragments including the SAD and MH2 revealed that the SAD contacts a Smad4 specific sequence in the MH2 domain [Shi Y;# 278] [Qin B #253]. This stabilizes a glutamine-rich α-helical extension termed the TOWER which, together with the proline rich SAD, form a transcriptional activation surface.

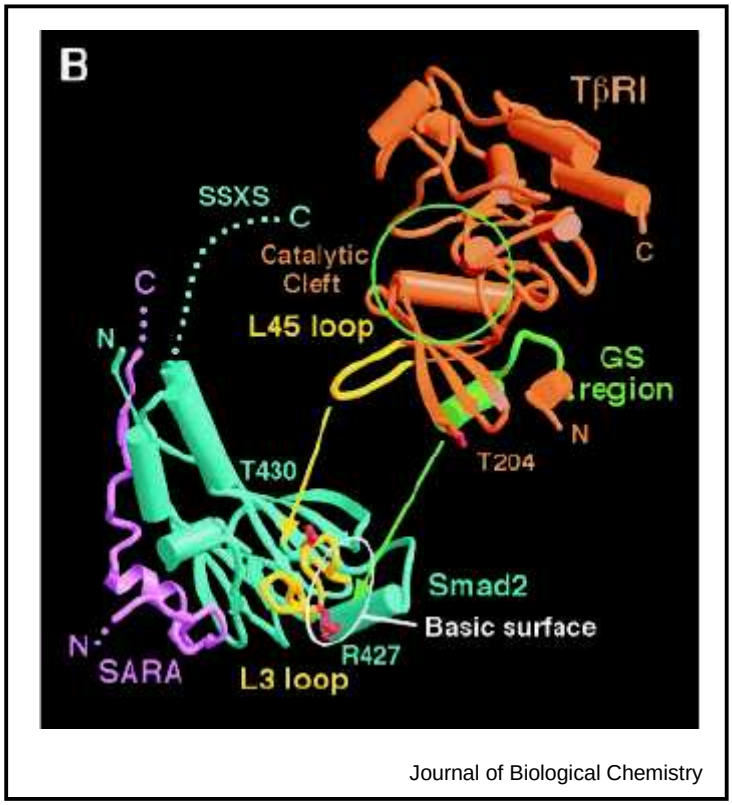


Figure 4: A proposed model for Smad2-TβRI interaction. TβRI, shown in red, interacts with Smad2, shown in green (still bound to SARA shown in blue), through two interfaces: With the phosphorylated GS region binding to the highly basic surface patch toprovide affinity, and the L45 loop recognizing L3 loop to provide specificity [Wu G #344]. The SSXS motif hereby comes into close proximity to the Catalytic Cleft.

1.2.4.3 *Smad-interacting proteins*

[Itoh S #134; Padgett RW #234]

	MH1	**linker**	**MH2**
Function	Nuclear import Cytoplasmic anchoring DNA-binding Transcription	Ubiquitination	Oligomerization Cytoplasmic anchoring Transcription
Regulatory phosphorylation	PKC (Smad2/3)	ERK (Smad1-3)	Type I receptors (Smad1,-2,-3,-5,-8)

	MH1	**linker**	**MH2**
Cytoplasmatic adaptors/effectors	Calmodulin (Smad1-4) Filamin (Smad1-6) Importin-β1 (Smad3)	Filamin (Smad1-6) ARIP (Smad3) β-Catenin? (Smad4) Microtubules? (Smad2-4) TAK1? (Smad6)	Axin, Axil (Smad 2/3) Dab2 (Smad2/3) SARA (Smad2/3) STRAP (Smad2,-3,-6,-7)
Ubiquitination adaptors/substrates	HEF1 (Smad3), N-terminal	Smurf1 (Smad1,-5,-7) Smurf2 (Smad2,-3,-7)	HEF1 (Smad3), C-terminal SCF subunits APC subunits
Transcriptional coactivators	PX HBV (Smad4)	Swift? (Smad1,2)	MSG1 (Smad4) P300/CBP (Smad1-4) P/CAF (Smad1-4)
Transcriptional repressors	HDAC? (Smad3) Hoxc-8 (Smad1)	Hoxc-8 (Smad1) SNIP1? (Smad 1,2,4)	SIP1 (Smad1-3,5) Ski (Smad2-4) SnoN (Smad2-4) TGIF (Smad2) Tob (Smad1,4,5,8)
Transcription factors	ATF2 (Smad3,4) Jun, JunB, JunD (Smad3,4) Lef1/Tcf (Smad2,-3) Sp1, Sp3 (Smad2-4) TFE3 (μE3) (Smad3,-4) VDR (Smad3) YY1 (Smad1,-3,-4)	p52 (NFkB)? (Smad3) Gli3 ΔC-ter (Smad1.4) HNF-4	AR (Smad3) BF-1 (Smad1-4) E1A (Smad1-3) ERα (Smad2-4) Evi-1 (Smad3) FAST (Smad2,3) Fos (Smad3) GR (Smad3) Lef1/Tcf (Smad2,3) Menin (Smad2,3) Milk (Smad2) Mixer (Smad2) OAZ (Smad1,-4) Runx/CBFα/AML (Smad1-4)

AR	androgen receptor, nuclear hormone receptor
ATF-2	activating transcription factor-2
BF-1	Brain factor-1 oncoprotein [Rodriguez C #265]
E1A	Early region of adenovirus binding transcription factor 1A
ERα	estrogen receptor-alpha [Matsuda T #205]
Evi-1	Evi-1 oncoprotein
FAST	Forkhead activin signal transducer
Gli3ΔC-ter	glioblastoma Kruppel zink finger transcription factor, deleted at C-terminus
GR	Glucocorticoid receptor, nuclear hormone receptor
HEF-1	Human enhancer of filamentation [Liu X #185] [Kardassis D #145]
HDAC	Histone deacetylase activity [Liberati NT #182]
Hoxc-8	homeobox c-8 transcriptional repressor
Lef1/TCF	lymphoid enhancer-binding factor1/T cell specific transcription factor1; high-mobility group transcription factor that normally binds β-catenin in response to Wnt signaling; interacts with Smad3/4 on the Xwin promoter [Labbe E #164]
Menin	multiple endocine neoplasia-type1 tumor suppressor protein [Kaji H #143]
Milk	Mix-1 related homeobox transcription factor[Hayes SA #109]
Mixer	homeobox transcription factor
MSG1	melanocyte specific gene1, transcriptional coactivator [Yahata T #352]
NFkB	B-cell specific nuclear factor binding to the intronic κ light chain; transcriptional enhancer
OAZ	olfactory factor O/E-1 associated zinc finger protein
p/CAF	[Itoh S #133]
pX HBV	pX oncoprotein of hepatitis B virus [Lee DK #170]
Runx	runt domain transcription factor [Ji C #139; Zaidi SK #364]
SIP	Smad interacting protein1, zinc-finger/homeodomain repressor [Verschueren K #321; Cacheux V #27]
SNIP1	Smad nuclear interacting protein1, association with Smad- and p300 transcriptional corepressor [Kim RH #152]
Sp1/Sp3	Specificity protein 1, zink-finger transcription factor [Feng XH #80; Pardali K #237]

STRAP WD domain protein, binds both MH2 of Smad7 and the typeI receptor [Datta PK #58]

Swift Xenopus BRCA1 C-terminal domain nuclear protein [Shimizu K #282]

TAK1 TGF-β activated kinase-1 [Yamaguchi K #354; Brown JD #22]

TFE3 transcription factor recognizing the immunoglobulin enhancer motif μE3

Tob Transducer of ErbB-2, APRO/Btg family of anti-proliferative factors [Yoshida Y #363]

VDR vitamin D receptor, nuclear hormone receptor

YY1 Yin yang 1, zink-finger transcription factor [MacLellan WR #197; Pulleyn LJ #252]

Accessory/scaffolding proteins

Several accessory or scaffolding proteins are reported to interact with the Smad proteins, one of major importance being **SARA** (Smad anchor for receptor activation). SARA is a cytoplasmic protein that specifically interacts with inactive Smad2 and the receptor complex (Figure 1 and Figure 5). This allows SARA to assist in the receptor-mediated phosphorylation of Smad2 by the type I-receptor [Tsukazaki T #314]. The interaction of Smad2 with SARA also inhibits its nuclear import [Xu L #350]. SARA contains a FYVE domain, and this motif is known to bind PI-3-phosphate. Thus SARA might anchor Smad2 to the inner leaflet of the cytoplasmic membrane or to endosomal vesicles. An other FYVE domain containing protein, **Hrs**, was also shown to assist Smad2 signaling and to cooperate with SARA [Miura S #212]. Several additional adaptor proteins (Figure 5) link Smad2/3 to the receptor complex and thus faciliate TGF-β signaling: Disabled-2 (**Dab-2**) and **Axin**, a negative regulator of Wnt signaling [Furuhashi M #87; Hocevar BA #117]. **Microtubules** also seem to play an important role in TGF-β signaling. They are able to anchor inactive Smads in the cytoplasm [Dong C #69], and disruption of the microtubule network leads to aberrant and constitutive activation of the Smad-pathway. It might be possible that the microtubules serve as highways of intracellular Smad movement. **ARIP**s (Activin receptor interacting proteins) associate with Smad2 and enhance Smad2-mediated responses to activin [Tsuchida K #313]. ARIPs, together with GIPC discussed earlier, are PDZ-domain proteins that are in general able to act as multiprotein-complex organizing centres. [Harris BZ #106]. **Filamin**, known as an actin binding factor and important scaffolding protein, is also observed to associate with the Smad proteins. Thus, it positively regulates Smad signaling [Sasaki A #274]. **TRAP1** (TGF-βRI associated protein-1) associates with Smad4 and is proposed to serve as a Smad4 anchor that

lies proximal to the receptor complex and might assist formation of R-Smad/Co-Smad hetero-oligomers [Wurthner JU #346]. The detailed integration of the TGF-β pathway into the cellular signaling network is reviewed in Lutz *et al*. [Lutz M #193].

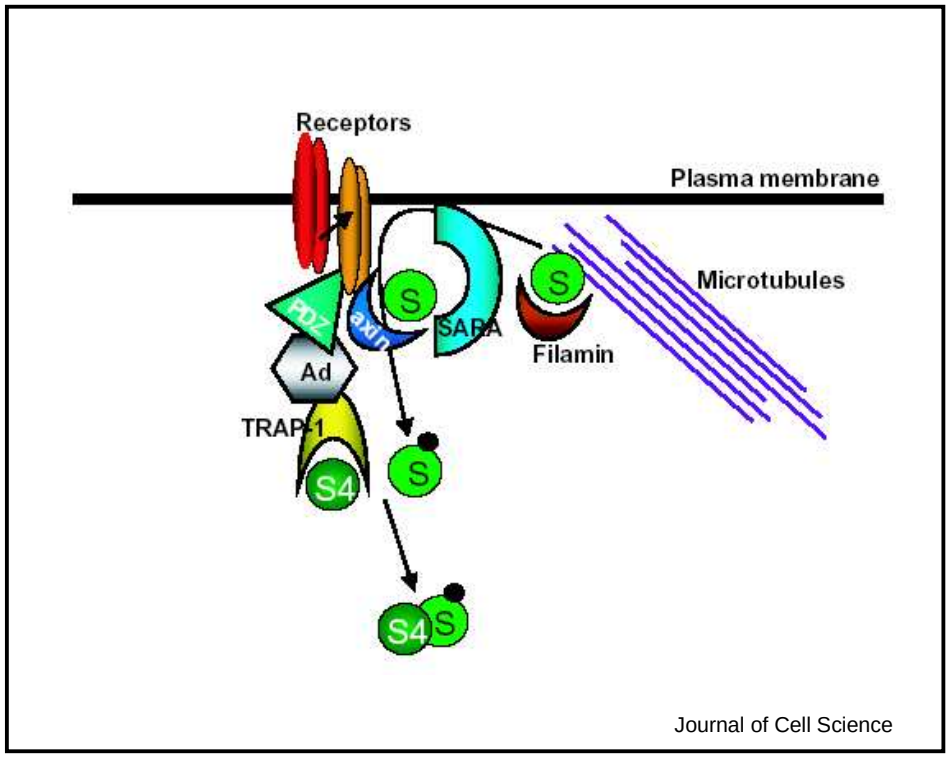


Figure 5: Smad signaling centres. Representation of early signaling events of the Smad pathway. A possible but not yet fully experimentally proven signaling scenario is shown, initiating at the plasma membrane. R-Smads (S) that are anchored to microtubules or filamin become mobilized towards SARA and the receptors. There, multiprotein centres are organized with the aid of scaffolding proteins containing PDZ domains such as ARIPs (PDZ), additional adaptors (Ad) and R-Smad and Smad4 (S4) anchors-activators such as axin and TRAP-1, respectively. This leads to R-Smad phosphorylation and R-Smad–Co-Smad oligomerization. A similar signaling scenario might become organized in early endosomes, immediately after receptor-mediated endocytosis [Moustakas A #218].

1.2.4.4 *Smad phosphorylation and activation*

Phosphorylation of the C-terminal serine residues in R-Smads by type I receptor kinases is a crucial step in TGF-β family signaling [Macias-Silva M #196; Abdollah S #1; Souchelnytskyi S #290]. The two most C-terminal serine residues are phosphorylated and, together with a third unphosphorylated serine, form an evolutionary conserved SSXS-motif in all R-Smads (Figure 3). Substrate specificity is determined by the L45 loop in the type I receptors, and, primarily, by the L3 loop in the MH2 domain of the R-Smads (see model in Figure 4). Thus, TGF-β and activin receptors phosphorylate Smad2 and Smad3, while BMP receptors phosphorylate Smad1, -5 and -8 [Chen YG #45]. The consequence of R-Smad

phosphorylation is the formation of heterooligomeric complexes together with the Co-Smad Smad4 as shown in Figure 1. Although phosphopeptide maps of ectopically expressed R-Smads are rather simple [Macias-Silva M #196; Abdollah S #1; de Caestecker MP #60], analysis of endogenous mammalian R-Smads reveals more than 10 different phosphopeptides [Souchelnytskyi S #290; Yakymovych I #353]. Thus, endogenous Smads might also be phosphorylated by other kinases, as further explained in the introduction section 1.2.5. The Smads contain phosphorylation sites for ERK [Kretzschmar M #158] and PKC [Yakymovych I #353]. Erk phosphorylates serine residues in the linker regions of Smad1 [Kretzschmar M #158], Smad 2 and Smad3 [Kretzschmar M #159]. Substitution of these serines by negatively charged residues inhibits nuclear translocation of Smads and thus TGF-β signal transduction. Upon treatment of cells with EGF or PDGF, phosphorylation of Ser240 was observed. PKC phosphorylates Smad2 at Ser47 and Ser110, and Smad3 at the analogous Ser37 and Ser70 [Yakymovych I #353]. Phosphorylation of Smad3 by PKC blocks DNA-binding, and thus transcriptional regulation. This inhibits TGF-β induced apoptosis and increases susceptibility of the cells to loss of contact inhibition. De Caestecker *et al*. demonstrated, that Erk phosphorylates Smad2 in responses to EGF or hepatocyte growth factor (HGF) at the C-terminal SSXS motif and thereby activates the Smad pathway [de Caestecker MP #60]. The molecular mechanism of synergistic activation of Smad2/3-mediated transcriptional responses by two other kinases, MEKK-1 and JNK, which phosphorylate unknown residues outside the SSXS-motif, remains unknown so far [Brown JD #22; Engel ME #77].

Phosphorylation of Smad4 has not been reported yet in mammalian cells, however, one of the Smad4 isoforms (Smad4b) is phosphorylated in *Xenopus*-cells. In contrast, Smad4a is not phosphorylated [Howell M #123; Masuyama N #204]. The biological relevance of this selective Smad4 isoform phosphorylation is not known up to now.

Phosphorylation of inhibitory Smads, Smad6/7, is observed, although the phosphorylating kinases are not characterized yet [Imamura T #126] [Pulaski L #251]. Smad7 gets phosphorylated at Ser249, and this phosphorylation depends on the proliferation status of the cells, but not on TGF-β receptor signaling [Pulaski L #251]. This phosphorylation regulates the transcriptional activity of Smad7, as Smad7 fused to the DNA-binding domain of GAL4 induced transcription from a reporter with mutated TATA minimal promoter in a Ser-249-dependent manner. Moreover, a reporter with the SV40 minimal promoter was inhibited by GAL4-Smad7, and this effect was also dependent on Ser-249 phosphorylation [Pulaski L #251].

1.2.4.5 *Smad oligomerization*

Biochemical and structural evidence suggest that the phosphorylated C-terminal tail of the R-Smads interacts with the L3-loop of an other Smads in a specific manner. This interaction is sufficient to cause oligomerization of the Smads [Correia JJ #53]. In an unphosphorylated state, the Smads exist as monomers. Upon phosphorylation, the R-Smads form Homo-oligomers, which then recruit one Smad4 protein as a Co-Smad [Kawabata M #148; Correia JJ #53]. Many evolutionary conserved residues are located in the loop-helix region and the three-helix bundle of the Smads. These regions make contacts and thus enhance the formation of hetero-oligomers [Shi Y;# 278]. In the cytoplasm, inactive Smads are intrinsically auto-inhibited through an intramolecular interaction between the MH1 and MH2 domains [Hata A #107] which prevents spontaneous oligomerization. Tada *et al.* report that Smad4 also contains a specific loop in its MH2 domain which is able to confer auto-inhibition in the absence of TGF-β signaling [Tada K #301]. Upon phosphorylation of the R-Smads by the TGF-β receptors, a conformational change is induced that relieves the auto-inhibition. In addition, this conformational change may expose hidden epitopes on the surface of the phosphorylated Smads, thus enabling the Smads to interact with Smad-binding proteins important for nuclear transport or the regulation of transcription. Several experiments indicate that the oligomerized Smad proteins may exist as trimers [Kawabata M #148; Shi Y;# 278]. However, also the existance of dimeric Smads (Smad2/4) has been reported [Wu JW #345]. Thus, even the Smad stoichiometry may be a means to confer specificity of TGF-β signaling.

1.2.4.6 *Nucleocytoplasmic shuttling*

All R-Smads, mammalian Smad4 and *Xenopus* Smad4a are located in the cytoplasm. In contrast, I-Smads and *Xenopus* Smad4b are found in the nucleus [Itoh S #135; Howell M #123; Masuyama N #204; Itoh F #132]. It is described that the nuclear translocation of R-Smads is independent of Smad4, whereas translocation of Smad4 seems to require the presence of activated R-Smads [Liu F #184; Hoodless PA #120]. The MH1 domain of all Smads contains a typical lysine-rich motif that, in the case of Smad1 and Smad3, has been shown to act as NLS (nuclear localization signal) [Xiao Z #347; Xiao Z #348]. In Smad3, C-terminal phosphorylation results in conformational changes that expose the NLS. Thus, importin-β1 may bind and can now mediate nuclear import, as described in [Xiao Z #347; Kurisaki A #162] and Figure 6 (pathway2). Smad2, in contrast, containing the same lysine-rich sequence in its MH1 domain, is released from SARA after receptor-mediated

phosphorylation and then translocates into the nucleus, as decribed as pathway1 in Figure 6. This nuclear transport mechanism is independent of any cytosolic factor, but requires the MH2 domain [Xu L #350]. This difference between Smad 2 and Smad3 is due to the presence of exon3 in the MH1 domain of Smad2, which is lacked by Smad3 [Kurisaki A #162].

The identification of alternatively spliced Smad4 variants in *Xenopus* led to the discovery that Smad4 constitutively enters the nucleus. Its cytoplasmic localization in untreated cells is thus due to active nuclear export [Pierreux CE #247; Watanabe M #333]. This export is mediated by a unique leucine-rich NES (nuclear export signal) localized in the linker region of Smad4. The export is catalysed by the exportin Crm1, as describes as pathway3 in Figure 6. Smad4 therefore continuously shuttles between the nuclear and cytoplasmic compartments of the cell. Smad1 has also been shown to be imported ligand-dependently and exported constitutively [Xiao Z #348]. The constitutive export is dependent on a NES contained in the MH2 domain. This NES is N-terminal to the L3 loop (conserved in all Smads), but seems to be active only in selected Smad proteins. Smad2/3 also exits the nucleus, but only after prolonged treatment with TGF-β [Pierreux CE #247]. So far, the NES remains undiscovered in Smad2/3.

I-Smads are constitutively subjected to nuclear import and are in turn exported from the nucleus upon TGF-β/BMP signaling [Itoh S #135; Itoh F #132].

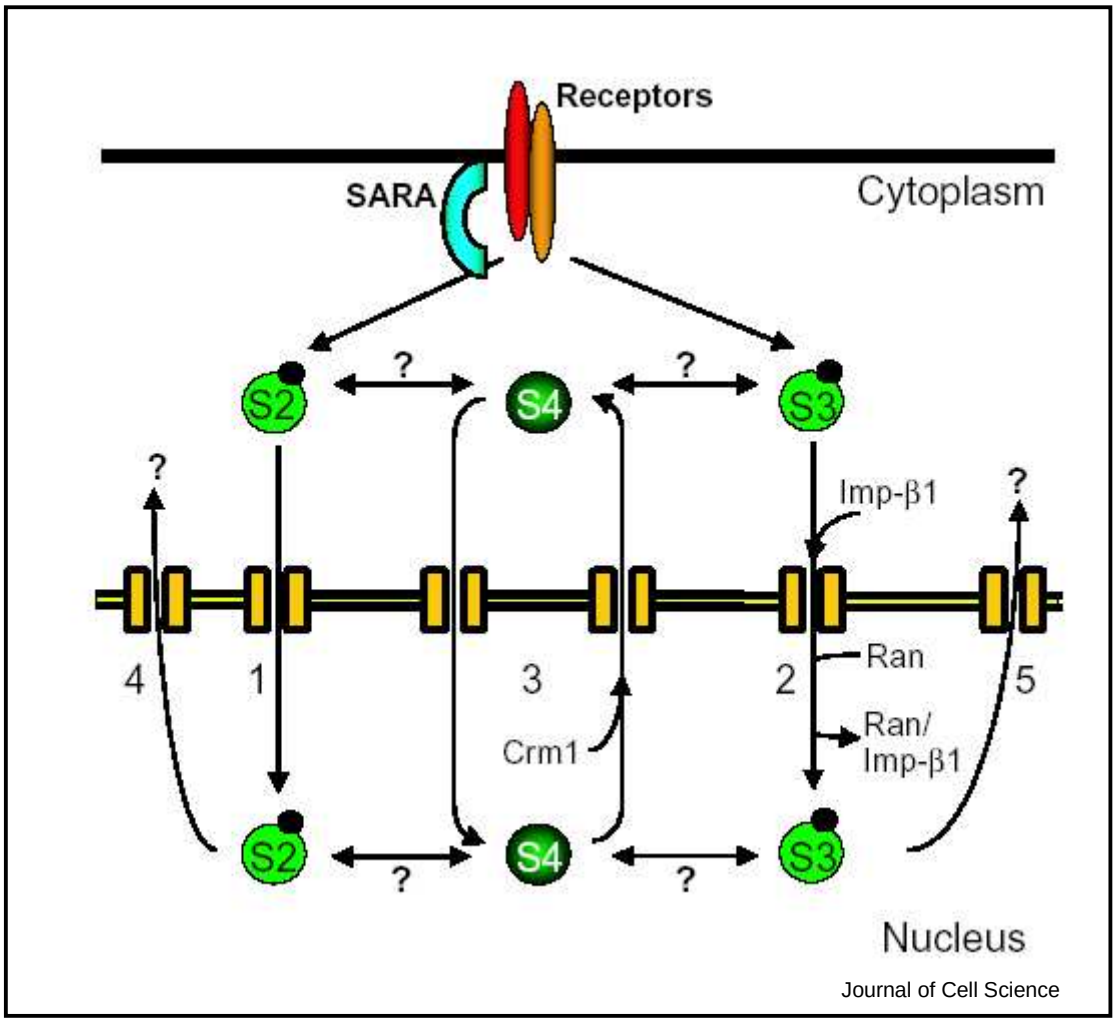


Figure 6: Smad nucleocytoplasmic shuttling. The five parthways that are shown are Smad2 nuclear import after release from SARA (pathway 1), Smad3 nuclear import mediated by importin-β1 and Ran (pathway 2), Smad4 shuttling mediated by the exportin Crm1 (pathway 3), and the putative Smad2 (pathway 4) and Smad3 (pathway 5) export pathways marked with question marks. Horizontal double arrowhead lines indicate the possibility of Smad oligomerization in the cytoplasm or nucleus. Smad2 (S2), Smad3 (S3) and Smad4 (S4) are shown as monomers and the actual stoichiometry of the Smad complexes is not depicted. Small black circles indicate the phosphate modifications of the common SSXS motif [Moustakas A #218].

1.2.4.7 *Nuclear signaling*

Transcriptional activity is observed with most Smad proteins [Itoh S #134; Massague J #203] [Pulaski L #251]. Smad3 and Smad4 can bind directly, though with low affinity, to SBEs (Smad-binding elements). SBEs contain the minimal sequence motif 5´-CAGAC-3´, which is bound by the Smad β-hairpin structure in the MH1 domain. Additional sequences may contribute to the binding, such as the α-helix 2 in the Smad3 protein [Kusanagi K #163].

Smad2, in contrast, is not able to bind directly to the SBE due to its unique exon3-coded sequence [Yagi K #351]. Smad2/4 complexes for example bind to Fast-1, which directs them to Activin responsive elements (ARE) of the Mix.2 promoter in Xenopus [Chen X #43; Chen X #44]. In this complex, Fast-1 and Smad4 contact the DNA, and Smad2 binds both Smad4 and Fast-1. Likewise, Smad2 associates with transcription factors of the Mix-family, in particular Mixer, Milk and Bix3 [Germain S #91]. Smad3 and Smad4 associate also with GC-rich motifs in the promoter regions of certain genes [Labbe E #165]. Likewise, BMP-responsive Smads bind to SBEs through conserved β-hairpin structures [Li W #180]. In addition to activation of gene expression, Smads are also able to induce the repression of certain genes. However, here the DNA binding motifs do not resemble SBEs or GC-rich sequences [Alliston T #5; Chen CR #39]. Smad3 is reported to associate with histone deacetylase (HDAC) through its MH1 domain [Liberati NT #182]. Alternatively, Smads can interact with with Co-repressors that recruit HDAC, including TGIF [Wotton D #341] and the proto-oncogens SnoN and Ski [Sun Y #298; Sun Y #299; Wang W #332; Liu X #186]. SnoN is an interesting example for a nuclear feedback loop [Stroschein SL #294]. The basal levels of SnoN keep TGF-β responsive genes in a repressed state. Upon TGF-β signaling, the Smads are entering the nucleus and then target SnoN for ubiquitination followed by degradation. This mechanism allows the Smads to activate transcription of target genes. One gene activated by Smads is SnoN itself. As soon as the nuclear Smad-levels decline, SnoN is repressing the genes again.

The transactivation activity of Smads is located in the MH2 domain and is mediated by association with p300 and P/CAF (p300 and CBP associating factors) families [Itoh S #133; Itoh S #134] (Figure 1). A crucial role in transactivation is proposed for Smad4, involving the unique Smad-activation domain (SAD). This domain is thought to enhance the association of Smad4 with CBP/p300 [de Caestecker MP #61; Chacko BM #32].

1.2.4.8 *Smad degradation*

A common mechanism for the regulation of protein levels is their subsequent ubiquitination and degradation [Ciechanover A #49]. In the first step of this enzymatic cascade, the highly conserved 76aa protein ubiquitin is activated and attached to an E1 (ubiqitin activating enzyme) through a thiolester bond. The ubiqitin is then transferred to a E2 (ubiquitin-conjugating enzyme) which functions together with an E3 ligase to transfer ubiquitin to the substrate. The polyubiquitinated substrate is then recognized by the 26S proteasome and is degraded. E3 ubiquitin ligases play a critical role in this process by recruiting specific substrates into the ubiquitin machinery. One class of E3 are directly involved in the ubiquitin transfer and contain a HECT ubiquitin ligase domain. However, a second class of E3s are multicomponent complexes that do not contain intrinsic ligase activity but rather act to specifically recognize and recruit target proteins in a complex containing an E2 enzyme [Hershko A #112].

One class of Hect-domain ubiquitin ligase of the E3 type has been shown to interact with the Smad proteins and is supposed to induce their degradation: The Smurfs. The basal level of Smad1 is regulated by Smurf1 in the cytoplasm [Zhu H #369] of unstimulated cells. C-terminally phosphorylated Smad2 is thought to be the target of Smurf2 after exerting its function in the transcriptional complex [Wotton D #341; Zhang Y #366]. Likewise, nuclear Smad3 is degraded after completion of its transcriptional role, mediated however though SCF/Roc1 E3 ligase [Fukuchi M #86]. The degradation of Smad3 takes place in the cytoplasm, with the SCF/Roc1 complex serving as nuclear export machinery. Protesomal degradation of Smad4 is observed in tumor cells, but the specific mechanism remains unsolved [Xu J #349].

1.2.5 Cross-talks of TGF-β with other signaling pathways

1.2.5.1 *MAP kinase pathways*

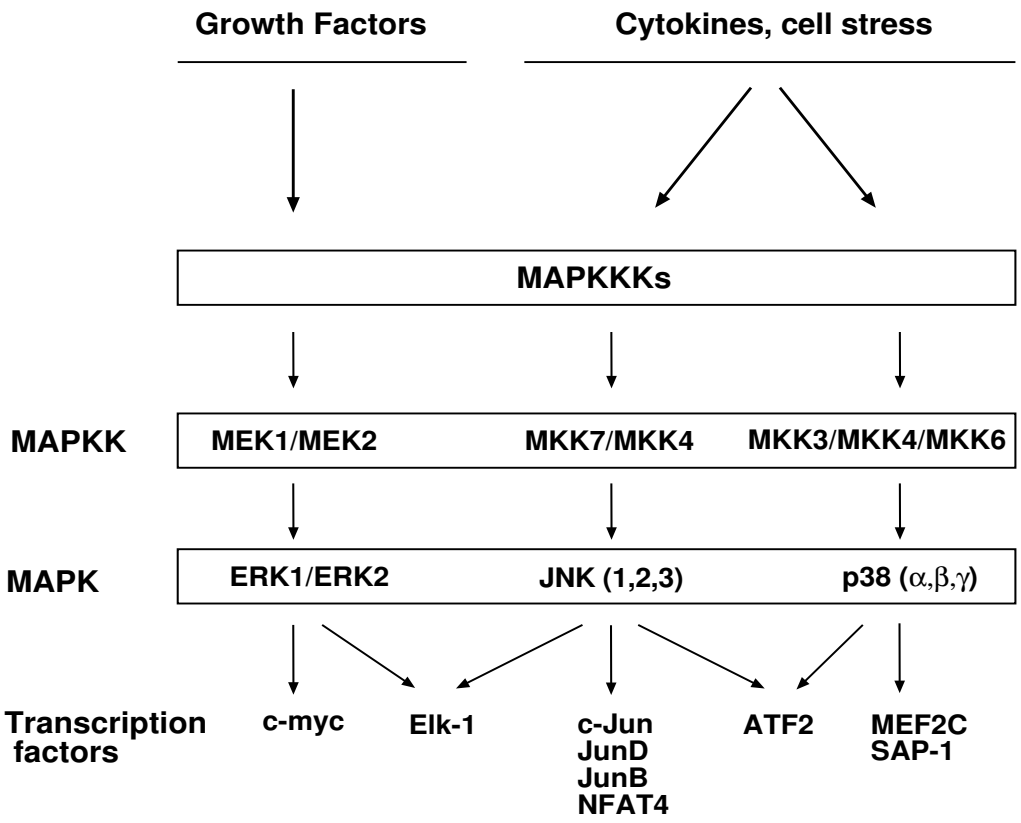


Figure 7: MAP kinase pathways. MAP kinases can be activated either by growth factors or cytokines and cellular stresses. The signal transduction ultimatively leads to the activation of transcription factors such as c-myc, elk-1 and c-jun.

Multiple crosstalk pathways influence TGF-β signal transduction, including the MAP kinases [Wrana JL;# 342]. At least three MAP-kinase pathways are known, as illustrated in Figure 7. The extracellular signal regulated kinases 1 and 2 (ERK1/2), the c-Jun NH2-terminal kinase (JNK) pathway and the p38 pathway [Robinson MJ #264], which all are involved in T cell activation [Su B #295; DeSilva DR #66]. JNK is activated in response to a variety of cellular stresses, DNA damage and cytokines [Minden A #211; Pombo CM #249; Verheij M #320]. JNK are activated by phosphorylation of tyrosine and threonine residues catalyzed by

SEK1/MKK4 [Moriguchi T #214; Cuenda A;# 56], which in turn can be activated by TGF-β-activated kinase-1 (TAK1) [Ip YT #130]. The JNK pathway has also been shown to play important roles in the activation of T cells, as knockout mice lacking individual *jnk* genes and transgenic mice overexpressing a dominant negative JNK protein have demonstrated a role for the JNK pathway in thymocyte apoptosis, T cell proliferation and T cell differentiation [Dong C #70; Sabapathy K #268]. It is known that Smad signaling can converge with the JNK pathway on the level of AP1-containing regulatory elements JNK [Zhang Y #367; Liberati NT #181]. It has been shown in several cell types that TGF-β activates ERK through the Ras pathway (Figure 8) [Reimann T #259; Axmann A #11].

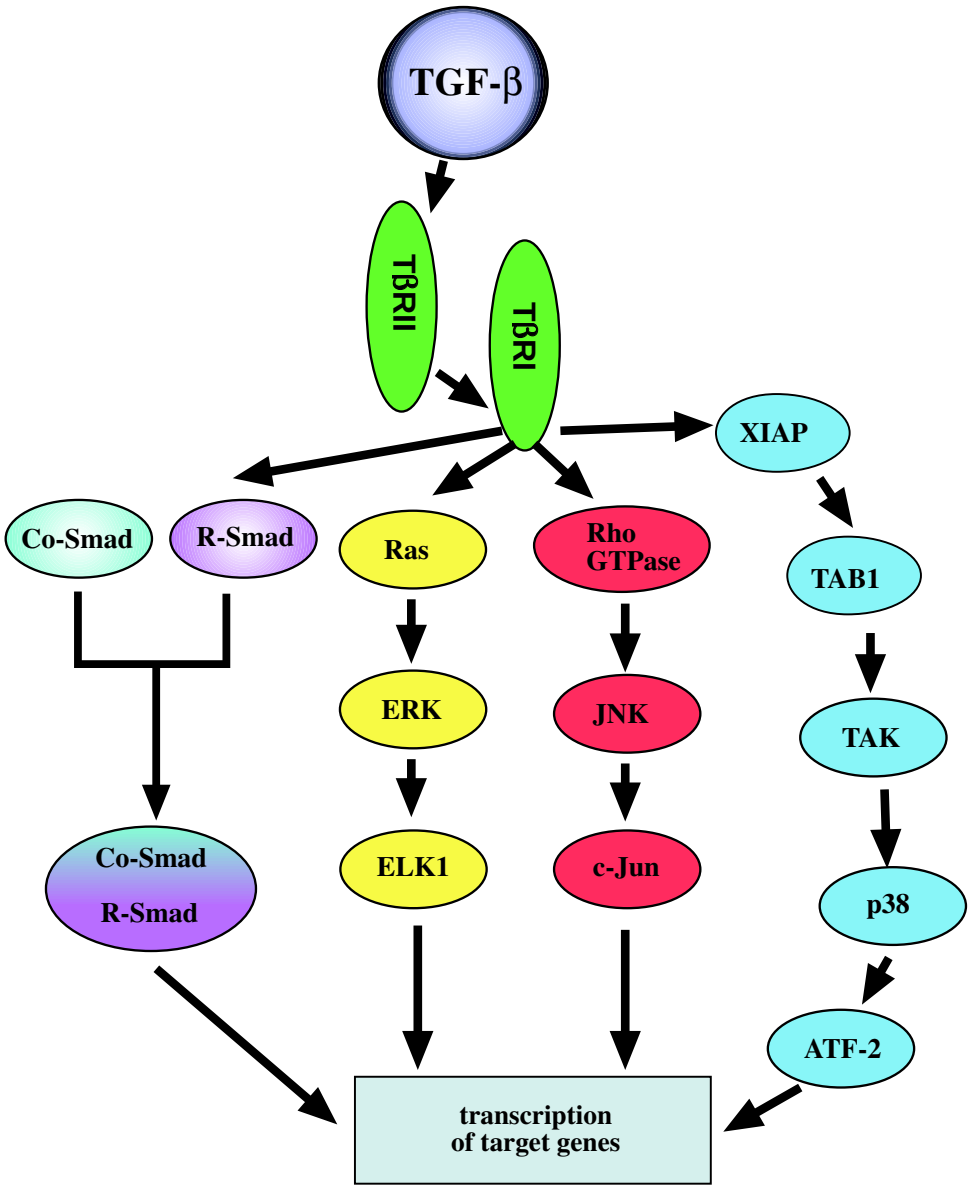


Figure 8: Schematic drawing of TGF-β crosstalks. As discussed in the text, TGF-β is able to activate other pathways in addition to the Smad pathway. TGF-β may induce transcription of target genes via c-Jun, ELK or ATF-2. Abbreviations: ERK: Extracellular regulated kinase; JNK: c-Jun-N-terminal kinase; XIAP: X-linked inhibitor of apoptosis protein; TAB1: TAK-1 binding protein; TAK: TGF-β activated kinase; ATF-2: Activating transcription factor-2

1.2.5.2 *Pathways other than MAP kinases*

Several crosstalks distinct from the MAP kinase pathways may influence the TGF-β signal transduction on the level of cytosolic factors or nuclear factors. Activation of Smads by phosphorylation at the SSXS motif is for example induced by treatment of cells with EGF

(Epithelial growth factor) or HGF (Hepatocyte growth factor) [Kretzschmar M #158; de Caestecker MP #60] (Figure 9). Although some of these phosphorylation events most likely occur at MAPK or ERK phosphorylation sites present in the linker region, activation of Smad2, possibly via phosphorylation at the SSXS-motif, was also observed [de Caestecker MP #60]. Overexpression of constitutively activated mitogen-activated protein kinase kinase kinase-1 (MEKK-1) in endothelial cells may also lead to activation of Smads [Brown JD #22]. So far it remains unsolved if Smad activation by these pathways involves serine-threonine kinase receptor signaling or occurs through some as yet uncharacterized mechanism.

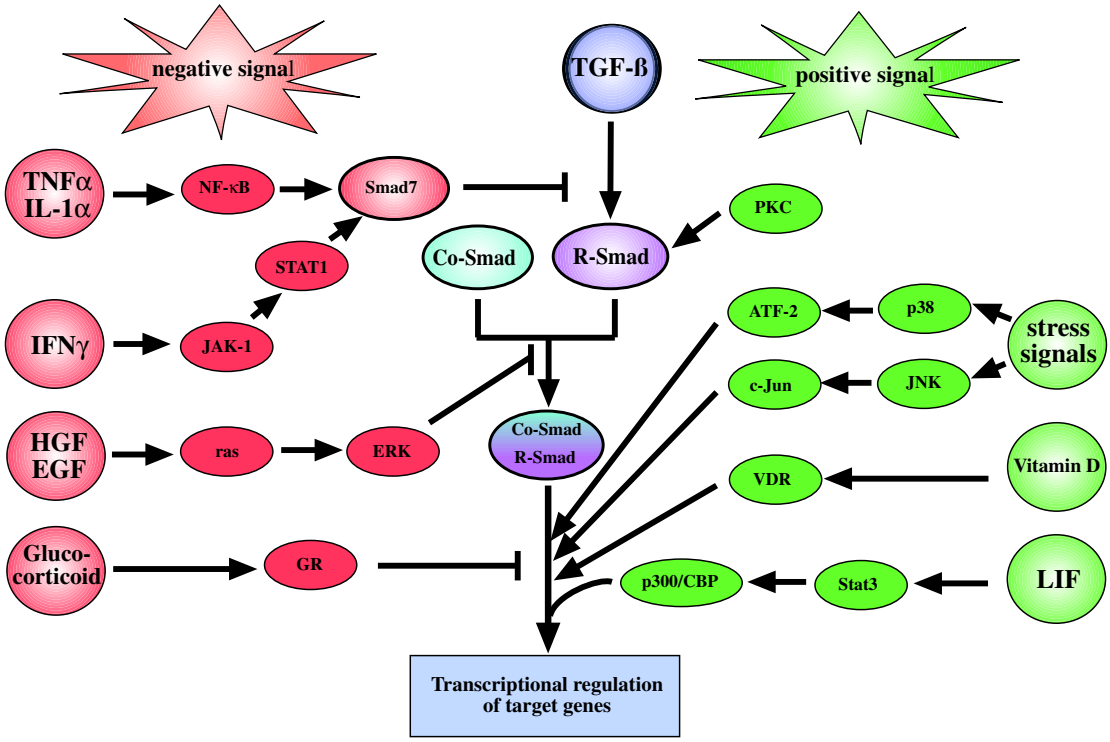


Figure 9: Signal transduction pathways influencing the Smad pathway. Some pathways negatively regulate the Smad pathway, while others positivly influence Smad signal trnasduction. Cytokines like TNFα or IFNγ block the Smad pathway early at the level of R-Smad phosphorylation. Other growth factors like EGF inhibit the Smad oligomerization. Most pathways, including negative regulation by glucocorticoid and positive regulation by stress signals or vitamin D, function at the level of transcriptional regulation.

In the nucleus, the Smads associate with the transcriptional coactivators CBP and p300. These function as coactivators for a plethora of other transcription factors, including CREB, AP1, steroid hormone or nuclear receptors, STATs (signal transducers and activators of transcription), MyoD, NFκB and p53. CBP may thus provide a link between the Smad

pathway and the other signal transduction pathways. Indeed, the viral oncogene E1A, that binds to a region of CBP close to the Smad binding region, blocks Smad-CBP interaction and thus blocks TGF-β signaling [Topper JN #310]. Furthermore, Smads may also modulate STAT signaling by interacting with p300. Smad signaling can also interact with signals for the vitaminD receptor (VDR) (Figure 9). Analysis of a vitaminD response element (VDRE) linked to a reporter gene revealed that the VDRE was responsive to TGF-β2 [Yanagisawa J #356]. In the absence of vitaminD, a VDRE reporter gene was unresponsive to TGF-β, but in the presence of vitaminD, which activated the promoter, TGF-β induced additional activity. Consistent with these observations, the Smad3 MH1 domain interacted weakly with the VDR in the absence of vitaminD. Smad4 seems not to bind the VDR and does not affect the activation of the VDRE, which suggests that enhancement of vitaminD-regulated promoters by TGF-β may not require Smad4 activity. How Smad3 functions to enhance vitaminD-dependent transcription is still unclear, but because Smad3 can bind to CBP/p300, it may act by bringing more coactivators into the DNA-bound complex [Wrana JL;# 342].

1.3 Biological effects

1.3.1 Cell cycle control

1.3.1.1 *Cell cycle regulation*

Cell cycle progression is regulated by cyclin dependent kinases (cdks), which are activated by cyclin binding, and inhibited by the cdk inhibitors [Donovan J #71]. G1 to S-phase progression is regulated by D-type cyclins, E-type cyclins and cyclin-A-associated cdks. G2 and M-phases are regulated by B-type cyclin-associated kinases. Both E-type and D-type cdks contribute to phosphorylation of the retinoblastoma protein (pRb). Phosphorylation of pRb in late G1 allows activation of genes required for S-phase. Cdk activation requires phosphorylation of a critical threonine. Two mammalian kinases are known to activate cdk *in vitro*: CyclinH/cdk7 (CAK) and Cak1p. They are active throughout the cell cycle, but their access to cdks is inhibited by p27. Cdk4 is inhibited by INK4 family members, including $p15^{INK4B}$. The KIP family (kinase inhibitor protein) consists of three members, $p21^{WAF1/Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. The KIPs bind to a broader spectrum of cdks than the INK4s. $p27^{Kip1}$ was first identified as a protein whose binding to cyclin E-cdk2 complexes was increased by TGF-β. $p27^{Kip1}$ is high in G0 and early G1, but decreases during G1 to S phase transition [Slingerland J #286].

1.3.1.2 *Cell cycle arrest by TGF-β*

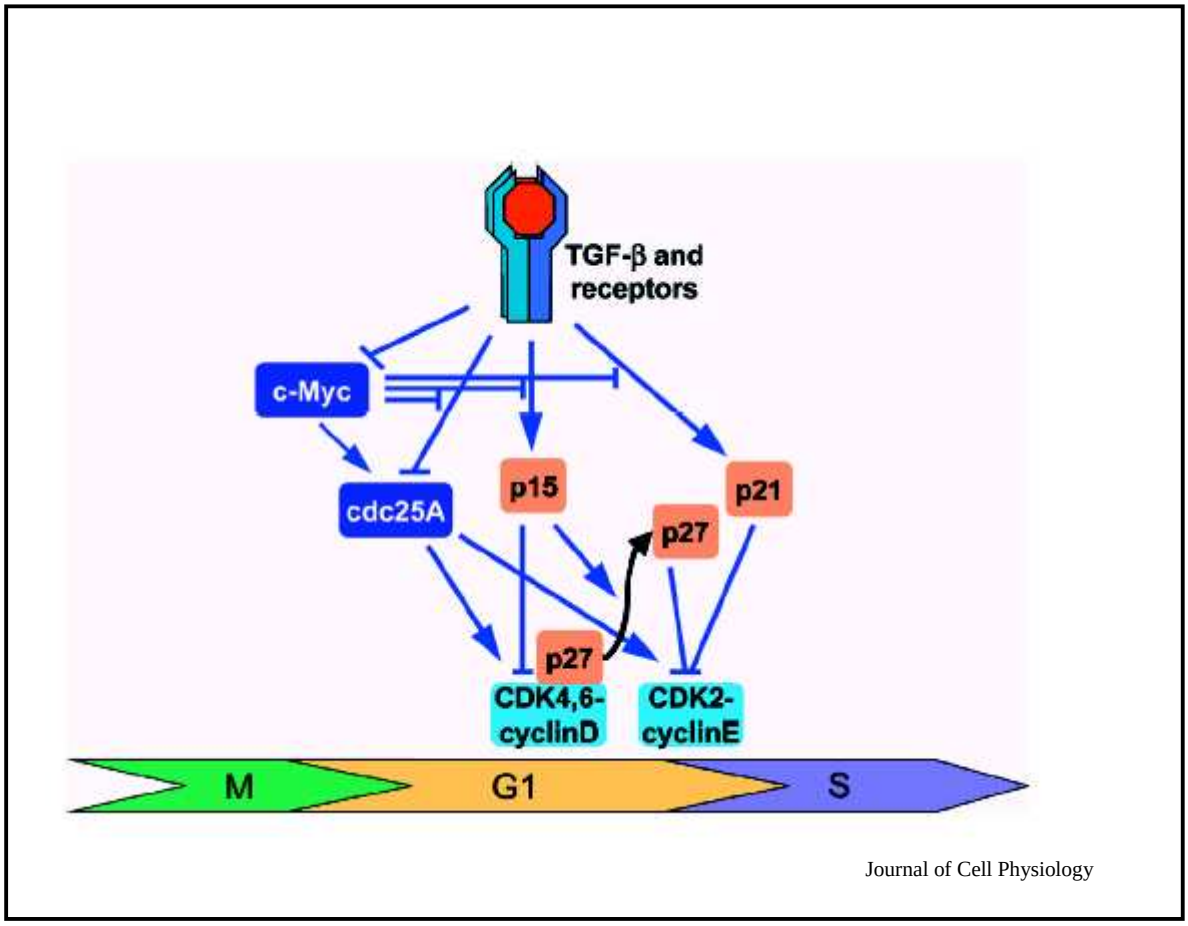


Figure 10: Gene regulation in TGF-β induced cell cycle arrest. In many cell types, TGF-β causes a G1 arrest of cell cycle progression by inhibiting the expression of c-Myc, inducing the expression of $p15^{INK4b}$ and $p21^{CIP/WAF1}$ expression, and/or inhibiting the expression of cdc25a [Ten Dijke P #308].

Cells are sensitive to TGF-β during a certain period in early G1, until they reach the "restriction point" about 6 -10h after G0 release [Laiho M #166]. When TGF-β is added after this time point, cells complete the cell cycle, but arrest at the following G1 phase. It was observed [Laiho M #166] that TGF-β inhibits pRb phosphorylation when it is added in early G1. This is achieved by multiple mechanisms leading to pRb dephosphorylation, most prominently by increasing p27 binding to cyclin E-cdk2. In many cells, TGF-β causes rapid inhibition of *c-myc* transcription [Coffey RJ, Jr. #51; Pietenpol JA #248]. Transcriptional regulation by the c-myc protein is required for G1 to S phase transition, and *c-myc* overexpression confers resistance to TGF-β [Alexandrow MG #4]. At the same time, TGF-β downregulates the cyclins themselves, e.g. cyclin A or cyclin E [Geng Y #90]. TGF-β is

thought to induce inhibitors of cdks (Figure 10). p15 is upregulated by TGF-β [Hannon GJ #105], and it stabilizes the p15 protein, leading to inhibition of cdk4 and cdk6. The p21 gene is also thought to be induced by TGF-β, as the promoter contains Sp1 sites, and overexpression of Smad4 induces p21 mRNA [Li JM #179; Hunt KK #125]. TGF-β also regulates cdk2 phosphorylation. In MvLu cells, TGF-β inhibits cdk2 in part by preventing phosphorylation of cdk2 on Thr160 [Koff A #156], either by inducing p27 [Kato JY #147] (which inhibits CAK access to cyclin-bound cdks) or by inhibiting the enzymatic activity of Cak1p [Nagahara H #221].

1.3.2 Regulation of early development

1.3.2.1 *Expression of TGF-β in the early* Xenopus *embryo*

The members of the TGF-β superfamily that are important in early *Xenopus* embryos belong to two subfamilies: The BMP family, in particular BMP2, -4,and -7, and the Activin family, including members of Nodal (Xnr1-6, Xenopus nodal related), Vg1 and activin itself. The activin related ligands are required for mesoderm formation and endoderm specification, for gastrulation movements and for left-right asymmetry [Osada SI #232]. The BMPs control the fundamental decision between formation of neural versus other ectodermal cell fates, and are also involved in patterning ventral and lateral mesoderm [Graff JM;# 98]. Moreover, TGF-β superfamily members have the potential to act as morphogens: Different doses of ligand turn on particular combinations of downstream genes, and thus enabling different groups of cells to adopt distinct fates [Gurdon JB #101; Wilson PA #340]. The activin-related members of the TGF-β superfamily are produced in the vegetal cells. They are zygotically expressed, and their synthesis requires the maternal transcription factor VegT, which is localized to the vegetal half of the embryo [Kofron M #157].

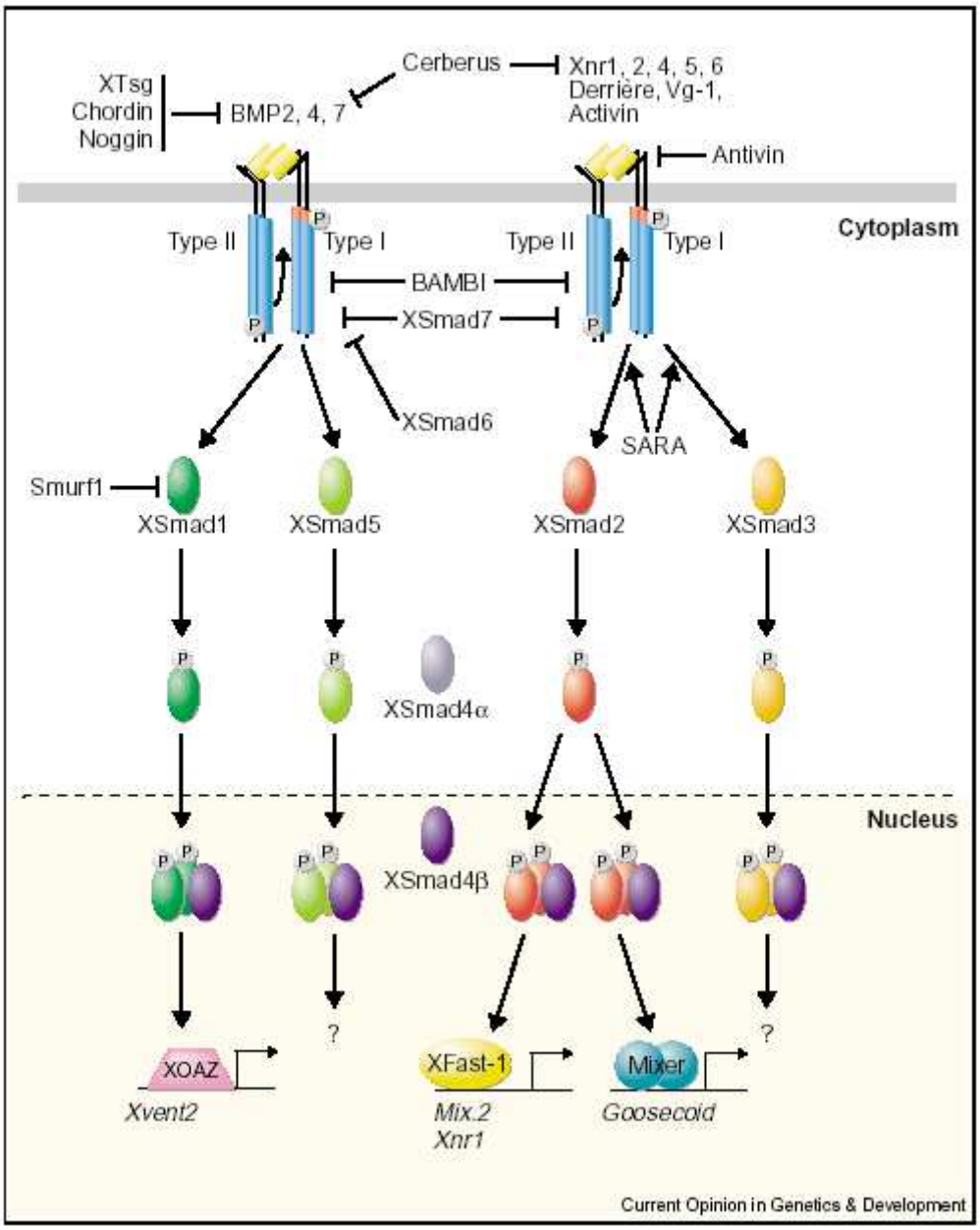


Figure 11: TGF-β signaling pathways in early *Xenopus* development. The BMP pathway is shown on the left and the Activin-like signaling pathway is on the right. The BMP type II receptor is XBMPRII, and two BMP type I receptors are known, a *Xenopus* ALK3 and XALK2. The Activin type II receptor is XActRIIB and the type I receptor is XALK4. Multiple ligands are thought to interact with the receptor pairs. Receptor activation leads to phosphorylation and activation of R-Smads, which are XSmad1 and XSmad5 for the BMP receptors and XSmad2 and XSmad3 for the Activin receptors. XSmad2 and XSmad3 are thought to be recruited to the receptors by the membrane-associated protein, SARA. The R-Smads then form complexes with co-Smads, which are the constitutively nuclear Xsmad4β at late blastula/early gastrula stages and XSmad4α at later stages, which is probably predominantly cytoplasmic in uninduced cells. Activated Smad complexes are recruited to DNA via specific transcription factors such as XOAZ for Smad1/Smad4 complexes and XFast-1 or Mixer for Smad2/Smad4 complexes. No recruiting transcription factors are yet known for Smad5/Smad4 or Smad3/Smad4 complexes. The pathway is

further regulated at different points as shown. Ligand antagonists function extracellularly, probably preventing ligand binding to the receptor. The pseudoreceptor BAMBI is incorporated into receptor complexes but does not signal. Inhibitory Smads (XSmad6 and XSmad7) can act at the level of the receptors or in the case of Smad6, can also compete with Smad4 for activated Smad1. Smurf1 is an E3 ubiquitin ligase that targets Smad1 for degradation [Hill CS;# 114].

Mesoendoderm-inducing TGF-β family members Xnr1,-2,-4,-5 and -6 are expressed dorsally. This way, a dorsoventral gradient of activin-related TGF-β signaling molecules is initiated. The gradient is further specified by the expression of inhibitors. For example, Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signaling and is essential for head-formation [Piccolo S #246]. Cerberus acts by binding to the ligands and inhibiting their interaction with their receptors. The activity of the BMP ligands is also graded throughout the embryo, being higher ventrally than dorsally. The lowest levels are observed in dorsal ectoderm which is fated to become neural tissue [Graff JM;# 98]. BMP4 transcripts become localized to the ventral side of the gastrulating embryo. Expression of BMP2, -4 and -7 is mainly uniform in the ectoderm and mesoderm of late blastulae [Hemmati-Brivanlou A #111]. Their activity is thought to be modulated along the dorsoventral axis by the potent BMP antagonists Chordin and Noggin, which act as inhibitors [Jones CM #140] by binding the BMPs and prevent their interaction with the receptors. A further regulation step is found at the levels of receptors. The pseudoreceptor BAMBI (BMP and Activin membrane-bound inhibitor) is related to the type I receptors, but lacks the intracellular kinase domain [Onichtchouk D #230]. This way, it inhibits BMP and Activin signaling by competing out the type I receptors in the activated receptor complexes. Additionally, the expression of inhibitory Smads may contribute to pattern formation in the early *Xenopus* embryo [Nakayama T #226].

1.3.2.2 *Early development in mice*

Numerous targeted gene disruptions in mice have revealed the important role of the TGF-β signaling pathway in murine early development:

Targeted protein	**Phenotype**	**References**
TβRI	Lethal around midgestation, defects in yolk sac vascularization and lack of circulating red blood cells	[Larsson J #169]

Targeted protein	Phenotype	References
TβRII	Lethal around E10.5 due to defects in yolk sac hematopoiesis and vasculogenesis	[Oshima M #233]
TGF-β1	Mice die postnatally (app. 4 weeks of age) from multifocal inflammatory ractions; 50% die at E10.5 due to defective yolk sac hematopoiesis and vasculogenesis	[Shull MM #284][Kulkarni AB #161] [Letterio JJ #176] [Christ M #47] [Letterio JJ #175] [Diebold RJ #68]
TGF-β2	Death due to congenital cyanosis before or during birth, malformations of eye, spinal column, inner ear, lung, limb, urogenital tract; cleft palate and heart defects	[Sanford LP #272]
TGF-β3	Death within 24h of birth, cleft palate and delay in pulmonary development/lung maturation	[Kaartinen V #142; Proetzel G #250; Taya Y #307]
Smad2	Embryonic lethal (before E8.5) due to defects in mesoderm induction, anterior-posterior and left-right patterning, extra-embryonic tissues and endoderm formation	[Nomura M #229; Waldrip WR #328; Weinstein M #335; Heyer J #113; Tremblay KD #311]
Smad3	Viable, but varying phenotypes including defects in T cell and splenocyte activation; metastatic colon cancer, accelerated wound healing, degenerative joint diseases	[Ashcroft GS #9; Yang X #360]
Smad4	Embryonic lethal (E6.5-8.5), defects in gastrulation and anterior development, epiblast proliferation and egg cylinder formation. Heterocygotes have intestinal tumors	[Sirard C #285; Takaku K #305; Yang X #361; Takaku K #304]
Smad5	Embryonic lethal (E9.5-11.5); Defects in angiogenesis, vasculogenesis, left-right-axis formation and primordial germ cell development	[Chang H #33; Yang X #359; Chang H #35; Chang H #34]
Smad6	Viable, but multiple cardiovascular abnormalities	[Galvin KM #88]

1.3.3 Extracellular matrix formation

TGF-β is one of the most potent regulators of the production and deposition of extracellular matrix (ECM). It stimulates the production and affects the adhesive properties of the extracellular matrix by two major mechanisms [Massague J;# 201]. First, TGF-β stimulates fibroblasts and other cells to produce ECM proteins and cellular adhesion proteins, including collagen, fibronectin and integrins. Second, TGF-β decreases the production of enzymes that degrade the extracellular matrix, including collagenase and heparinase, and increases the production of proteins that inhibit ECM producing enzymes, including plasminogen-activator-inhibitor type I (PAI-1) and tissue inhibitor of metalloprotease. Although the signaling pathways by which TGF-β controls the cell cycle have been analysed in detail, the signaling pathways involved in extracellular matrix regulation by TGF-β are less clear. Recent studies have implicated that the protein kinase A pathway is playing a role in mediating TGF-β stimulation of the α1 chain of type I collagen and of fibronectin. It was found that TGF-β stimulates protein kinase A activation by *in vitro* kinase assays specific for PKA as well as by nuclear accumulation of the catalytic subunit of PKA [Wang L #330]. Inhibiting the PKA pathway by overexpressing the inhibitory peptide of PKA (PKI) attenuates TGF-β-induced stimulation of fibronectin mRNA expression. A specific PKA inhibitor does not affect Smad2 phosphorylation, but inhibits TGF-β induced CREB phosphorylation [Wang L #330]. Inhibiting PKA also reduces TGF-β induced stimulation of the α1-I-collagen mRNA expression. Other pathways implicated in extracellular matrix accumulation by TGF-β are, for example, the MAP-kinase pathway. In a leukemic cell line TGF-β was found to stimulate fibronectin via the JNK-pathway [Hocevar BA #116]. In human skin fibroblasts Smad3 was found to regulate TGF-β induction of α2(I) collagen production [Chen S #40]. In humans, overproduction of TGF-β leading to extensive extracellular matrix production is though to cause diseases such as diabetic kidney disease.

1.3.4 Wound healing

A surprising finding in the Smad3 null mice was their ability to heal wounds more rapidly than wild-type animals, with homozygotes exhibiting a more pronounced phenotype than heterozygote animals [Ashcroft GS #9]. There were fewer monocytes in the wound area, and the direct contribution of Smad3 to reduction of fibrosis was also proven. The normal induction of matrix proteins could be restored by either the introduction of wild-type monocytes to the wound or by exogeneous adminitration of TGF-β. The results clearly

demonstrated the importance of TGF-β as chemoattractant for monocytes. In addition to presence of lower numbers of inflammatory cells, the wounds of Smad3 mutants exhibited an increased rate of re-epithelialization. This was the result of an increased keratinocyte proliferation rate [Ashcroft GS #9].

1.3.5 Angiogenesis

TGF-β directly stimulates angiogenesis *in vivo*, and the stimulation can be blocked by TGF-β antibodies [Pepper MS;# 241]. Expression of the TGF-β receptor endoglin is greatly enhanced during angiogenesis [Burrows FJ #24]. Also, Smad5 has been implicated in the angiogenic process because embryos that lack Smad5 failed to develop blood vessels in the yolk sac and in the embryo proper [Chang H #33; Yang X #359]. In addition, tissue derived from Smad5 deficient embryos failed to support the formation of vascular networks by wild type endothelial cells *in vitro*. An interesting observation is that similar phenotypes were reported from embryos deficient in TGF-β and in embryos that lacked the TβRII [Dickson MC #67; Goumans MJ #97]. Indeed, TGF-β is normally expressed in endothelial and mesenchymal cells and is known to affect interactions between cells that are necessary for blood vessel formation. Angiogenesis requires extensive interactions between endothelial cells and extracellular matrix, pericytes or smooth muscle cells [Folkman J #84; Risau W;# 260]. Smad5 is expressed in both endothelial cells of blood vessels and their surrounding mesenchymal cells at early stages of angiogenesis [Yang X #359], suggesting that Smad5 may mediate the interaction between the endothelium and mesenchyme during the formation of blood vessels.

1.3.6 Immune function

The immunosuppressive effects of TGF-β can be deducted from the phenotype of the TGF-β1 null mice. These mice exhibit a phenotype that can be largely attributed to an overactive immune system [Shull MM #284; Kulkarni AB #161]. These mice succumb to a multi-tissue inflammatory disease, produce autoimmune antibodies and die in the first weeks of life. Most prominent are the inflammatory lesions in their hearts and lungs. Although evidence points towards an auto-immune like etiology, the pattern of immune responses was atypical. The heart and the lung were the primary targets, the response was evident within days after birth, and although lymphocytes appeared active in the invaded tissues, they were non-responsive to TCR stimulation and mitogens *in vitro* [Christ M #47; Chen W #42; Chen W #41]. In addition to an autoimmune-like phenotype, the spleen and thymuses of those TGF-β null mice showed

diminished size and cellular content. The lymphocytes were depleted, and interference with selection and self-tolerance had occured. Apoptotic death was substantially escalated in TGF-β null thymuses [Chen W #41]. More than twice as many T cells were undergoing apoptosis in the thymus in the absence of TGF-β. These data indicate that TGF-β1 must somehow protect activated thymocytes from death. Also, by some mechanisms TGF-β1 appears to be involved in the regulation of the expression of T cell death receptors [Cerwenka A #30; Cerwenka A #31; Genestier L #89; Chen W #41]. In the TGF-β1 null mouse, peripheral CD4+ T cells are more susceptibel to Fas-mediated death and TNFα is the preferential perpetrator of CD8+ T cell death.

Immune system dysfunction could be suspected in the Smad3 null mice based on the loss of TGF-β mediated growth inhibition of primary splenocytes. In addition, the high level of Smad3 expression in the spleen and thymus also suggests a role for Smad3 in immune function [Yang X #360]. Smad3 null mice do, in fact, exhibit a phenotype consistent with an overactive immune system. These mice develop inflammation in the stomach, mucosa and pancreas, with infiltration of T cells and neutrophils [Yang X #360]. In contrast to this phenotype is the increased susceptibility of these mice to infection. Specifically, Smad3 null mice develop large bacterial subcutaneous and mucosal abscesses [Yang X #360]. The occurrence of these abscesses is, in part, attributed to a loss of migration to TGF-β of the Smad3 null neutrophils.

In addition to knockout phenotypes, numerous influences of TGF-β on immune cells have been reported. TGF-β is known to impair the response of tumor specific CTL [Inge TH #127; Vanky F #319; McCartney-Francis NL #208; Cook G #52]. Nevertheless the data concerning TGF-β effects on CTL appeares inconsistent. It was shown that TGF-β secreted by carcinoma cells counteracts the activation and inhibits the function of cytotoxic lymphocytes [Nagy N #224], but TGF-β is also reported to have costimulatory effects on proliferation of murine splenocytes [Lee HM #171]. This regulative role of TGF-β on CTL seems to be distinct from the influence on CD4+ T cells according to observations made in transgenic mice. Transgenics, expressing dominant negative TβRII in T cells, developed a proliferative disorder affecting only CD8+ T cells [Lucas PJ #191].

1.3.7 Apoptosis

1.3.7.1 *Overview*

The apoptotic cascade can be divided into three phases: initiation, integration and execution. In the initiation phase, apoptosis is triggered by stress signals or specific factors acting through a subset of receptors: Fas-R, TNF-R and p75-NTR. During the integration phase, signals from several signaling pathways are balanced, and the decision is made regarding whether the execution of cell death should be initiated. Signaling pathways involved are the MAP kinase pathways (JNK, p38, ERK), acting inpart through Jun/AP-1 activation. During the execution phase, proteases of the caspase family are activated following cytochrome c release from the mitochondria. This step is promoted by activated Bax, and blocked by Bcl-2/Bcl-XL. The caspases then degrade specific substrates leading to the self-destruction of the cell.

1.3.7.2 *TGF-β induces apoptosis in cells of the immune system*

TGF-β induces growth arrest and apoptosis in lymphocytes of human and mouse origin [Kehrl JH #150; Wahl SM #327; Chaouchi N #36; Lomo J #188]. The mouse pre-B-lymphoma cell line WEHI 231 responds to TGF-β in a dose dependent manner [Brown TL #23]. Upon TGF-β treatment cell growth is inhibited, but with increasing doses of TGF-β, a substancial number of cells die by apoptosis. A broad-spectrum caspase inhibitor, but not specific caspase inhibitors, can completely block this apoptotic cell loss. Whereas cellular viability is maintained, the growth arrest persists, thereby distinguishing the growth inhibitory effects from an apoptotic TGF-β pathway. In the same cell line, CD40 stimulation inhibits TGF-β induced apoptosis [Patil S #240]. Analysis of this phenomenon has revealed an upregulation of Smad7 in response to CD40 stimulation. This activation of Smad7 is dependent on NFκB. The induction of apoptosis has also been shown in the IL-2 dependent cell lines OVA-7 and CTLL-2 [Weller M #337]. Cycloheximide prevents TGF-β induced apoptosis in CTLL-2 cells, but not in OVA-7 cells, indicating that protein synthesis is needed in the CTLL-2 cell line. *Bcl-2* and *c-myc* mRNA levels remain constant in this process, thus distinguishing it from cell death induced by IL-2 deprivation. In this case, the levels of *Bcl-2* and *c-myc* mRNA are strongly reduced. L1210 leukemic cells respond to TGF-β with a partial cell cycle arrest at the G1/S transition, and also with induction of apoptosis [Motyl T #217]. Apoptosis is accompanied by two phases of generation of reactive oxygen species: A rapid

(60min) and a delayed (24h/48h) phase after TGF-β treatment. While Bcl-2 levels decrease, the level of bax protein increases. Thereby, the balance between death-inhibiting and death-promoting effects is shifted towards the latter. In another study, upstream caspases involved in TGF-β mediated cell death have been studied in detail [Inman GJ #129]. TGF-β mediated apoptosis of the Burkitts lymphoma cell line BL-41 involves the activation of caspases 2, 3, 7, 8 and 9. The authors show that activated caspase 8 is a prerequisite for activation of the other caspases. However, in this case activation of caspase 8 is independent of death-receptor activation by Fas or TNF-α. While Bcl-X_L and Bad protein levels were reduced after 24h, the levels of bax in contrast were unaltered. In contrast to the pro-apoptotic effects of TGF-β in cell lines, TGF-β knockout mice have a smaller thymus and spleen than their wild-type littermates [Shull MM #284; Christ M #47]. This indicates a depletion of T cells, possibly through induced apoptosis. Recent findings [Chen W #41] have shown that TGF-β1 is localized at the mitochondria. Presumably, this mediates protection from T cell apoptosis.

1.3.7.3 *Apoptosis in the nervous system*

The fine tuning of neuron survival and neuronal death is a prerequisite for the accurate development, maintenance and repair of the nervous system. In ontogenetic neuron death, the role of TGF-β was demontrated by application of TGF-β neutralizing antibodies to chick embryos *in ovo* [Krieglstein K #160]. Thus, the death of dorsal root and spinal chord moto-neurons was abrogated in large parts. In another study it was shown that TGF-β is essential for regulation of apoptosis in the central retina [Dunker N #75].

1.3.7.4 *Smads in apoptosis*

As ist was shown in several studies, the Smad proteins play important roles in the induction of apoptosis of cell lines *in vitro*. The overexpression of Smad4, for example, induces apoptosis in transiently transfected MDCK cells [Atfi A #10], and coexpression of both Smad3 and Smad4 can even enhance the apoptotic effect. Others report on strong induction of cell death by Smad3, but not Smad2 [Yanagisawa K #357]. Yamamura *et al.* found that in Hep3B cells the overexpression of dominant negative (DN) Smad3 inhibited TGF-β induced apoptosis [Yamamura Y #355]. The same effect was observed upon overexpression of Smad7. In this mechanism, an interaction of Smad proteins with the AP1 transcription factor was detected. Inhibition of AP1 also caused blockage of TGF-β mediated apoptosis. Daxx is an adaptor protein which is normally associated with the Fas receptor and mediates the activation of JNK and Fas-induced apoptosis. Recently, Daxx has been shown to be an essential component of

the TGF-β signal transduction pathway [Perlman R #242]. Daxx binds to the cytoplasmic domain of the TβRII, and Daxx antisense treatment of murine hepatocytes results in inhibition of TGF-β mediated apoptosis. The induction of apoptosis and the activation of JNK by TGF-β application can be abolished by overexpressing a dominant negative c-terminal part of Daxx, indicating that JNK activation via Daxx is an important upstream event during TGF-β mediated apoptosis.

Recently a new downstream effector important for TGF-β mediated apoptosis was identified: ARTS (apoptosis-related protein in TGF-β signaling pathway) [Larisch S #168]. ARTS has been located in mitochondria, and sensitizes cells to TGF-β stimulated apoptosis, whereas it only weakly affects fas or TNF-α mediated cell death. Upon TGF-β-application, ARTS is released from the mitochondria and migrates to the nucleus. Inhibition of the ARTS protein was shown to inhibit TGF-β mediated apoptosis significantly [Larisch S #168].

1.3.8 Cancer development

1.3.8.1 *Disruption of cell cycle control*

Altered cdk inhibitor expression

Dysregulation of INK4 family may contribute to TGF-β resistance in cancer. In human tumors, deletion of p15 often accompanies p16 deletion due to their proximity on chromosome 9p [Cairns P #28]. Silencing of p15 through hypermethylation of the promoter region is often observed in leukemias and results in TGF-β resistance [Batova A #12]. The antiproliferative role of p27 is frequently disrupted in human cancers. Although mutations in the p27 gene are rare, one mechanisms for downregulation of p27 is accelerated proteolysis [Catzavelos C #29; Slingerland J #286]. Some cancer types express stable, but inactive p27 in the cytoplasm [Orend G #231]. Also, altered KIP function has beed observed in TGF-β resistant cancers, such as prostate cancer cell lines [Cipriano SC #50] and breast cancers [Tsihlias J #312].

Activation of c-myc and Ras

TGF-β growth inhibition resistant cells often fail to downregulate c-myc [Malliri A #198]. Moreover, in numerous cancers, oncogenic activation of c-myc impairs TGF-β responsiveness through a number of mechanisms, for example by increasing G1 cyclin levels, and by indirect regulation of cyclin D1, E and A expression [Shibuya H #281; Jansen-Durr P #138]. Additional mechanisms link c-myc with cyclinE-cdk2 activation, as overexpression of c-myc may induce a factor that binds p27 and inhibits association with cyclinE-cdk2 [Vlach J

#322]. Overexpression of activated Ras has been shown to abrogate the anti-proliferative effect of TGF-β [Filmus J #82]. Oncogenic activation of Ras is a common feature in many human cancers, and thus can be linked to TGF-β resistance. Ras is able to interfere with Smad2 signal transduction [Kretzschmar M #159], and Ras can increase cyclin D1 levels [Aktas H #3]. Ras activation also accelerates p27 degradation [Kawada M #149]. Activation of Ras may also be indirect, as it was shown in breast cancer cells overexpressing EGF and ErbB2, which both activate the Ras effector PI-3 kinase [Ram TG #254].

1.3.8.2 *Metastasis and angiogenesis*

As described in section "ECM production", the net effect of TGF -β production is increased production of extracellular matrix. In cancer cells the production of TGF-β is increased, which increases the invasiveness of the cells by increasing their proteolytic activity and promoting their binding to cell-adhesion molecules [Machara Y #195]. Also, TGF-β directly stimulates angiogenesis *in vivo*, and the stimulation can be blocked by TGF-β antibodies [Pepper MS;# 241]. In parallel, expression of the TGF-β receptor endoglin is greatly enhanced during angiogenesis [Burrows FJ #24]. Thus, stimulation of angiogenesis might be another mechanism by which TGF-β stimulates the growth of late-stage tumors.

1.3.8.3 *TGF-β receptor mutations*

Mutations in the TGF-β signaling pathway were initially demonstrated in the type II receptor, when several cancers were found to have mutations in or loss of expression of this receptor. The coding region of the gene for this receptor has a sequence of 10 consecutive adenine nucleotides. The addition or deletion of an adenine within this region results in the production of an truncated, functionally inactive receptor. Such mutations are found in colon, gastric, endometrial and other cancers [Markowitz S #200; Myeroff LL #220]. The same mutations have been found in the tumors of patients with hereditary nonpolyposis cancer, a genetic disorder due to a germ-line mutation of DNA mismatch repair genes. Other mutations interfering with the function or expression of the type II receptor have been described. Loss of expression of TβRII has been correlated with progression to more malignant phenotypes in gastric cancer and some T cell lymphomas [Knaus PI #155; Taipale J #302]. In addition, reexpression of the type II receptor restores TGF-β sensitivity and reduces tumor formation in cells lines that have lost their sensitivity to TGF-β due to mutations in TβRII [Sun L #297].

1.3.8.4 *Smad mutations*

Smad4

Human SMAD4 is located in chromosome 18q21.1, where loss of heterozygosity (LOH) is frequently demonstrated in a wide variety of tumors, including pancreatic, colon and gastric adenocarcinomas [Hahn SA #104; Howe JR #121]. Smad4 was first cloned as a tumor suppressor that is deleted in in about 50% of pancreatic cancers [Hahn SA #103]. Mutations in SMAD4 have also been identified in about 30% of colon carcinomas and less than 10% of other cancers [Nagatake M #223; Schutte M #277]. It was reported that germline mutations of human SMAD4 contribute to familial juvenile polyposis, an autosomal dominant disorder characterized by predisposition to colon carcinomas and gastrointestinal cancers [Howe JR #122]. Smad4 +/- heterozygotic mice develop gastric polyps when the animals are 6-12 months of age, and with increasing age the polyps developed to tumors [Takaku K #305; Takaku K #304]. Smad4 would appear to function in a similar fashion to p53, in that haploinsufficiency at the p53 locus is sufficient for tumorigenesis without LOH.

Smad3

Different laboratories have reported different phenotypes for Smad3 mutants [Zhu Y #370; Datto MB #59; Yang X #360]. One group reports that animals lacking Smad3 developed metastatic colon cancer at high frequency. The incidence of cancer was background dependent, as 100% of the mice developed cancer, but that the incidence dropped to 30% when the strain was outbred on a C57BL/6 background [Zhu Y #370]. Other groups have not found colon cancer in their mice [Datto MB #59; Yang X #360].

2 The immune system

2.1 Overview

Our environment contains a great variety of infectious microbes – viruses, bacteria, fungi, protozoa and multicellular parasites. These can cause disease, and if they multiply uncontrolled, they will eventually kill their host. But most infections in normal individuals are short-lived and cause little permanent damage. This is due to the immune system, which combats infectious agents. Any immune response involves, firstly, recognition of the pathogen or other foreign mateial, and secondly, mounting a reaction to eliminate it. The different types of immune responses fall into two categories: Innate (or non-adaptive) immune responses, and adaptive immune responses. Adaptive immune responses are highly specific for a particular pathogen, and adaptive immune resonse improves with each successive encounter with the pathogen.

2.1.1 Cells of the immune system

Immune responses are produced primarily by leucocytes, of which there are different types. One important group of leukocytes are the phagocytic cells such as the monocytes, macrophages and polymorphnuclear neutrophils. These cells bind to microorganisms, internalize them and kill them. Since they use primitive non-specific recognition systems, which allow them to bind to a variety of microbial products, they are mediating innate immune responses. They are acting as a first line of defence against infection. Another important set of leukocytes are the lymphocytes. These cells are central to all adaptive immune responses, since they specifically recognize individual pathogens. The two basic categories here are T cells and B cells. B cells combat extracellular pathogens by releasing antibodies. T lymphocytes have a wider range of functions as discussed below.

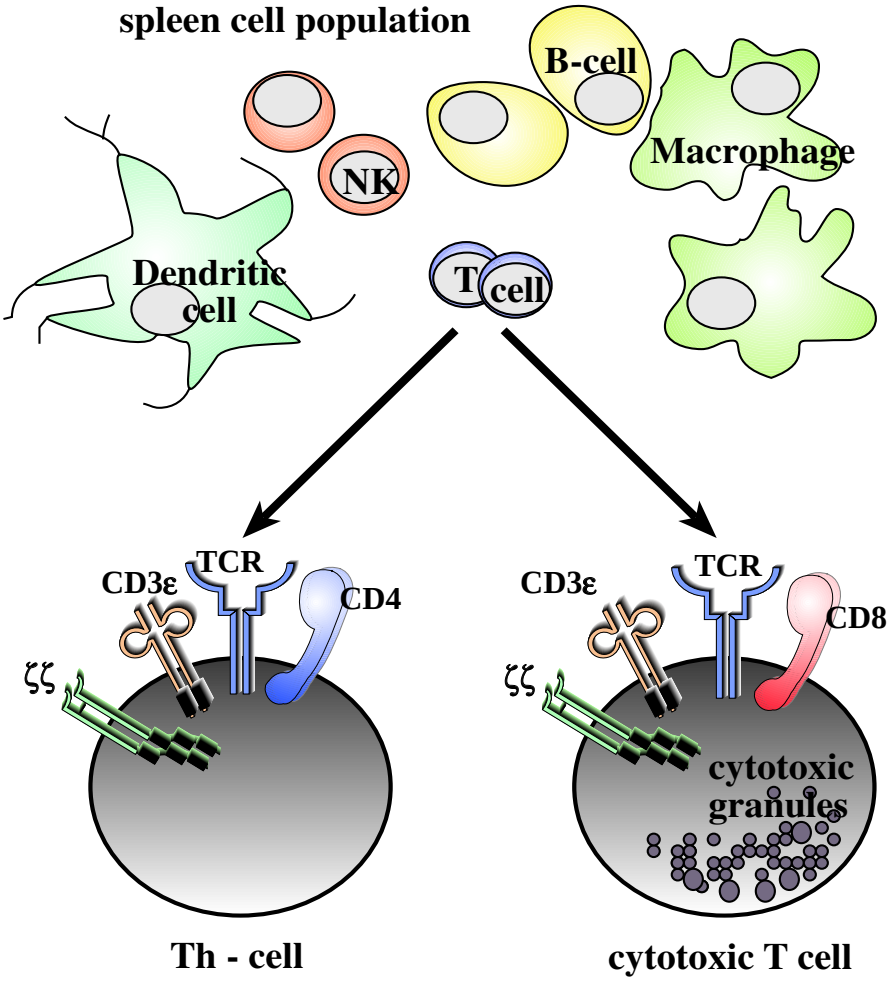


Figure 12: Cells of the murine spleen. The spleen cell population contains distinct types of immune cells. Macrophages and dendritic cells present antigen to the T cells on their MHC class II molecules. Natural killer cells (NK) are a type of cytotoxic cell, and B cells produce antibodies. The spleen also contains two types of T cells: Th cells and cyotoxic T cells. The Th cells recognize MHC class II, bear the CD4 coreceptor and provide help to macrophages, cytotoxic cells and B cells. Cytotoxic T cells recognize antigens in the context of MHC class I, bear the CD8 coreceptor and kill virus-infected cells and tumor cells by exocytosis of their cytotoxic granules containing perforin. 2/3 of all T cells are CD4+, and 1/3 is CD8+.

2.1.2 The Mayor Histocompatibility Complex

T cells recognize antigens only in association with Major Histocompatibility Complexes (MHC) on the surface of host cells. In humans, the MHC is named HLA (Human leucocyte antigen). MHC can be divided in two subgroups. MHC class I is present on all body cells. It binds cytoplasmic endogeneous peptides derived from self-peptides or foreign antigens being present in the cytoplasm. Peptides of 8 or 9 aa length fit into the binding cleft of MHC class I.

MHC class II is predominantely present on phagocytic cells. It binds longer peptides that are derived from foreign antigens that have been endocytosed and processed in phagosomes. Binding pockets within the binding cleft of the MHC molecules are able to accommodate different peptides depending on the haplotype. Since MHC molecules are highly polymorphic, and since a cell expresses several different MHC molecules, cells can present many different antigenic peptides to T cells.

2.1.3 Interleukins

Interleukins are a subgroup of cytokines that are predominantely produced by leukocytes. They are necessary for lymphocyte proliferation, differentiation and activation. A selection of the most important cytokines is shown in the following table.

cytokine	cellular source	effects on immune functions
IFNγ	T cells, NK cells	immunoregulation, antiviral action, B cell differentiation
IL-1 (α/β)	Monocytes, DC, B cells, MΦ, endothelial/epithelial cells	inflammation, fever
IL-2	T cells, NK cells	proliferation, activation
IL-3	T cells	pan-specific colony stimulating factor
IL-4	T cells	proliferation and differentiation
IL-5	T cells	differentiation
IL-6	T cells, fibroblats, B cells, MΦ	differentiation, acute phase protein synthesis
IL-8	MΦ, skin cells	chemotaxis

Table 1: Important cytokines, their sources and actions.

2.2 T cells

Several types of T cells (Figure 12) are known which have a variety of functions. T helper cells (Th) interact with B cells and help them to divide, differentiate and produce antibodies, or interact with mononuclear phagocytes to help them to destroy intracellular pathogens. Cytotoxic T lymphocytes (CTL) are responsible for the destruction of host cells which are infected by viruses or other intracellular pathogens, and they are able to destroy malignant host cells

2.2.1 T cell surface molecules

TCR

The defined T cell lineage marker is the T cell receptor complex (TCR). The T cell receptor is a heterodimer of the disulphide linked α and β chain. The amino acid variability of the TCR resides in the N-terminal domain of the α and β subunits, which are homologous with the variable domains of the immunoglobulins. The TCR is associated with a series of polypeptides, collectively called CD3 (Figure 12). The CD3 proteins include the γ, δ,and ε chains. These are present as monomers non-covalently associated with the disulphide-linked αβ TCR heterodimer, whereas two copies are present of the CD3ε molecule. The ζ chain is present as a disulphide-linked homodimer. Some TCR complexes show a η - ζ heterodimer instead of the ζ–ζ- homodimer. The immunoreceptor tyrosine-based activation motifs in the cytoplasmic tains of the CD3 and ζ chains are conserved sequences that include sites for tyrosine phosphorylation.

CD4/8

TCR+ cells can further be divided into two distinct populations (Figure 12), CD4+ T helper cells and CD8+ cytotoxic T cells. CD4 and CD8 are glycoproteins that bind to non-polymorphic regions of the MHC molecule. Both CD4 and CD8 perform a combination of adhesive and signaling functions, which greatly enhance the sensitivity of T cells to antigen. Since they are associated with the TCR, they are often called co-receptors. Approximately 65% of peripheral T cells express CD4, and 35% express CD8.

Th and CTL can be further divided into subtypes. Th1 produce predominantely IFNγ and IL-2. They activate macrophages and are involved in the killing of intracellular pathogens. Th2 cells produce IL-4 and IL-5 and induce antibody production, combatting extracellular pathogens. Th3 cells are thought to be suppressor cells, producing TGF-β. Tc1 are thought to produce also IL-2 and IFNγ , while Tc2, similar to Th2, are thought to secrete IL-4 and IL-5. The generation of Tc1 versus Tc2 is thought to be dependent on TGF-β [Erard F #78].

CD28

In addition to TCR engagement, T cells depend on other costimulatory signals in order to become activated. The most prominent costimulatory molecule is CD28. Its counterreceptors are found on B cells and are named B7-1 and B7-2. CD28 costimulation prolongs and augments the production of IL-2 and other cytokines

2.2.2 T cell development

After the precursor cells have been released from the bone marrow, they migrate to the thymic subcapsular zone as immature CD4-CD8- double negative (DN) cells. There they undergo selection and maturation. Besides the lymphocytes at various stages of differentiation, the thymus is composed of multiple supporting stromal cells [Surh CD #300; Rathmell JC #256;# 257]. The thymic lobes are enclosed in a connective tissue capsule below which a network of epithelial cells, mesenchymal fibroblasts, macrophages and dendritic cells are found which participate in thymocyte education. Until fetal day 14 or 15, thymic development involves mostly triple negative cells (CD4-CD8-TCR-). After day 15, TCR expression and the first expression of CD4 and CD8 can be detected. Over the next few days, double positive (DP) cells dominate with maturation to CD4+CD8- or CD8+CD4- single positive (SP) cells [Ellmeier W #76]. By fetal day 20 or at birth, the thymus contains all stages of developing T cells. Typically, the majority (80%) of normal thymocytes are DP, express low levels of TCR, and are found in the cortex of the thymus. This DP population normally contains most of the thymic apoptotic cells. More than 95% of these T cells die due to their failure to get positively selected. Within 3-4 days after generation, the DP T cells will undergo apoptosis unless they express a TCR that can interact with an MHC molecule on stromal cells, implicating the TCR and activation as the basic requirement for selection. In the context of the TCR, the interaction with MHC-I or II shapes the lymphocyte’s phenotype and provides positive selection and escape from apoptosis. If their TCRs overreact to thymic self-antigens complexed with MHC-I, DP cells die through the process of negative selection (tolerance induction). Since only a few percent escape selection with their lives, their maturation to CD4+CD8- or CD4-CD8+ SP thymocytes in the medulla enables longevity and export from the thymus.

2.2.3 T cell signal transduction

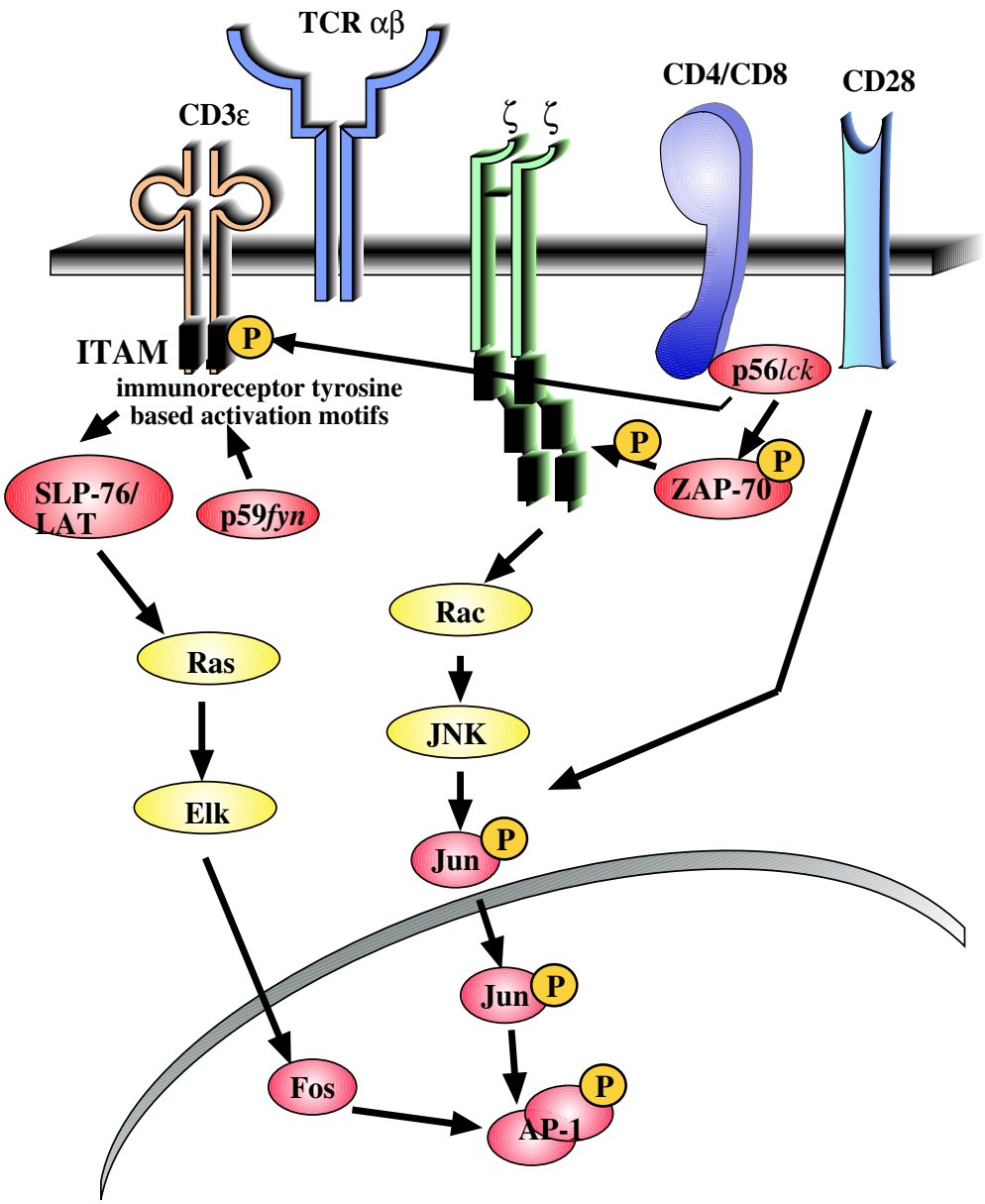


Figure 13: T cell signal transduction as described in the text.

As illustrated in Figure 13, the earliest intracellular event that occurs in response to TCR binding of ligands is the protein-tyrosine-kinase (PTK) mediated phosphorylation of tyrosine residues within the immunoreceptor tyrosine-based activation motifs (ITAMs). These ITAMs are present in the cytoplasmic tails of the CD3ε and ζ chains of the CD3 complex. Once the ITAMs are tyrosine-phosphorylated, they become specific docking-sites which bind

cytoplasmic proteins with SH2 (src-homology) domains. One is the zeta-associated protein-70 (ZAP-70), which is a member of a family of PTKs distinct from the Src family. ZAP-70 itself has to be phosphorylated in order to bind, and this is most likely achieved by $p56^{lck}$, which is associated with CD4/CD8. $p56^{lck}$ is also able to directly phosphorylate the ITAMs, as it is also known for $p59^{fyn}$. SLP-76 (Ship-like protease) then activates the Ras/raf pathway onwards from CD3ε ITAMs [Su B #296], whereas the ζ chain-ITAMs transduce the signal via Rac. One of the most important costimulatory pathways is the CD28 molecule, which binds B7-1 and B7-2 expressed on APCs. CD28 is able to directly activate the JNK pathway after T cell stimulation. In the nucleus, Jun/Fos heterodimers are formed and act as transcription factors.

2.2.4 Activation via single-chain antibodies

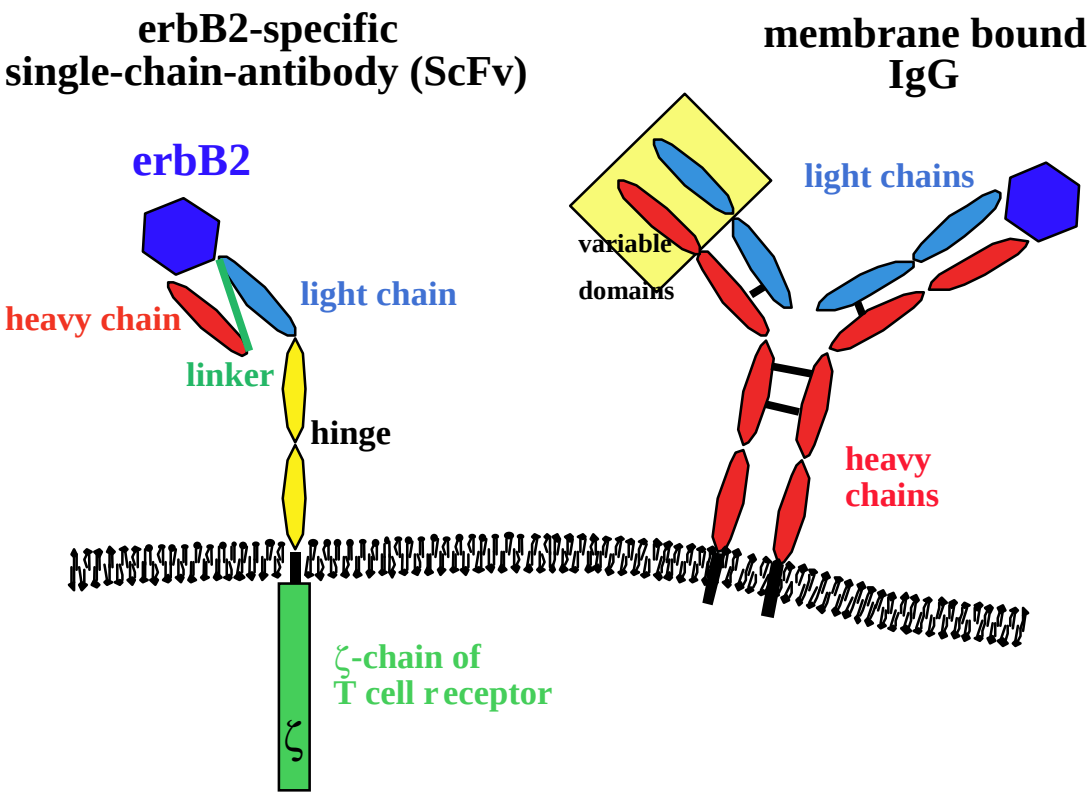


Figure 14: Illustration of single-chain antibody in contrast to membrane-bound IgG. The ScFv contains the linker-coupled variable domains of the light chain and the heavy chain of an erbB2 specific antibody. Via a hinge region and a transmembrane domain, it is coupled to the signal transducing ζ-chain of the CD3-TCR-complex. In contrast, the IgG consists of the di-sulphide linked heavy chain and light chain, anchored to the plasma membrane via a transmembrane domain.

Malignant transformation of body cells is very often accompanied by abnormalities in antigen processing and MHC presentation. This hinders T cells to properly recognize tumor antigens such as erbB2 or Melan-A (specifically expressed in melanoma) on the surface of cancer

cells. The erbB2 proto-oncogene encodes a transmembrane tyrosine kinase, and the erbB2/HER2 receptor protein belongs to the epidermal growth factor (EGF) receptor family [Dougall WC #73]. In cancer, the HER2 gene is amplified and/or overexpressed and leads to transformation [Guy TC;# 102]. To enable the cytotoxic T cells to be targeted MHC independently to malignant cells, the T cells can be grafted with chimeric receptors [Stancovski I #293; Altenschmidt U #6] (Figure 14), the binding domain is a single-chain antibody fragments (ScFv). The activation domain may for example consist of the ζ–chain of the CD3 TCR associated complex. The target cell lysis is then independent of TCR, independent of coreceptors, and is not restricted by the MHC molecules, but correlates with the expression of antigens recognized by the erbB2-specific binding domain of the single-chain antibody (Figure 15).

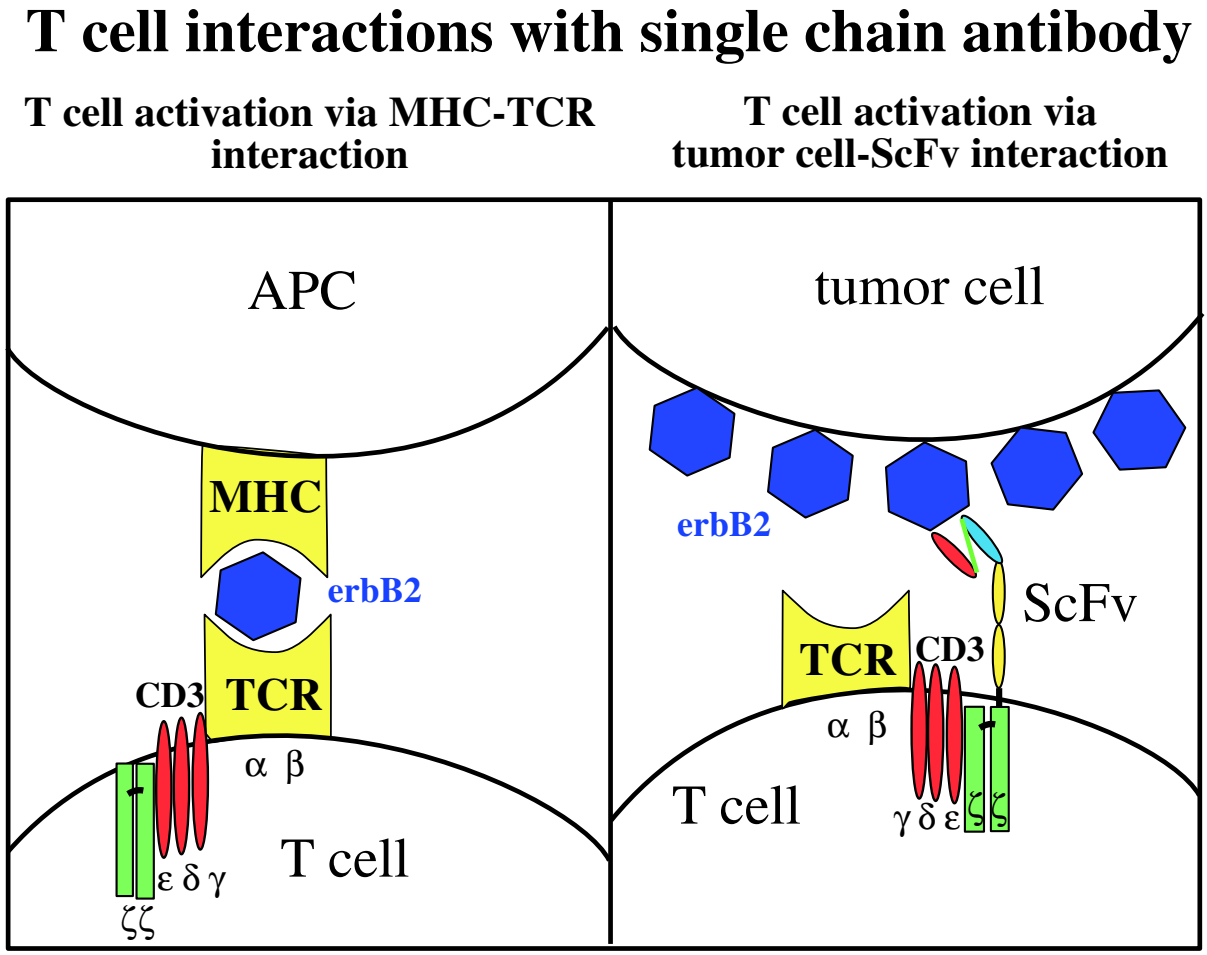


Figure 15: Recognition of target cells by T cells. In normal CTL, the erbB2 (by antigenic fragments) is recognized by the specific TCR in the context of MHC molecules, and via the CD3 complex the signal is transduced to the nucleus. T cells grafted with the ScFv, in contrast, directly recognize the erbB2 receptor on the surface of the tumor cells, and by heterooligomerization of the ScFv with other molecules of the CD3 complex the signal is transduced independent of TCR crosslinking.

2.2.5 Peripheral T cell activation

Those thymocytes successful in their education, maturation and survival migrate to their peripheral residence, the periarteriolar sheath (PALS) in secondary lymphoid organs. From there they enter the recirculating pool of long-lived lymphocytes. The T cells circulate, pass through peripheral lymphoid tissues unless or until encountering antigen-presenting cells which activate and alter their life-course. Homing patterns direct them to extralymphoid sites of activation where they proliferate and participate in host defence. Post-thymic expansion of antigen-experienced T cells is antigen-driven [Sprent J #292]. Preferential accumulation of lymphocytes in a specific tissue is guided not only by the adhesion molecules on the endothelium, but also the activation-induced expression of counter-receptors on the lymphocytes [Wahl SM #326]. These adhesion interactions provide the basis for specificity and targeted entry of activated lymphocytes. Adhesion molecule expression is highest on activate lymphocytes. Within the tissues, accumulation is not only dependent on recruitment, but also on proliferation, rate of cell-death and/or exit rate [Westermann J #338].

2.2.6 Cytotoxicity mechanisms

MHC restricted cytotoxic T cells are mostly CD8+ and therefore recognize antigens in association with MHC I. First, the cell binds to the target, followed by a Ca2+ dependent phase in which the vesicle contents of the cytotoxic granules are discharged. The vesicles contain perforin, a monomeric pore-forming protein related to the lytic component of the complement. The vesicles also contain a serine esterase that may be involved in the assembly of the lytic complex. In the presence of Ca+, the perforin monomers bind to the target cell membrane and polymerize to form transmembrane channels. Although in close contact with the perforin, the CTL survives and continues killing further targets. The autoprotection is believed to be due to a proteoglycan which is also present in the vesicles and which may bind and inactivate perforin. The killing seen by perforin is nevertheless distinct from complement killing, as perforin mediated killing is connected with apoptosis: DNA-fragmentation and disintegration of the cell into apoptotic bodies.

2.2.7 Activation induced cell death

After the elimination of an antigen or foreign agent, it is necessary to reduce the amount of antigen-reactive T cells. Activation-induced cell-death (AICD) is a means to accomplish this reduction and generally involves enhanced expression of death receptors including tumor necrosis factor receptor (TNFR) family members such as FAS, TNFR and TRAIL-R [Smith

CA #287; Wallach D #329]. Engagement of these receptors with their respective ligands, FasL, TNF or TRAIL initiates a series of events that tightly regulate cell death. In addition to antigen-driven T cell apoptosis, lymphokine withdrawal also promotes passive apoptosis. Although mature post-thymic T cells initially resist apoptosis, once they are activated and have proceeded through cell-cycle in response to IL-2, vulnerability to apoptosis is maximal. [Lenardo M #173]. Deletion of effector T cells is essential to clear the site, re-establish homeostasis and restore tissue-integrity. Otherwise, continuous recruitment and activation of T cells can be pathologic. Autoimmune diseases are often associated with aberrant apoptotic behaviour of T cells [Lenardo M #173].

II Results

The overall aim of this study was to shed light on TGF-β1 actions on immune cells in general and on cytotoxic T cells in particular, as functional immune responses are indispensable for combatting infectious diseases and malignancies. TGF-β1 itself plays pivotal roles in disease mechanisms such as cancer development and autoimmune reactions [Letterio JJ #177; Blobe GC #16]. Nevertheless, evaluating the current literature on specific effects of TGF-β on T cells, it becomes apparent that the proposed roles for TGF-β remain controversial, as it is already mentioned in the introduction. Some data suggest inhibition of cytotoxicity and proliferative responses by TGF-β in primary mixed lymphocyte reactions [Pardoux C #238], others report promotion of proliferative responses at low concentrations of TGF-β1 (0.1-1pg/ml), gaining more inhibitory properties when concentrations increase (1-10ng/ml) [McKarns SC #209]. In mice, bearing dominant negative (DN) TβRII transgenic T cells, only CD8+ cells appear to expand massively, but not CD4+ T cells [Lucas PJ #191]. These observations led us to the conviction that dissecting and analysing distinct molecular mechanisms of TGF-β action on T cells is fundamental to ultimately developing strategies to overcome fatal TGF-β effects in certain disease states. Therefore, we first focused on the different activation mechanisms and their influence on TGF-β sensitivity in chapter 1. The analyzed parameter hereby were proliferation (1.1), differentiation to CD4+ cells and CD8+ cells (1.2), target cell lysis (1.3), receptor surface expression (1.4) and Smad phosphorylation (1.5, 1.6 and 1.7). In chapter 2 the molecular mechanisms of the divergent sensitivity of differentially stimulated T cells to TGF-β1 are further analysed. In this chapter it is demonstrated that MAP kinase pathways are involved in conferring resistance to TGF-β1 mediated growth inhibition, as inhibtion of MEK-1 renders cells sensitive to TGF-β1 (2.1) and abrogates sustained phosphorylation of Smad2 (2.2) and JNK (2.4) in total splenocytes. However, inhibition of MEK-1 does not alter TGF-β1 sensitivity of purified CD8+ T cells (2.3). In chapter 3 attempts to alter the phenotype of TGF-β1 sensitive T cells is described. However, addition of αCD28 or IFNγ does not restore sustained Smad2 phosphorylation (3.1) and does not rescue cells from growth inhibition (3.2). In order to establish cellular systems that combine the TGF-β1 sensitivity/insensitivity profile upon divergent stimulation and the ability to be easily manipulated, numerous T cell lines were analysed according to the

relevant parameters listed above. In chapter 4, different cell lines are analysed for TGF-β1 growth sensitivity (4.1), surface expression of the type II receptor (4.2), ligand binding (4.3) and Smad2 (4.4) and JNK (4.5) phosphorylation. Leading from the description of molecular events up to active manipulation, in chapter 5 the cloning and expression of a chimeric dominant negative (DN) type II receptor is described. As explained in the introduction, the idea is to combine tumor-antigen specific single-chain antibodies that enable T cells to target tumor cells under circumvention of the MHC molecules with DN TGF-β receptors to render the cells resistant to tumor-produced TGF-β1. Here, we combined the DN type II receptor with a *Herpes simplex* thymidine kinase as suicide gene by recombinant PCR (5.1) and expressed the construct in cell culture (5.2).

1 Resistance to TGF-β1 mediated growth inhibition correlates with sustained Smad2 phosphorylation

As we concluded from the literature, the often reported contradictory data on inhibition versus stimulation versus indifferent responses of T cells towards TGF-β1 ([Pardoux C #238] [McKarns SC #209] [Lucas PJ #191]) might be accounted to a number of critical differences in study design, including the use of clonal T-lymphocytes, cell lines or primary T cells, either mixed lyphocytes or purified CD4+ or CD8+ cells, treatment duration as well as the supplementation of additional growth factors or cytokines to the cell cultures. To test the influence of different experimental conditions, we analysed both total murine spleen cells, enriched for T cells (35% CD4+ and 15% CD8+ T cells), and purified CD8+ T cell cultures (>95%). Both types of cultures were either stimulated as descrived in section "Materials & Methods" with IL-2 alone without TCR-activation, or stimulated polyclonally via TCR crosslinking (anti-CD3) plus IL-2, or by addition of lectin (ConA) plus IL-2. The utilized TGF-β1 concentration was hereby kept constant at 240pM, and treatment duration was restricted to 5 days (cytotoxicity assay) and 3 days (other assays).

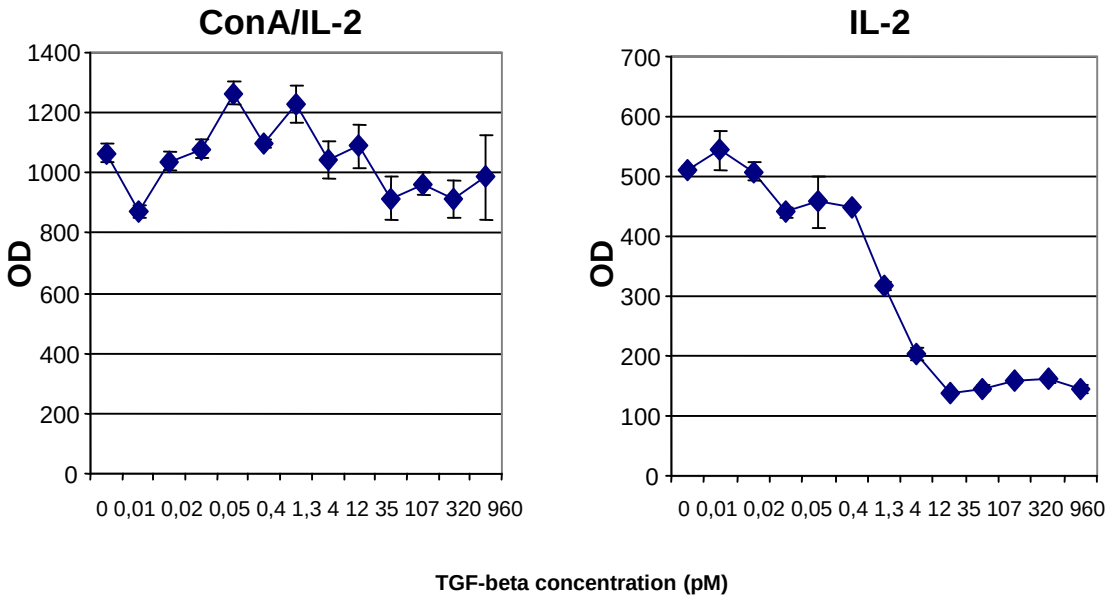


Figure 16: Dose response of total splenocytes to TGF-β1. Total splenocytes were stimulated with ConA/IL-2 or IL-2 and cultured in the presence of decreasing amounts of TGF-β1. Whereas in ConA/IL-2 stimulated cultures even concentrations up to 320pM TGF-β1 are not inhibiting the proliferation of cells, in IL-2 stimulated cells TGF-β1 is effective down to 0.05pM.

The effective TGF-β1 concentration was determined using a dose-response curve as depicted in Figure 16. However, much lower doses were effective on IL-2 stimulated cells. The treatment duration normally cannot extended to more than 5 days in total cultures, as after 4 days the total cell numbers decline due to apoptotic mechanisms (Figure 17A). In purified CD8+ T cells, however, the proliferation rate is lower, resulting in extended culture life (Figure 17B).

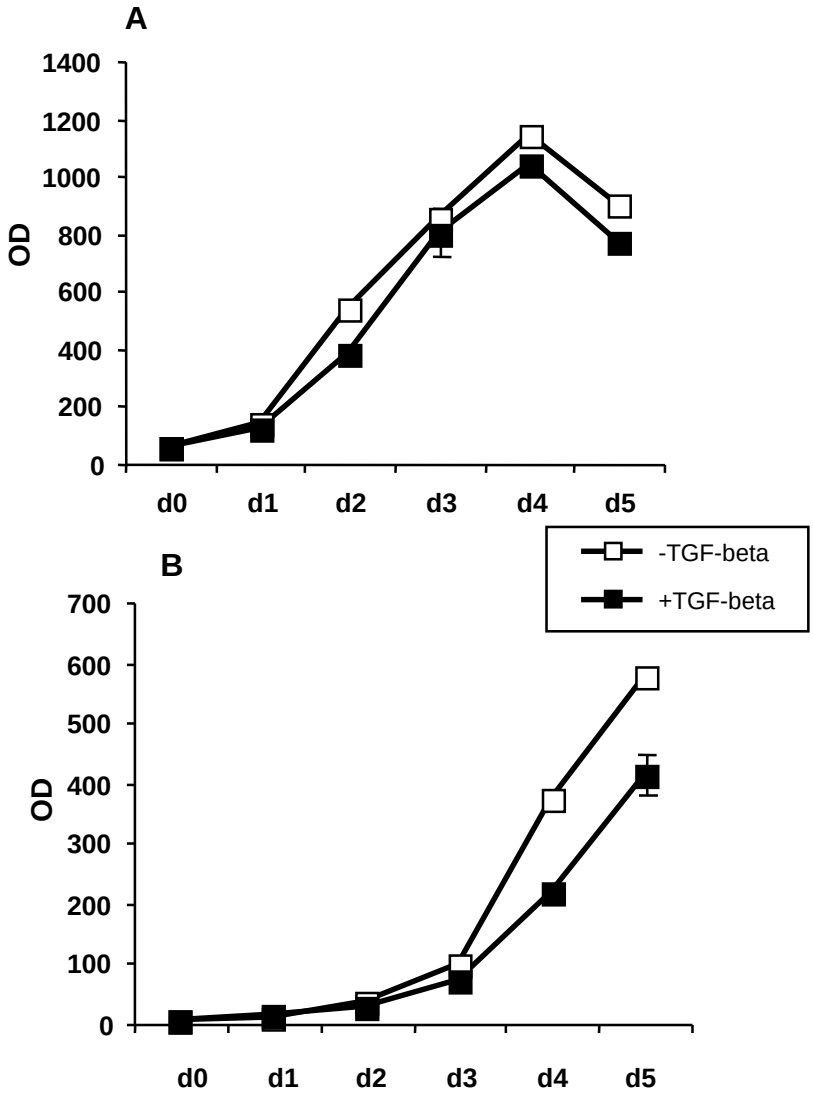


Figure 17: Time course of stimulated spleen cells. (A) shows the growth curve of total splenocytes stimulated with αCD3/IL-2 in the presence or absence of TGF-β1. The cells proliferate up to day4, then the population starts to decline, and cell death is observed. In (B) purified CD8+ T cells are shown, which exhibit a different growth dynamics: They take longer to proliferate the first days, but then proliferate up to day 5. Later on (data not shown), on days 6-7, cell death is observed in these cultures as well.

1.1 Long-term exposure to TGF-β1 affects proliferation of CTL dependent on stimulation and cellular environment

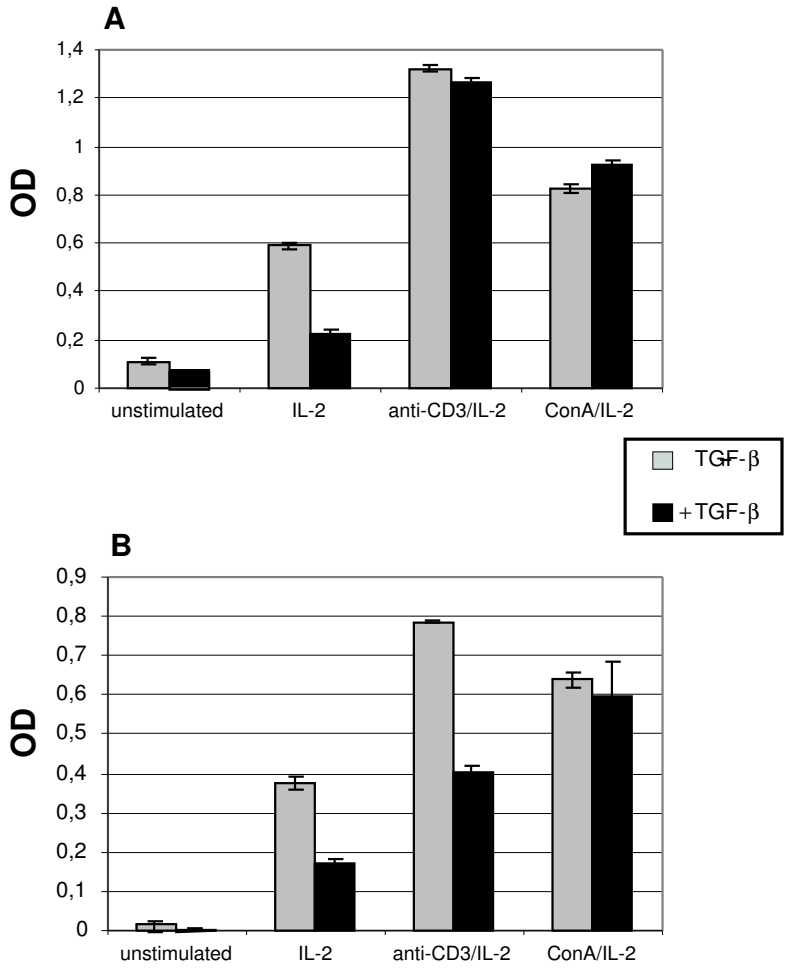


Figure 18: Effect of TGF-β1 on proliferation of primary murine splenocytes. (A) Splenocytes are enriched for T cells by passage over nylon wool columns (see section Materials and Methods). In (B) these cells are further enriched for CD8-positive cells as described in Materials & Methods, section IV. Cells were stimulated with recombinant IL-2 (500ng/ml), ConA (2µg/ml) + IL-2 (250ng/ml) or soluble anti-CD3 antibody (1µg/ml) + IL-2 (250ng/ml) and incubated for 3 days with or without addition of TGF-β1 (240pM). To assay for proliferation, cells were analysed using the CellTiter 96 Aqeous One Solution Reagent (Promega) as described in Materials and Methods. Dark bars and grey bars refer to cells cultured in the presence or absence of TGF-β1, respectively. The presented results are derived from one representative experiment out of three. SD was from triplicates.

Total splenocyte cultures and purified CD8+ T cell cultures were polyclonally activated with IL-2, IL-2 plus ConA or anti-CD3 as described in Materials and Methods, section IV, and cultured in the absence or presence of 240pM TGF-β1. After 3 days the cells were assayed for proliferation. Analysing total lymphocytes we found that cultures stimulated with IL-2

showed 60% reduction in total cell numbers upon TGF-β1 treatment (Figure 18). In contrast, proliferation of cells activated with αCD3/IL-2 or ConA/IL-2 was not negatively regulated by the addition of exogeneous TGF-β1 (Figure 18A). Surprisingly, TGF-β1 treatment of purified CD8+ CTL resulted in reduced proliferation for both IL-2 and anti-CD3/IL-2 treated cells (Figure 18B), while in ConA/IL-2 activated cells nearly no reduction was observed (Figure 18B). From the absence of dead cells as determined by staining with trypan blue (data not shown) we concluded that TGF-β1 did not act via increased apoptosis, but inhibited the proliferation of T cells.

1.2 Long-term exposure to TGF-β1 does not alter the proportion of CD8+ T cells in total splenocyte cultures

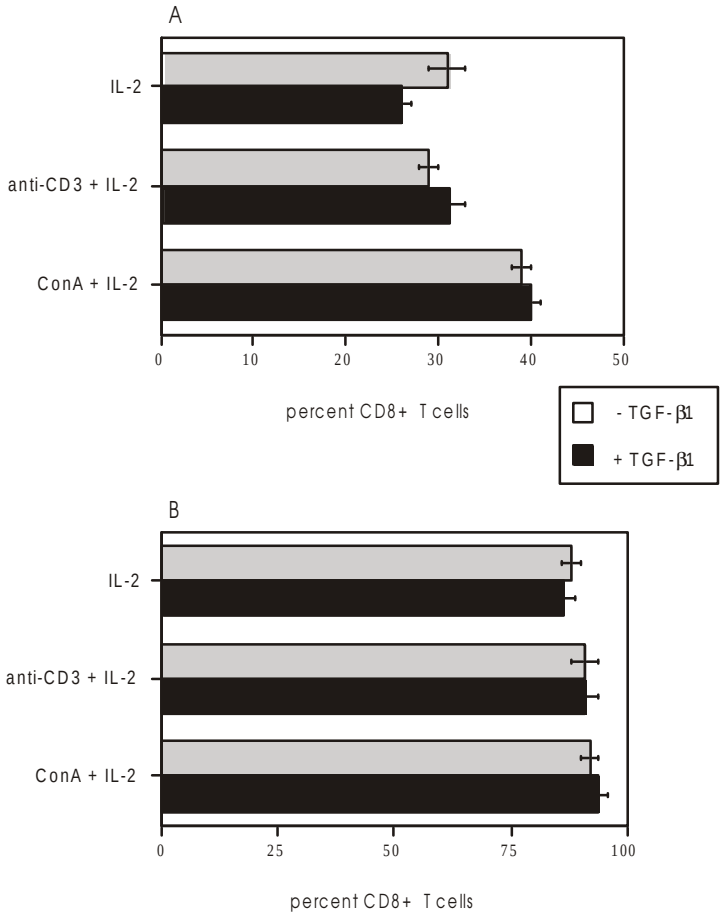


Figure 19: Effect of TGF-β1 on the percentage of CD8+ populations in cultures of primary splenocytes. (A) Splenocytes are enriched for T cells by passage over nylon wool columns. In (B) these cells are additionally enriched for CD8-positive cells. Cells were stimulated as described in Figure 18. After 3 days with or without TGF-β1 (240pM) the cells are stained with αCD3-FITC, αCD4 Cychrome and αCD8 PE and analysed by flow cytometry. In IL-2 and ConA/IL-2 cultures, percentages represent CD3+CD8+ double positive cells. Cells stimulated with αCD3 could not be stained with αCD3 antibodies, and thus values here include all CD8+ cells. Dark bars and grey bars refer to cells cultured in the presence or absence of TGF-β1, respectively.

As total splenocyte cultures consist of several cell types, we investigated the possibility that in total splenocytes stimulated with αCD3/IL-2 or ConA/IL-2 (Figure 18A) increased cell numbers were caused by other populations, while CD8+ T cells might be growth inhibited by TGF-β1 like in the purified cultures (Figure 18B). Thus we analysed the proliferation of

CD8+ cells within total splenocyte cultures by determining the percentage of CD8+ T cells by flow cytometry (Figure 19). As we show in Figure 19A, there is no change in the proportion of CD8+ CTL in total splenocyte cultures treated with TGF-β1. The proportion of CD8+ cells in purified CTL cultures is also independent of TGF-β1 (Figure 19B). Thus we concluded that TGF-β1 does not favor the outgrowth of other cell types in total splenocyte cultures.

1.3 Long-term exposure to TGF-β1 abolishes the lytic abilities of CTL

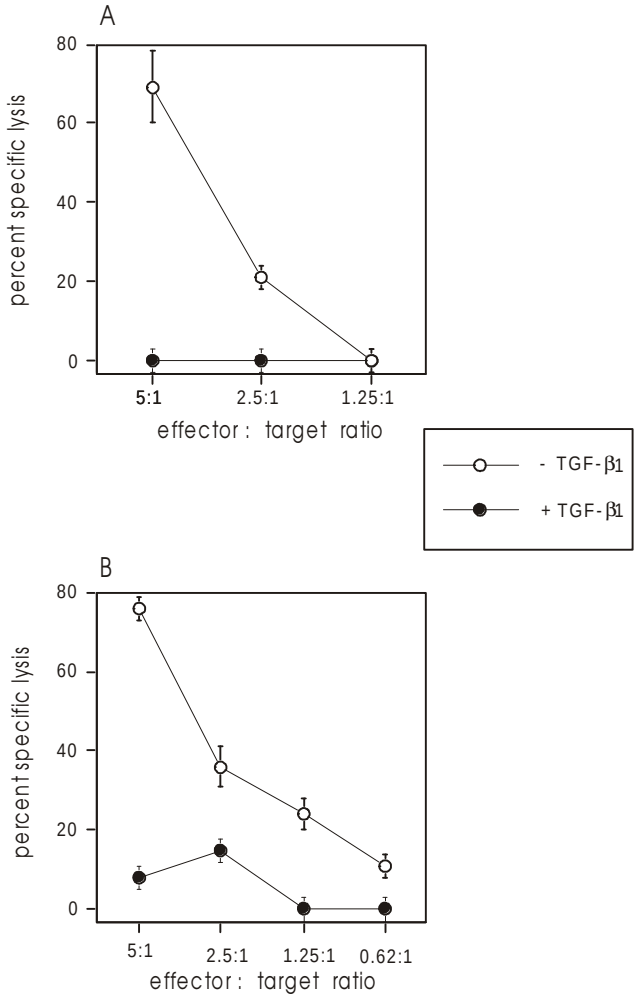


Figure 20: Long-term exposure to TGF-β1 abolishes lytic activity of cytotoxic T cells. Splenocytes are enriched for T cells by passage over nylon wool columns. Cells were stimulated with (A) IL-2 (500ng/ml) or (B) IL-2 (250ng/ml) + ConA (2µg/ml). After 5 days with or without TGF-β1 (240pM) the cells are plated with allogeneic tumor cells (P815) at different effector:target ratios as indicated. As control, syngeneic EL-4 cells were used as target cells. For calculating the specific lysis, the unspecific lysis obtained in wells with syngeneic target cells was subtracted from the lysis measured with allogenic cells.

Next, we studied cytotoxicity of T cells subjected to long-term treatment with TGF-β1. Here, we focused on IL-2 stimulated cells that were shown to be growth inhibited by TGF-β1, and ConA/IL-2 stimulated cells that did not show TGF-β1 sensitivity (Figure 18A). Splenocytes stimulated with IL-2 or ConA/IL-2 were cultured for 5 days in the presence or absence of 240pM TGF-β1. On day 5, the cells were plated with either allogeneic target cells (P815) to determine the specific lysis, or syngeneic target cells (EL-4) to assess the unspecific lysis. As the percentage of CD3+CD8+ T cells in total splenocyte cultures stimulated with ConA/IL-2 is approximately 10% higher than in IL-2 stimulated cultures (Figure 19A), the latter achieved lower specific lysis. As depicted in Figure 20A, TGF-β1 abrogates the lytic function of IL-2 stimulated total splenocytes. Interestingly, even cultures stimulated with ConA/IL-2 were found unable to specifically lyse allogeneic tumor cells in the presence of TGF-β1 (Figure 20B). The underlying mechanism of inhibition of lytic function by TGF-β1 thus seems to be different from inhibition of proliferation.

1.4 Long-term exposure to TGF-β1 does not downregulate TβRII on the cell surface

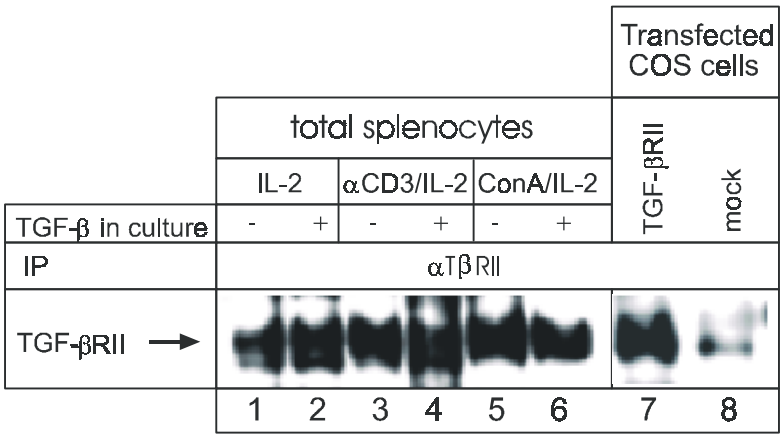


Figure 21: TβRII is not downregulated upon long-term exposure to TGF-β1. Total splenocytes were stimulated as described in Figure 18. Cell surface proteins were biotinylated as described in Materials and Methods, section IV. TβRII was immunoprecipitated with anti-TβRII [Moustakas A #219]. Additionally, COS-cells were transfected with TβRII or mock DNA and likewise biotinylated and immunoprecipitated as positive (lane 7) and negative (lane 8) control.

Next, we wanted to elucidate if the described unresponsiveness of total splenocytes to TGF-β1 mediated growth inhibition is caused by downregulation of TβRII. We therefore analysed surface expression of TβRII using cell surface protein biotinylation. Equal numbers of total

splenocytes were stimulated as described and cultured in the presence or absence of TGF-β1. After biotinylating cell surface molecules, cell lysates were subjected to immunoprecipitation with anti-TβRII [Moustakas A #219]. We found that in IL-2 (Figure 21, lanes 1 and 2), αCD3/IL-2 (Figure 21, lanes 3 and 4) and ConA/IL-2 (Figure 21, lanes 5 and 6) stimulated total splenocytes long-term exposure to TGF-β1 does not downregulate the surface expression of TβRII. COS-cells transfected with TβRII cDNA or mock [Aruffo A #8] serve as controls (Figure 21 lanes 7 and 8). Therefore, the mechanism of unresponsiveness to TGF-β1 has to be different from receptor downregulation.

1.5 Long-term exposure to TGF-β1 leads to deregulated phosphorylation of Smad2, dependent on T cell stimulation and cellular environment

To investigate the observed effects more closely, we next focused on TGF-β1 mediated signal transduction via Smad2 and Smad3. The cells were stimulated as described above and cultured in the absence or presence of 240pM TGF-β1. On day 3, TGF-β1 was thoroughly washed away and all cells were starved for 3 hours without TGF-β1. Cells were washed, pulsed for 15min with 240pM TGF-β1 or left untreated, cell lysates were prepared and analysed by western blotting. Smad2, phosphorylated at the C-terminal SSXS-motif was detected using anti-phospho-Smad2 antisera (αPS2) [Ishisaki A #131]. To ensure equal loading, total Smad2 was detected on the same blot using αSmad2 antisera as described by Nakao *et al*. [Nakao A #225].

1.5.1 Long-term exposure to TGF-β1 leads to sustained Smad2 phosphorylation in total splenocytes

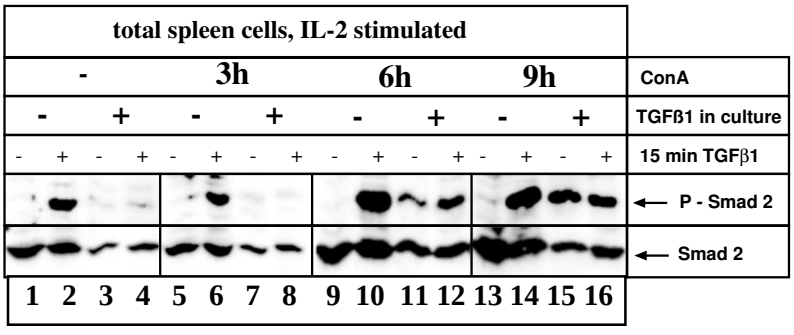


Figure 22: Addition of ConA leads to sustained Smad2 phosphorylation in IL-2 stimulated total spleen cells. Total splenocytes were stimulated with TGF-β and IL-2 as indicated, and lysed as described in

Materials & Methods, section IV. Total cell lysates were analysed by western blotting, first with αP-Smad2 antibody directed against the phosphorylated C-terminal SSXS-motif (upper panel), and second with αSmad2 antibody recognizing the linker region of Smad2 (lower panel).

Total splenocytes cultured for 3 days with ConA/IL-2 in the presence of TGF-β1 showed sustained phosphorylation of Smad2, as shown in Figure 25A, lanes 11 and 12, whereas total splenocytes cultures with IL-2 alone were found unable to phoshorylate Smad2 at all (Figure 25A lanes 3 and 4, and Figure 22). This sustained phosphorylation is observed after at least 6h of ConA-stimulation, as seen in Figure 22. Here, total splenocytes are stimulated for 3 days with IL-2 (250ng/µl). On day 3, 2µg/ml ConA was added. After 3 (lanes 5-8), 6 (lanes 9-12) and 9 (lanes 13-16) hours, as indicated in Figure 22, the cells were starved for 3h and lysates were prepared as described in Materials & Methods, IV3.4.1. After 3h of ConA, still no sustained Smad phosphorylation is observed, moreover, no Smad-phosphorylation at all is observed in cells cultured in the presence of TGF-β. After 6h, weak sustained phosphorylation can be seen. After 9h of stimulation with ConA, the total splenocytes express equal sustained Smad2 phosphorylation as compared to cells cultured for 3 days in the presence of ConA/IL-2 and TGF-β1 (Figure 25A, lanes 11 and 12). This leads to the assumption that translation might be a prerequisite for aquiring the sustained Smad2 phosphorylation.

1.5.2 ConA treatment alone does not lead to Smad2 phosphorylation

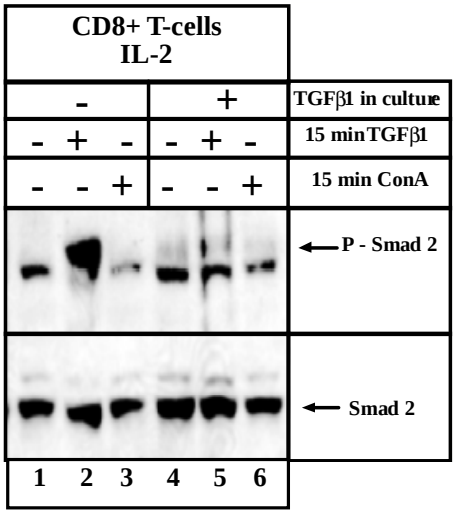


Figure 23: ConA alone does not lead to Smad2 phosphorylation.

To analyze whether ConA administration alone is sufficient to induce Smad2 phosphorylation, total splenocytes were stimulated with IL-2 with or without addition of

TGF-β1. After 3h of starving in fresh media on day 3, the cells received either TGF-β1 or ConA for 15min. As it is seen in Figure 23, the addition of TGF-β1 to cells that had not seen long-term TGF-β1 leads to phosphorylation of Smad2 (Figure 23, lane 2). In contrast, ConA alone is not able to phosphorylate Smad2 (Figure 23, lane 3). As already shown in Figure 22, lanes 3, 4, 7 and 8, upon long-term exposure to TGF-β1 no Smad2 phosphorylation can be observed in Figure 23, lanes 4-6. Thus, it is unlikely that ConA crosslinks the TGF-β-receptors, and Smad2 is phosphorylated upon ConA-induced crosslink of the TGF-β receptors. Therefore, other pathways have to be induced by ConA, leading to sustained Smad2-phosphorylation in total splenocytes after 6h of continuous ConA stimulation (Figure 22).

1.5.3 Stability of the sustained phosphorylation

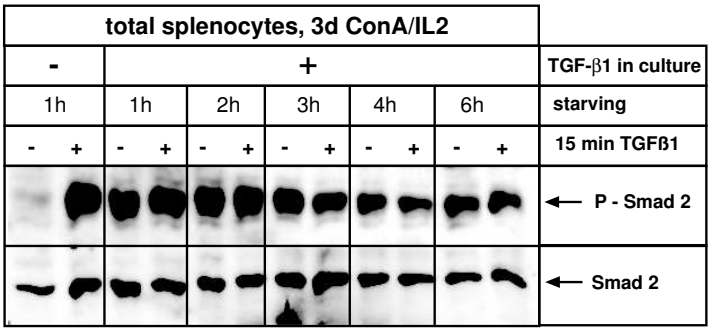


Figure 24: The sustained phosphorylation is stable for 6 hours. The total splenocytes are stimulated with ConA/IL-2 as described. On day 3, the cells are starved in fresh media for the indicated time points, and after application of 15min TGF-β1 cellular lysates are prepared and subjected to western blotting as described.

To analyze the stability of the observed sustained phosphorylation, ConA/IL-2 stimulated cells that had received long-term TGF-β1 were starved in fresh media for up to 6h. As ist is seen in Figure 24, the sustained phosphorylation is stable for 6 hours. After 6h, the sustained phosphorylation slightly declines. However, even after 24h the sustained phosphorylation is still weakly present (data not shown). This excludes the possibility that the sustained phosphorylation is caused by residual TGF-β1 still bound to the receptors.

1.5.4 Deregulated phosphorylation of Smad2 is dependent on T cell stimulation and cellular environment

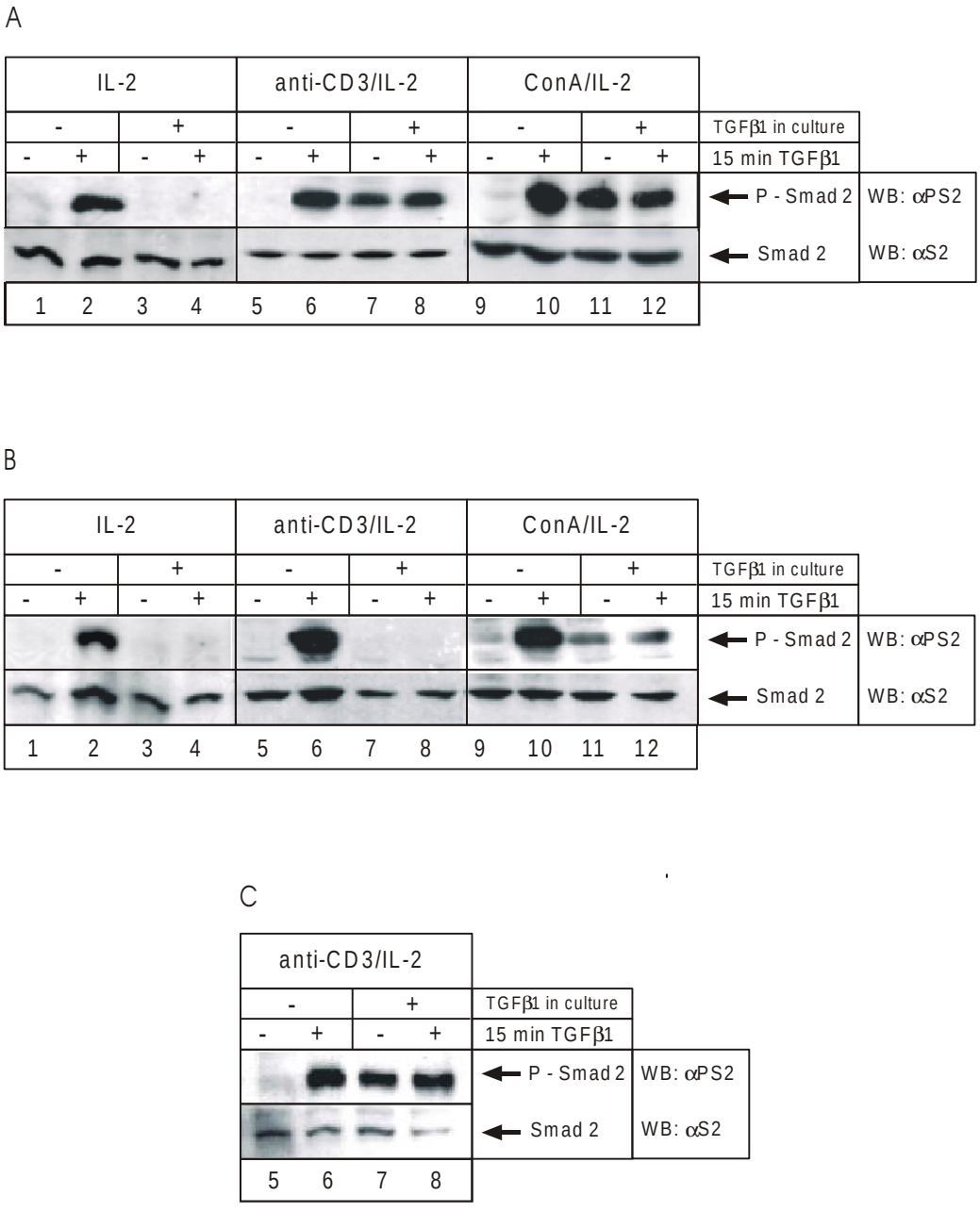


Figure 25: Long-term exposure to TGF-β1 causes deregulated Smad2-phosphorylation in primary mouse splenocytes. In (A) splenocytes are enriched for T cells by passage over nylon wool columns. In (B), these cells are enriched for CD8-positive cells. In (C), CD8+ cells were purified from a three day total splenocyte culture. Cells were stimulated as described in Figure 18. Stimulated cells were cultured with or without 240pM TGF-β1. On day 3, equal cell numbers were starved for 3h and were then stimulated with 240pM TGF-β1 for 15 minutes or left untreated. Total cell lysates were analysed by western blotting, first with

anti-P-Smad2 antibody (αPS2) directed against the phosphorylated C-terminal SSXS-motif (A, B, and C upper panels), and second with anti-Smad2 antibody (αS2) recognizing the linker region of Smad2 (A, B and C, lower panels). The presented results are derived from one representative experiment out of three.

In total splenocytes as well as in purified CD8+ T cells not exposed to TGF-β1 for 3 days, a 15min pulse of TGF-β1 lead to a strong phosphorylation of Smad2 (Figure 25A and B, lanes 2, 6, and 10). However, long-term addition of TGF-β1 changes the Smad2 phosphorylation properties of these cells. Purified CD8+ T cells, as depicted in Figure 25B, stimulated with IL-2 alone (lanes 1-4) or αCD3/IL-2 (lanes 5-8) were unable to phosphorylate Smad2 when cultured in the presence of TGF-β1, although the amount of total Smad2 remains constant (lanes 1-8, lower panel). CD8+ T cells stimulated with ConA/IL2 show weak sustained phosphorylation of Smad2 (Figure 25B, lanes 11 and 12). To ensure that this sustained phosphorylation was not caused by residual TGF-β1 bound to the receptors, the cells were washed on day 3 and kept without TGF-β1 to determine the stability of this sustained phosphorylation. In total splenocytes, IL-2 stimulated cells show the same phosphorylation pattern as purified CD8+ cells (Figure 25A and 4B, lanes 1-4). However, the sustained phosphorylation in total splenocytes stimulated with ConA/IL-2 is much stronger than in purified CD8+ cells (Figure 25A and B, lanes 11 and 12). The most striking difference between total splenocytes and purified CD8+ T cells is observed in cells stimulated with αCD3/IL-2 and cultured in the presence of TGF-β1. Total splenocytes show strong sustained phosphorylation of Smad2. Surprisingly, purified CD8+ T cells under the same conditions are unable to phosphorylate Smad2 at all (compare Figure 25A lanes 7, 8 to Figure 25B lanes 7, 8). When we first cultured αCD3/IL-2 activated total splenocytes in the presence of TGF-β1 for 3 days and then purified CD8+ T cells from these cultures on day 3, these CD8+ T cells showed the sustained phosphorylation of Smad2 (Figure 25C, lanes 3 and 4) as seen in total splenocyte cultures. From this we conclude that CTL show deregulated Smad2 phosphorylation in response to long-term TGF-β1. This deregulated Smad2 phosphorylation differs dependent on the mode of stimulation as well as the cellular environment and correlates with TGF-β1 growth resistance.

1.6 Smad3 shows sustained phosphorylation comparable to Smad2

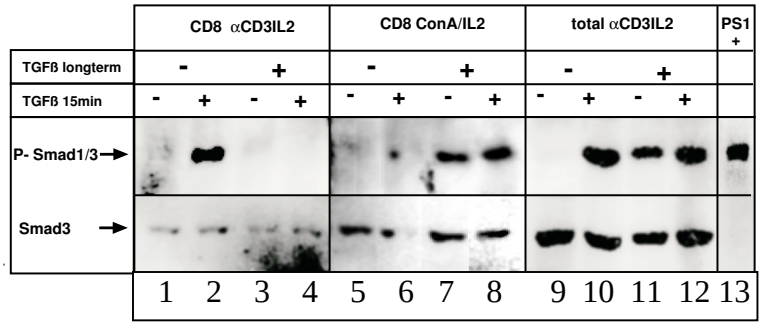


Figure 26: Sustained phosphorylation is also present in Smad3. Lanes 1-4 show CD8+ cells stimulated with αCD3/IL-2. Lanes 5-8 show CD8+ cells stimulated with ConA/IL-2. Lanes 9-12 show total splenocytes stimulated with αCD3/IL-2. In the upper panel, WB are probed with αP-Smad1/3, in the lower panel, the blots are reprobed with Smad3. Lane 13 shows P-Smad1/3 positive control using C2C12 cells, stimulated with BMP-2 to phosphorylate Smad1 (upper panel). The lower panel in lane 13 shows that αS3 does not crossreact with Smad1.

As not only Smad2, but Smad3 as well is implicated in regulating immune cell functions, especially as SMAD3 mutant mice generated by gene targeting die between 1 and 8 months due to a primary defect in immune function [Yang X #360], we also analyzed the primary T cells for Smad3 phosphorylation. The cells were stimulated as described, and on day 3 lysates were prepared and analyzed for phosphorylation of Smad3 [Nakao A #225]. P-Smad3 was detected using antisera against P-Smad1 cross-reacting with P-Smad3 as described by Dooley *et al.* [Dooley S;#72] and shown in Figure 27, lane 13, where lysates from C2C12 cells [Katagiri T;#146], stimulated with BMP2, were probed with αPS1/3. In contrast, unphosphorylated Smad1 is not detected by αS3 (Figure 27, lane 13, lower panel). In purified CD8+ T cells, sustained Smad3 phosphorylation is observed in cells stimulated with ConA/IL-2 (Figure 27, lanes 7 and 8) upon long-term exposure to TGF-β1, comparable to Smad2 (Figure 25B, lanes 11 and 12). Stimulation of CD8+ T cells with αCD3/IL-2 and TGF-β1, however, renders Smad3 unable to become phosphorylated (Figure 27, lanes 3 and 4), equal to Smad2 (Figure 25B, lanes 7 and 8). Like Smad2 (Figure 25A, lanes 7 and 8), Smad3 as well shows strong sustained phosphorylation upon long-term exposure to TGF-β1 in total splenocytes stimulated with αCD3/IL-2 (Figure 27, lanes 11 and 12).

1.7 Cellular distribution of Smad2, Smad3 and Smad4 upon long-term exposure to TGF-β1

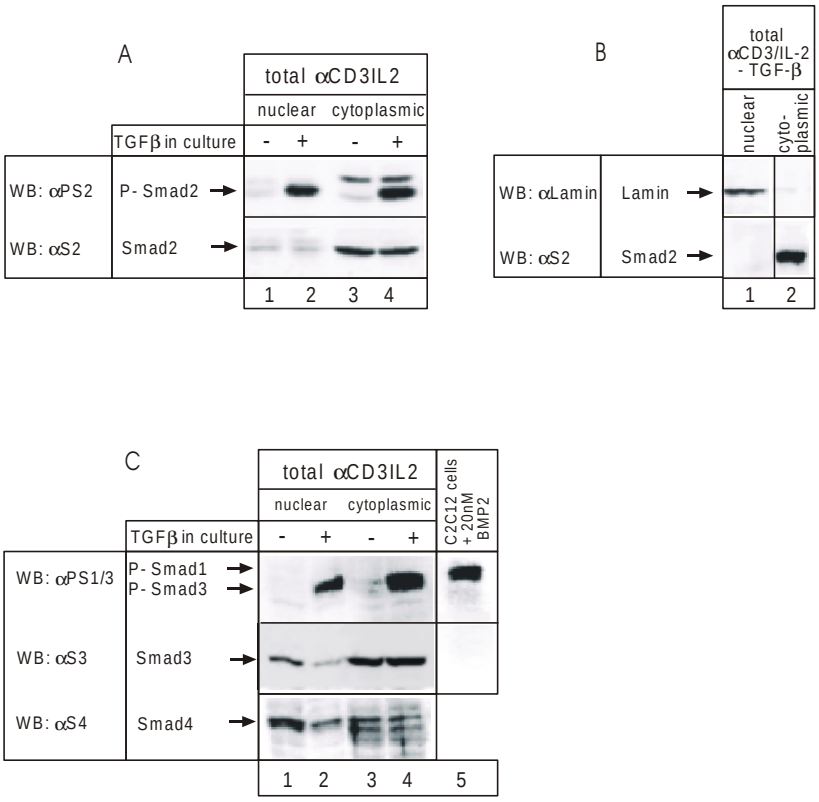


Figure 27: Localization of Smad2, Smad3 and Smad4 in different cellular compartments. In A, B and C total splenocytes are stimulated with αCD3/IL-2 as described in Figure 18 and fractionated into cytoplasmic and nuclear compartment on day 3. In (B) the quality of subcellular fractionation was verified using nucleus-specific lamin antisera. (A) shows the cytoplasmic and nuclear localization of Smad2 and P-Smad2. In (C), the localization of Smad4, Smad3 and P-Smad3 is shown. (C), lane 5, shows P-Smad1/3 positive control using C2C12 cells, stimulated with BMP-2 to phosphorylate Smad1 (upper panel). The middle panel in (C) shows that αS3 does not crossreact with Smad1, while αPS1/3 crossreacts between P-Smad1 and P-Smad3.

Next, we wanted to determine if the unresponsiveness to TGF-β1 is caused by retention of Smads in the nucleus, leading to impaired signal transduction. Therefore, we fractionated anti-CD3/IL-2 stimulated total splenocytes in nuclear and cytoplasmic compartments. Figure 27A shows the cellular distribution of phosphorylated Smad2 (P-Smad2) by western blotting. P-Smad2 (upper panel) is seen in the cytoplasmic (lane 4) as well as in the nuclear (lane 2) compartment after long-term treatment with TGF-β1. To control the quality of cellular fractionation performed for this experiment, we probed the extracts with antiserum against

nucleus-specific lamin protein (Figure 27B, upper panel). Next, we examined the distribution of Smad3 [Nakao A #225] and Smad4 [de Winter JP #62] in these cells (Figure 27C). upon stimulation with TGF-β1 (Figure 27A and C, lanes 2 and 4). Without TGF-β1 treatment, neither Smad2 nor Smad3 are present in the nucleus, in contrast to Smad4. Smad4 is detected in nucleus and cytoplasm regardless of TGF-β1 exposure (Figure 27C, lower panel).

We conclude from these data that unresponsiveness to TGF-β1 is not caused by the lack of Smads in the cytoplasmic compartment.

2 MEK-1 is an important mediator of growth resistance toTGF-β1

In the former experiments described in the previous chapter we could show that TGF-β treated CTL co-cultured with other splenocytes are not growth inhibited and show sustained Smad2 phosphorylation, in contrast to purified CTL. To further investigate the signal transduction pathway conferring resistance to TGF-β1 mediated growth inhibition, we focussed on TGF-β crosstalk pathways shown to play important roles is T cell signaling: the JNK and ERK1/2 MAP (view section I1.2.5)

2.1 Inhibition of MEK-1 renders total splenocytes sensitive to TGF-β1 mediated growth inhibition

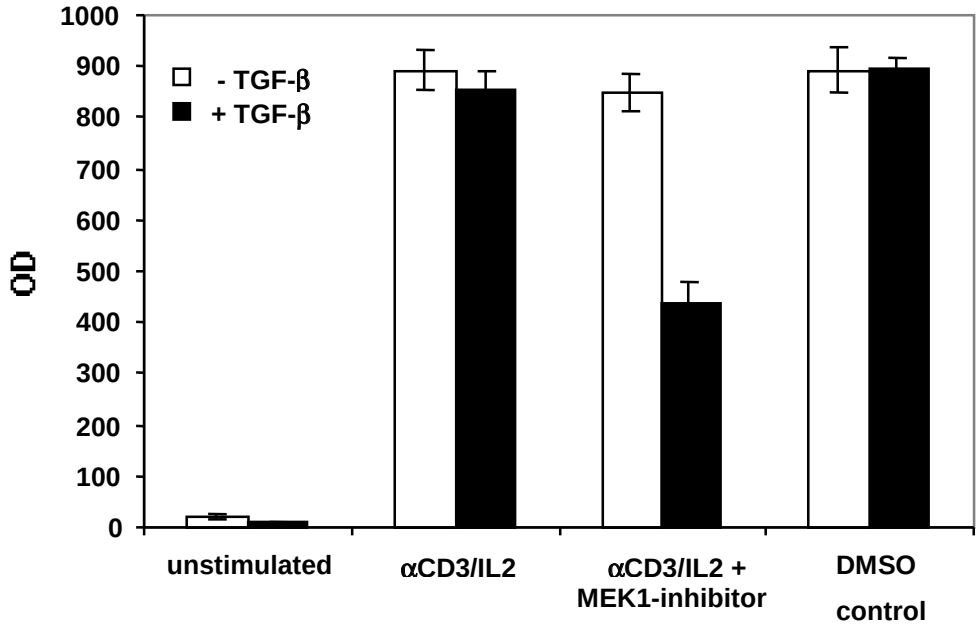


Figure 28: Inhibition of the MEK-1 pathway renders total splenocytes sensitive to TGF-β1 mediated growth inhibition. Murine total splenocytes and CD8+ T cells were prepared and stimulated for 3 days with αCD3/IL-2 +/- TGF-β1 as described in Figure 18. PD98059 was used in at a final concentration of 50µM, and was given 1h prior to αCD3/IL-2 and TGF-β1. As PD98059 is dissolved in DMSO, control cells received equal amounts of DMSO, but exhibited no impaired proliferation. To assay for proliferation, cells were analyzed using using the CellTiter 96 Aqueous One Solution Reagent (Promega) as described in Materials & Methods (section IV3.5.4). Dark bars and grey bars refer to cells cultured in the presence or absence of TGF-β1, respectively.

Murine total splenocytes and CD8+ T cells were harvested, stimulated with αCD3/IL-2 and cultured for 3 days in the presence of TGF-β1 as described. The cells received a MEK-1 inhibitor, PD98059 or were left untreated. After 3 days the cultures were assayed for proliferation. As shown in Figure 18, total splenocytes with intact MEK-1 signal transduction pathway are not growth inhibited by long-term exposure to TGF-β1. In contrast, purified CD8+ T cells show a strong reduction in proliferation when exposed to long-term TGF-β1 (Figure 18). Surprisingly, upon blockage of MEK-1 in total splenocytes (Figure 28), these cells become sensitive to TGF-β1 mediated growth inhibition and thus resemble purified CD8+ T cells with respect to their proliferation properties. Cells that received equal amounts of DMSO as a control were also unaffected by TGF-β1, as shown in Figure 28.

2.2 Inhibition of MEK-1 abrogates sustained phosphorylation of Smad2

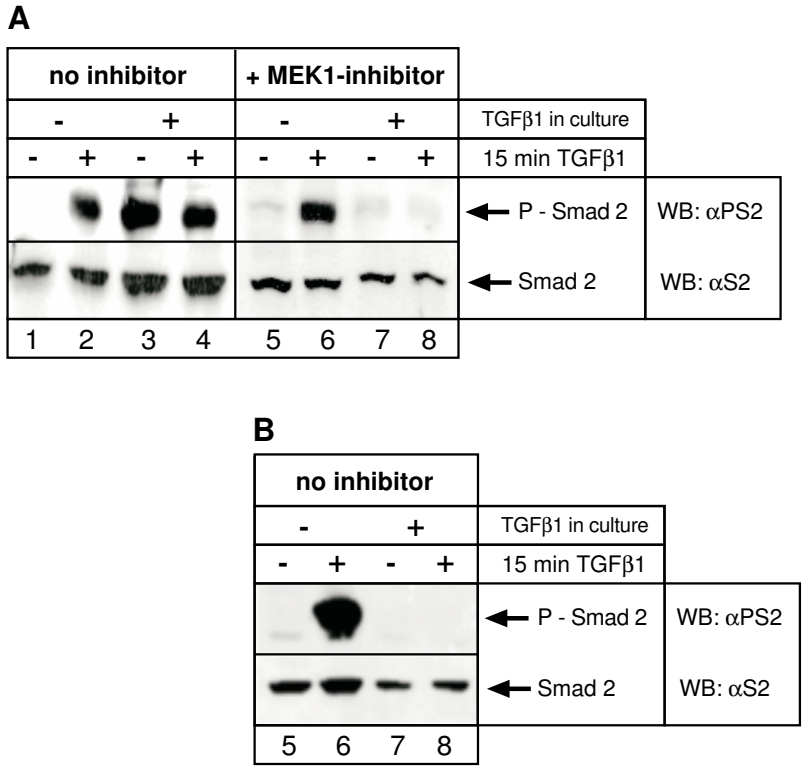


Figure 29: MEK-1 inhibitor abrogates sustained Smad2 phosphorylation in αCD3/IL-2 stimulated total splenocytes. Murine total splenocytes and CD8+ T cells were prepared and stimulated with αCD3/IL-2 as described in Figure 18. Addition of TGF-β1 and PD98059 was as described in Figure 28. Control cells received equal amounts of DMSO but exhibited no alterations in Smad2 phosphorylation (data not shown). On day 3, equal cell numbers were starved for 3h and were then stimulated with 240pM TGF-β1

or received no stimulus. Total cell lysates were analyzed by western blotting, first with α-P-Smad2 antibody (αPS2) directed against the phosphorylated C-terminal SSXS-motif (upper panel), and second with α-Smad2 antibody (αS2) recognizing the linker region of Smad2 (lower panel).

As resistance to TGF-β1 mediated growth inhibition is connected with sustained phosphorylation of Smad2 as described in section 1, we next tested whether inhibition of MEK-1 alters the Smad2 phosphorylation pattern of total splenocytes as well. Therefore we stimulated total splenocytes with αCD3/IL-2 and cultured these cells in the presence or absence of TGF-β1 and MEK-1 inhibitor. In the absence of MEK-1 inhibitor but presence of TGF-β1 sustained Smad2 phosphorylation is present in total splenocytes (Figure 29A lanes 3 and 4), whereas no phosphorylation is observed in CTL (Figure 29B, lanes 7 and 8). Upon addition of PD98059 to total splenocytes (Figure 29 lanes 5-8) the sustained phosphorylation of Smad2 in TGF-β1 long-term cultures is completely abrogated (Figure 29 lanes 7 and 8). However, Smad2 phosphorylation of cells cultured in the presence of MEK-1 inhibitor but in the absence of long-term TGF-β1 remains intact (Figure 29 lane 6). This suggests that short-term TGF-β mediated Smad2-phosphorylation to be different from phosphorylation obtained after long-term culture with TGF-β1. As shown in Figure 28 and Figure 29, inhibition of the MEK1 pathway in total splenocytes resembles TGF-β1 responsiveness in CD8+ T cells, suggesting that the MEK-1 pathway is required for growth resistance to TGF-β1 and concomitant sustained Smad2-phosphorylation.

2.3 Inhibition of the MEK pathway does not influence TGF-β1 sensitivity in purified CD8+ T cells

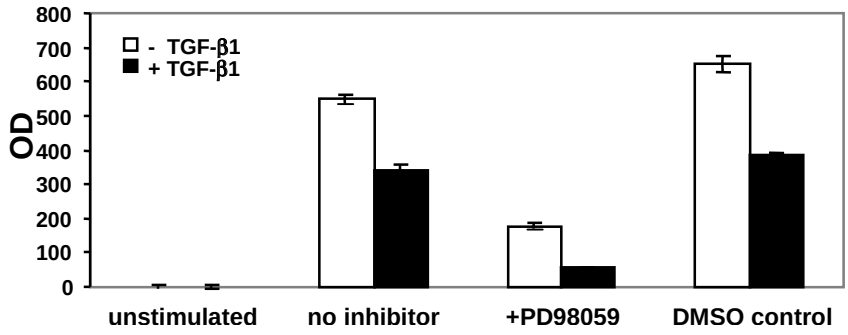


Figure 30: Addition of the MEK1-inhibitor PD98059 does not alter TGF-β1 sensitivity in purified CD8+ T cells. The CTLs are purified as described in materials and methods, section IV3.5.2 and stimulated for 3 days with αCD3/IL-2 in the presence or absence of 240pM TGF-β1. PD98059 is added on day 0, 1hour

prior to αCD3/IL-2. On day 3, total cell numbers are determined using the CellTiter proliferation assay (Promega).

As inhibition of the MEK1 pathway alters TGF-β1 sensitivity in total splenocytes (Figure 28 and Figure 29), we next wanted to determine if inhibition of the MEK pathway has effects on purified CTL as well. Therefore, we purified CD8+ T cells as described, and added 50µM PD98059 1h prior to αCD3/IL-2 and TGF-β1. As shown in Figure 30, CTL thus stimulated are growth inhibitied by TGF-β1 regardless of MEK1-inhibition. Upon blocking the MEK1 pathway, the overall proliferation is lower. Cells that received equal amounts of DMSO reacted as cultures that received no PD98059. We therefore conclude, that inhibition of the MEK1 pathway sensitizes formerly resistant total splenocytes to TGF-β1 mediated growth inhibition.

2.4 JNK-phosphorylation is crucial for resistance to TGF-β1 mediated growth inhibition

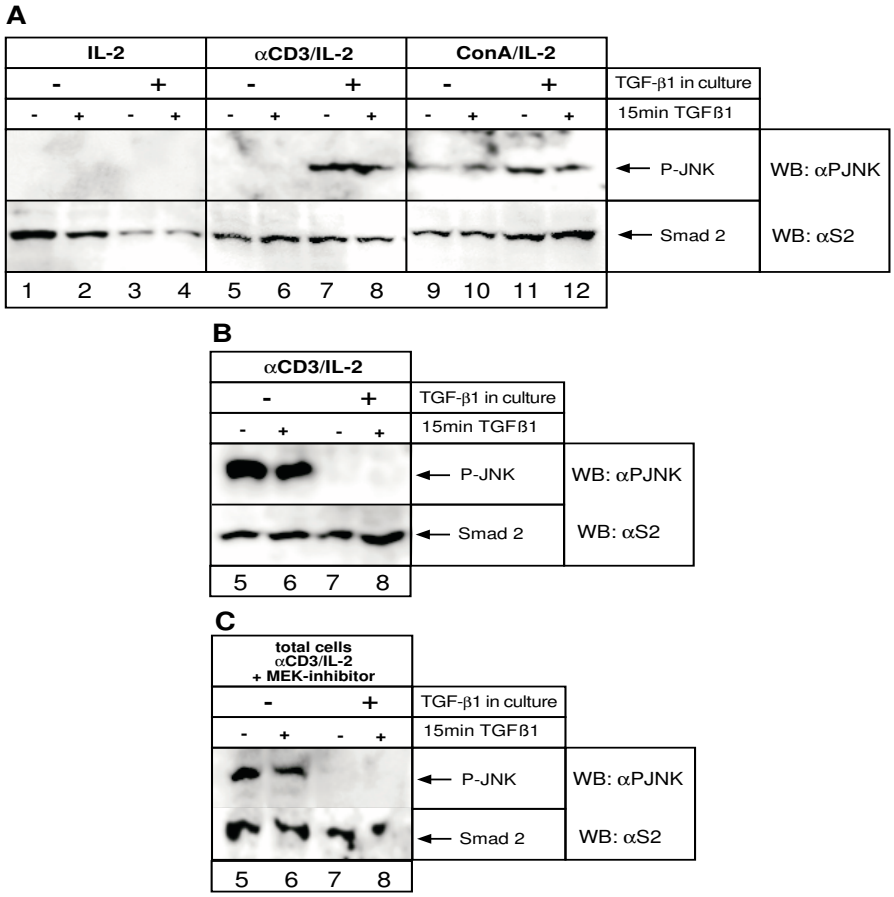


Figure 31:**Long-term exposure to TGF-β1 alters JNK-phosphorylation patterns dependent on cell type and activation of MEK-1. Murine total splenocytes (Figure 31A and C) and CD8+ T cells (Figure 31B) were prepared and stimulated with αCD3/IL-2 as described. (Figure 31C) PD98059 (MEK-inhibitor) was**

used as desribed for Figure 28. On day 3, equal cell numbers were starved for 3h and were then stimulated with 240pM TGF-β1 or received no stimulus. Total cell lysates were analyzed by western blotting, first with α-P-JNK antibody (αPJNK, upper panel), and second with α-Smad2 antibody (αS2, lower panel).

Total splenocytes (Figure 31A) and CD8+ T cells (Figure 31B) were prepared and stimulated for 3 days with αCD3/IL-2 +/- TGF-β1 as described. Figure 31C shows total splenocytes stimulated with αCD3/IL-2 and cultured in the presence of MEK-1 inhibitor. As shown in Figure 31 (lanes 1-4) splenocytes stimulated with IL-2 alone did not exhibit JNK-phosphorylation. In contrast, splenocytes stimulated with αCD3/IL-2 showed JNK-phosphorylation after long-term exposure to TGF-β1 (Figure 31A, lanes 7 and 8). Splenocytes stimulated with ConA/IL-2 showed JNK-phosphorylation regardless of exposure to TGF-β1 (Figure 31A, lanes 9-12). Analyzing purified CD8+ T cells we found that stimulation with αCD3/IL-2 was sufficient to induce JNK-phosphorylation (Figure 31B, lanes 5 and 6), but this phosphorylation was abrogated upon long-term exposure to TGF-β1 (Figure 31B, lanes 7 and 8). As described in Figure 18, total splenocytes stimulated with αCD3/IL-2 or ConA/IL-2 were not growth inhibited by long-term TGF-β1, whereas IL-2 stimulated total splenocytes and CTL stimulated with αCD3/IL-2 were growth inhibited. Taken together, these data suggest that resistance to TGF-β1 mediated growth inhibition correlates with activation of the JN-Kinase, while inactive JN-Kinase correlates with sensitivity to TGF-β1 mediated growth inhibition. Upon inhibition of the MEK-1 pathway (Figure 31C, lanes 5-8), the JNK-phosphorylation pattern of total splenocytes converts to a CTL-like pattern (compare Figure 31A, lanes 5-8 with Figure 31, lanes 5-8). We conclude from these results that MEK1 is a central player in regulating TGF-β mediated responses in CTL. Stimuli leading to MEK1 activation result in resistance to growth inhibition by TGF-β, sustained Smad2 phosphorylation and sustained JNK-phosphorylation in total splenocyte populations.

3 Purified CD8+ T cells cannot be rescued from TGF-β mediated growth inhibition by addition of αCD28 and IFNγ

Next, we attempted to revert the phenotype described for purified CTLs when cultured long-term in the presence of TGF-β1. As the total splenocytes were found to be resistant to TGF-β1 mediated growth inhibition (Figure 18) and showed sustained Smad2 phosphorylation (Figure 25), we supplemented the CTL cultures with stimuli that were potentially responsible

for the total cell phenotype. As it is known that IFNγ is able to block the signal transduction of TGF-β [Ulloa, 1999#373], and IFNγ is produced in high amounts by CD4+ T cells (view introduction, Table 1), we added recombinant IFNγ to purified CTL cultures. And as TGF-β1 resistance is connected with the activation of the JNK pathway (Figure 31), and the JNK pathway itself is activated by crosslinking the CD28 coreceptor on T cells with B7-1/B7-2 on B cells (Figure 13), we also added soluble αCD28 antibody.

3.1 IFNγ or antibodies against CD28 do not restore sustained Smad2 phosphorylation

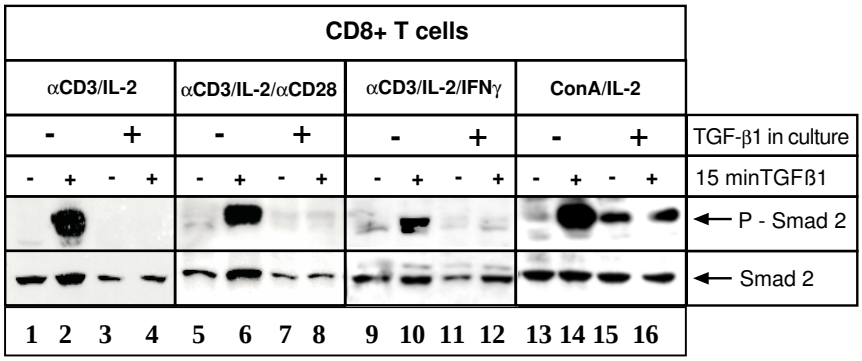


Figure 32: Addition of αCD28 or IFNγ does not lead to sustained Smad2 phosphorylation in purified CD8+ T cells. The cells were cultured with αCD3/IL-2, and received additionally either exogenous IFNγ or soluble αCD28 antibody that was reported to be stimulatory (information supplied by Pharmingen). On day 3, equal cell numbers were starved for 3h and were then stimulated with 240pM TGF-β1 or received no stimulus. Total cell lysates were analyzed by western blotting, first with α-Psmad2 antibody (upper panel), and second with α-Smad2 antibody (lower panel).

As shown in Figure 32, neither addition of αCD28 antibody (lanes 5-8) nor addition of IFNγ (lanes 9-12) cause the αCD3/IL-2 stimulated purified CD8+ T cells to exhibit sustained Smad2 phosphorylation. Although the cultures that received IFNγ showed a more activated morphology (data not shown), the supplements are not able to cause sustained Smad2 phosphorylation. In contrast, addition of ConA leads to sustained Smad2 phosphorylation (Figure 32, lanes 13-15).

3.2 IFNγ or antibodies against CD28 do not rescue sensitive T cells from TGF-β1 mediated growth inhibition

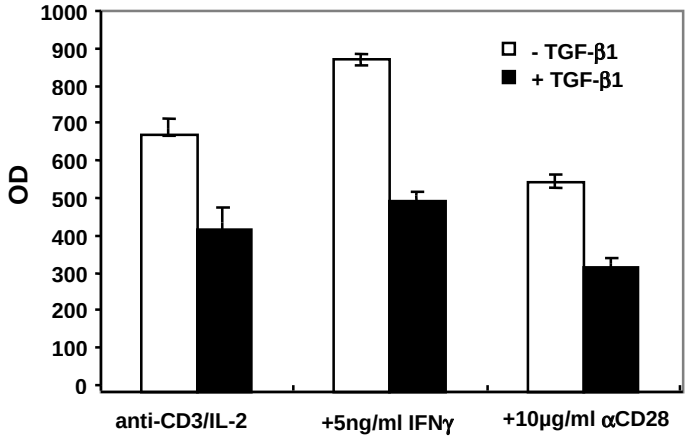


Figure 33: Addition of αCD28 or IFNγ does not rescue purified CD8+ T cells from TGF-β1 mediated growth inhibition. The cells were cultured with αCD3/IL-2, and received additionally either exogenous IFNγ or soluble αCD28 antibody. On day 3, total cell numbers were determined using the CellTiter proliferation assay (Promega)

As shown in Figure 33, neither addition of αCD28 antibody nor addition of IFNγ cause the αCD3/IL-2 stimulated purified CD8+ T cells to become resistant to TGF-β1 mediated growth inhibition. Although the cultures that received IFNγ showed a more activated morphology (data not shown) and a higher proliferation rate, the supplements are not able to render the cells resistant.

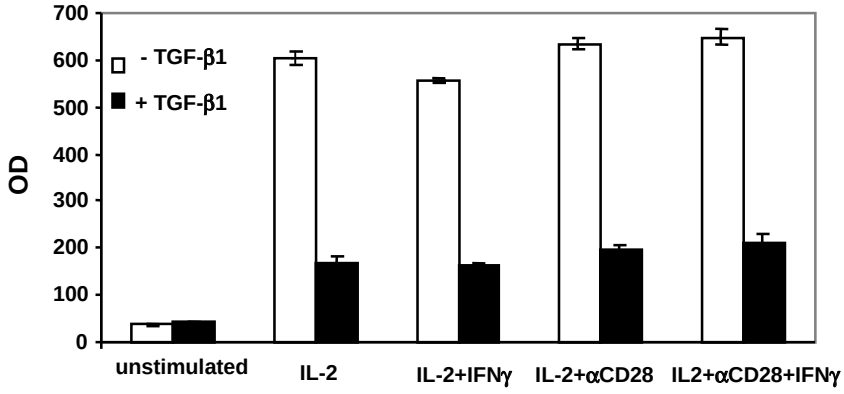


Figure 34: Addition of αCD28 or IFNγ does not rescue IL-2 stimulated total splenocytes from TGF-β1 mediated growth inhibition. The cells were cultured with αCD3/IL-2 and received additionally exogenous

IFNγ and/or soluble αCD28 Ab. On day 3, total cell numbers were determined using the CellTiter proliferation assay (Promega)

Comparable to the purified CTLs, addition of αCD28 or IFNγ did not have any effect on the TGF-β1 sensitivity of total splenocytes as well (Figure 34). Here, cultures were additionally stimulated with both IFNγ and αCD28, but still the IL-2 stimulated total splenocytes were growth inhibited by long-term TGF-β1. The mechanism by which either ConA (purified CD8+ T cells) and/or αCD3 stimulation in concert with other cells (total splenocytes) confers resistance to TGF-β1 mediated growth inhibition and sustained Smad2 phosphorylation has to be different from IFNγ/STAT pathways and CD28/JNK pathways or these pathways have to interact with yet unknown signal molecules. In further experiments we found out that supernatant from TGF-β1 resistant cultures does not suffice to induce sustained Smad2 phosphorylation in TGF-β1 sensitive cultures (data not shown), and therefore the mechanism is most likely to involve cell-cell contact, mediated by receptor crosslinking.

4 Analysis of established T cell lines

4.1 TGF-β1 growth sensitivity

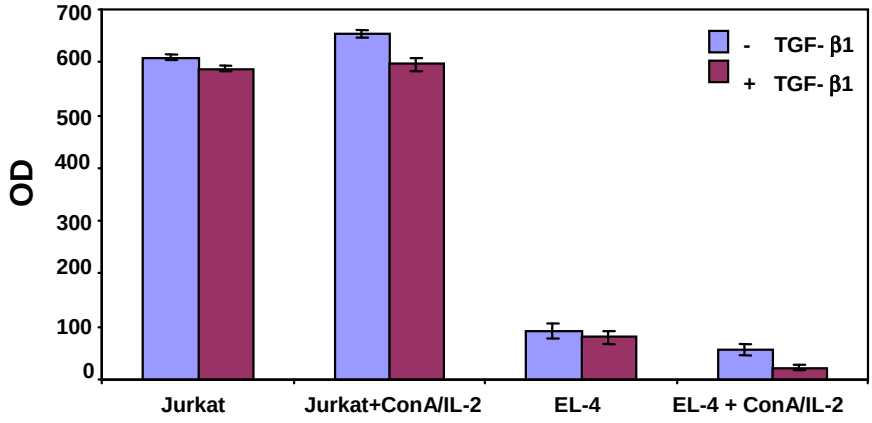


Figure 35: Effect of TGF-β1 on T cell lines. Jurkat cells are human T cell lymphoma cells, and EL-4 cells are murine T cell lymphoma. All cultures were either left untreated, and/or were stimulated with ConA/IL-2 and TGF-β1. After 3 days of culture, total cell numbers were determined using the CellTiter proliferation assay (Promega).

The analyzed cell lines include MD45, Jurkat, EL-4, P815, A301 and CTLL-2, all of which are characterized in chapter IV2.4.3. All these cell lines except the CTLL-2 were found

unresponsive to TGF-β1 mediated growth inhibition. In Figure 35, representative for all analyzed cell lines, the human T cell lymphoma Jurkat and the murine T cell lymphoma EL-4 is shown. Both cell lines are not significantly growth inhibited by TGF-β1, regardless of no stimulation versus ConA/IL-2 stimulation. Both cell lines are T helper cell lymphomas, and the data from these cell lines are consistent with the data received from the primary murine splenocytes. Activated cell pools which contain CD4+ T cells are not growth inhibited by TGF-β1. In contrast, the CTLL-2 cell line, which is characterized as murine CD8+ T cell lymphoma, is growth inhibited by TGF-β1 regardless of the type of stimulation (Figure 36). CTLL-2 cells stimulated with ConA/IL-2 showed higher total cell numbers as well.

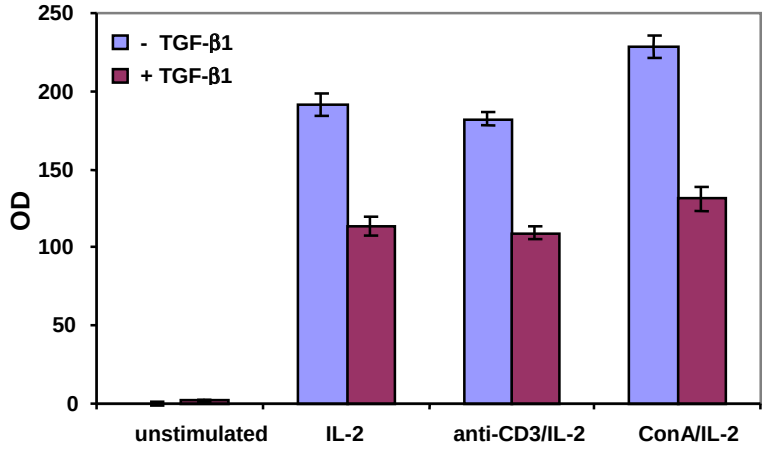


Figure 36: CTLL-2 are growth inhibited by TGF-β1. CTLL-2 cells were either cultured without stimulation (including no T-Stim supplement, as this contains ConA), or with IL-2, αCD3 and ConA as inducated in the figure. After 3 days, total cell numbers are determined using the CellTiter proliferation assay (Promega).

4.2 Expression of TβRII as detected by biotinylation

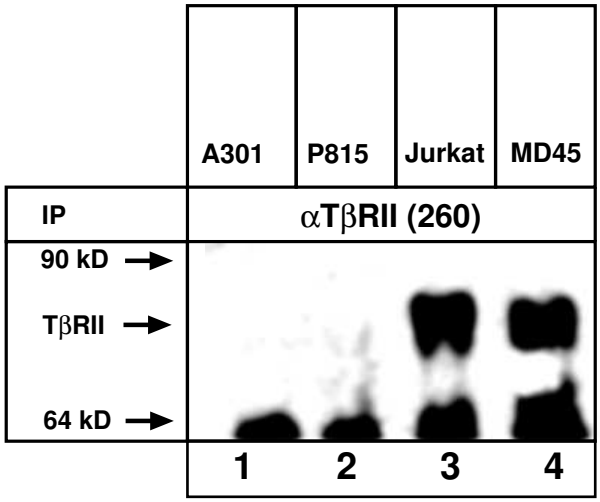


Figure 37: Expression of TβRII at the surface of immune cell lines upon long-term exposure to TGF-β1. The cell surface proteins of indicated cell lines were biotinylated as described in "materials and methods". TβRII was immunoprecipitated with anti-TβRII [Moustakas A #219].

Although A301 (human T cell lymphoma), P815 (murine mastocytoma), Jurkat (human T cell lymphoma) and MD45 (murine T cell hybridoma) were shown to be equally unresponsive to TGF-β1 mediated growth inhibition (Figure 35 and data not shown), their cell surface expression of TβRII is divergent (Figure 37). Whereas A301 and P815 do not express TβRII on their surface, and their TGF-β1 unresponsiveness is most likely founded on that fact, MD45 and Jurkat do express TβRII at their surface. The molecular mechanisms of unresponsiveness of MD45 and Jurkat to TGF-β1 therefore is suggested to be mediated by further downstream molecules.

4.3 Ability of TGF-β1 to bind to the receptors as detected by binding and crosslinking

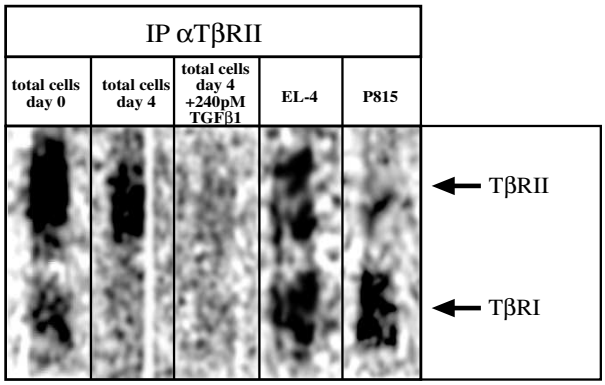


Figure 38: Binding and crosslinking of radioactive labelled TGF-β1 to the surface of cells. EL-4 and P815 cells were not stimulated. Total splenocytes were analyzed on day 0 and after 4 days of stimulation with ConA/IL-2 in the presence or absence of 240pM TGF-β1. TGF-β1 was iodinated and crosslinked to the receptors as described in Materials & Methods, chapter IV3.4.7 and IV3.4.8. The radioactive samples were immunoprecipitated with an antibody against the type II receptor, run on a SDS gel and detected by phosphoimaging.

As the TβRII of divergently stimulated primary splenocytes (Figure 21) was detected by biotinylation, and cell lines proved to differ in expression of TβRII on their surface (Figure 37), we next wanted to determine the ability of the ligand to bind to the receptors. As it is shown in Figure 38, in day 0 primary murine splenocytes TGF-β1 is bound to both TβRII and TβRI. After 4 days of stimulation with ConA/IL-2, the signal for the TβRI has disappeared. Moreover, if the cells are cultured in the presence of TGF-β1 for 4 days, both TβRI and TβRII are not detected in the crosslink. However, the TβRII can be detected by biotinylation in the same cells (Figure 21). From this we conclude that the long-term effect of TGF-β1 on murine T cell is to inhibit its own binding to the present TβRII. How this is accomplished remains to be resolved. EL-4 cells, although resistant to TGF-β1 mediated growth inhibition, are able to bind TGF-β1. P815 mastocytoma reveal conflicting data: TGF-β1 appears to be able to bind to the TβRI, although the TβRII is detected only in trace amounts. However, as the samples were subjected to immunoprecipitation agains the TβRII, the type II receptor has to be present in order to detect the type I receptor.

4.4 Phosphorylation of Smad2

As the analyzed cell lines showed divergent results with respect to their sensitivity to TGF-β1 mediated growth inhibition and the expression and binding capacities of their receptors, we next focused on the Smad signal transduction.

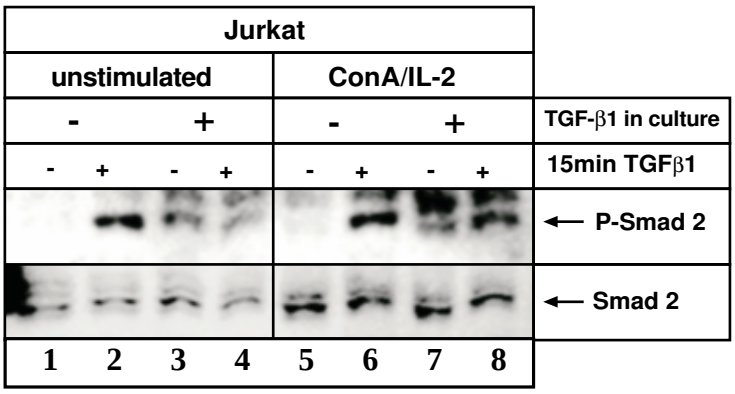


Figure 39: Smad2 phosporylation in Jurkat cells. Jurkat cells were either cultured without further stimuli or stimulated with ConA/IL-2 in the presence or absence of 240pM TGF-β1. Cellular lysates were prepared as described, and the lysates were blotted and probed against P-Smad2 and reprobed with Smad2.

Jurkat cells were found to phosphorylate Smad2 if cultured in the absence of TGF-β1 upon 15min TGF-β1 (Figure 39, lane 2 and 6). However, when the Jurkat cells are cultured in the presence of TGF-β1 (Figure 39, lanes 3, 4, 7 and 8), they show sustained Smad2 phosphorylation, whether they are stimulated with ConA/IL-2 (Figure 39, lanes 5-8) or not (Figure 39, lanes 1-4). This is consistent with the data from the proliferation assay (Figure 35), where ist was shown that Jurkats are not growth inhibited by TGF-β1 regardless of stimulation, and the data from primary splenocytes, where sustained Smad2 phosphorylation was found to be connected with resistance to TGF-β1 mediated growth inhibition (Figure 25).

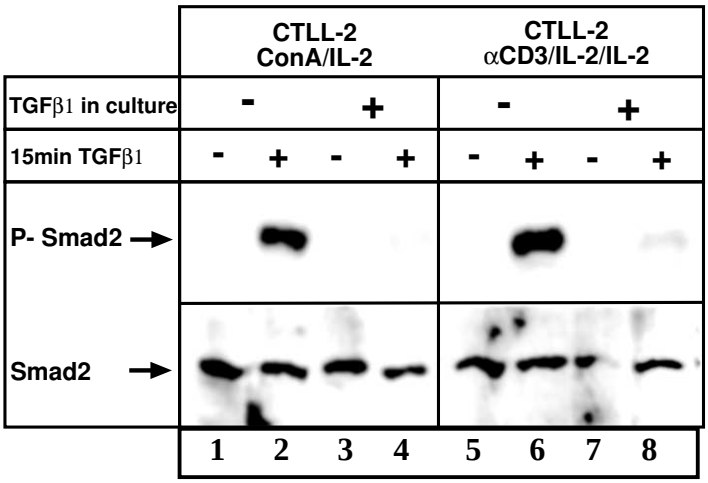


Figure 40: Smad2 phosphorylation in CTLL-2 cells. CTLL-2 cells were stimulated with ConA/IL-2 or αCD3/IL-2 in the presence or absence of 240pM TGF-β1. Cellular lysates were prepared as described, and the lysates were blotted and probed against P-Smad2 and reprobed with Smad2.

As it is shown in Figure 40, CTLL-2 cells cultured in the absence of TGF-β1 phosphorylate Smad2 upon a 15min TGF-β1 stimulus (lanes 2 and 6). However, if the CTLL-2 cells are cultured in the presence of TGF-β1 (Figure 40, lanes 3, 4, 7 and 8) they are unable to further Smad2 phosphorylation. This is consistent with the proliferation data as well, as CTLL-2 cells were found to be growth inhibited by TGF-β1 regardless of their stimulation (Figure 36), and in primary splenocytes incapability to phosphorylate Smad2 upon long-term exposure to TGF-β1 was connected with TGF-β1 mediated growth inhibition (Figure 25).

4.5 Phosphorylation of JNK

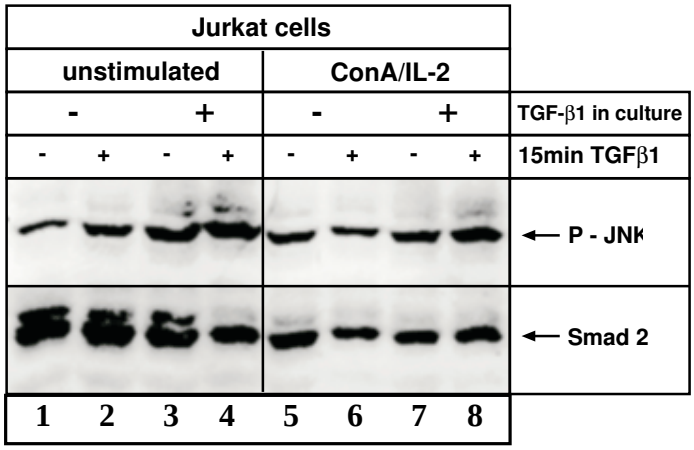


Figure 41: Phosphorylation of JNK in Jurkat cells. Jurkat cells were either cultured without stimulation or stimulated with ConA/IL-2 and cultured in the presence or absence of 240pM TGF-β1. Cellular lysates

were prepared as described, blotted and probed against P-JNK and reprobed against Smad2 as loading control.

In Jurkat cells JNK was found to be constitutively phosphorylated independent of ConA/IL-2 stimulation (Figure 41 lanes 5-8) or no stimulation (lanes 1-4), and also independent of TGF-β1 stimulation (Figure 41), in consistence with the described insensitivity to TGF-β1 mediated growth inhibition of Jurkat cells (Figure 35) and their expression of sustained Smad2 phosphorylation (Figure 39). Figure 31 shows JNK phosphorylation in primary splenocytes, and here the data are consistent as well. Primary cells resistant to TGF-β1 show JNK phosphorylation, and cells sensitive to TGF-β1 show no JNK phosphorylation.

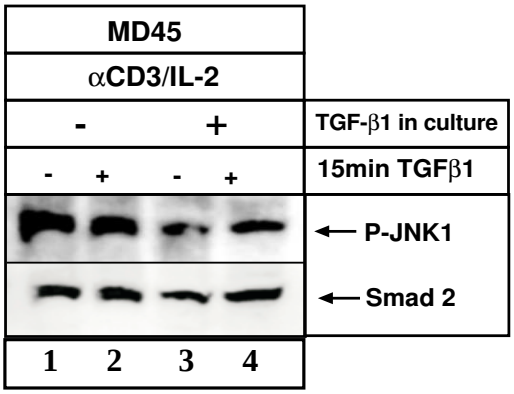


Figure 42: Phosphorylation of JNK in MD45 cells. MD45 cells were stimulated with αCD3/IL-2 and cultured in the presence or absence of 240pM TGF-β1. Cellular lysates were prepared as described, blotted and probed against P-JNK and reprobed against Smad2 as loading control.

Constitutive JNK phosphorylation is shown by MD45 hybridoma cells as well, regardless of TGF-β1 stimulation (Figure 42, lanes 1-4). MD45 cells are not growth inhibited by TGF-β1 (data not shown), but express the TβRII on their surface, and therefore resemble the the Jurkat cells in respect to their reaction to TGF-β1 (Figure 35, Figure 37 and Figure 41).

5 Cloning of the TβRIIΔcyt-Thymidine kinase construct

As described in the introduction, there is the possibility to use cytotoxic T cells in the elimination of cancer. Normal T cells are limited in this respect to cancer cells that present neoantigens on their MHC class I complexes. To circumvent this limitation, the usage of chimeric single chain antibodies have been a point of interest in recent years. Hereby, the ScFv constructs (Figure 14 and Figure 15) were introduced into primary T cells via retroviral-

mediated gene transfer. However, it is also reported [Altenschmidt U #6] that the provision of tumor cell specific T lymphocytes is not sufficient, as tumors secrete TGF-β and thereby inhibit the cytotoxic activity of the T cells. Inspired by this fact, we wanted to generate T cells which are designed to express a dominant-negative TβRII at the surface, which is trunkated by a stop-codon shortly after the transmembrane region, rendering them unresponsive to TGF-β1. This TβΔcyt had already been cloned by Marion Lutz in the laboratory of Dr. Petra Knaus. However, rendering T cells unresponsive to TGF-β might turn fatal, as this possibly creates lymphomas itself. The idea was now, to create a chimeric construct of this DN Tβdeltcyt, linked to the Herpes simplex virus thymidine kinase (HSV-TK). Upon addition of ganciclovir (GCV), a nucleoside analog, the TK becomes toxic: Phosphorylation of GCV is phosphorylated to its triphosphorylated form by the TK and host cell kinases. The triphosphorylated form acts as a chain terminator and interferes with DNA synthesis in replicating cells, what will eventually cause cell arrest or cell death [Matthews T #206]. Thus, linking this HSV-TK to the DN TβRIIΔcyt enables to eliminate the TGF-β resistant T cells after they killed the tumor cells simply by administration of ganciclovir. So the chimeric construct was cloned by recombinant PCR from the EGIRT-Tβdeltcyt and the FOV-7-TK.

5.1 Cloning of the TβΔcyt-TK by recombinant PCR

5.1.1 Cloning of TβΔcyt in pet3d

The construct EGIRT-TβΔcyt, containing an HA-tag, was provided by Marion Lutz. As first step, TβΔcyt (877bp) was cut from the vector using BamHI/NcoI and was cloned into the vector pet3d, which was opened with BamHI/NcoI as well. After ligation, the pet3d-TβΔcyt was transformed into *E. coli* C600. In this vector, however, the construct was in reverse direction and thus had to be cloned into pcDNAIII.

5.1.2 Cloning of TβΔcyt in pcDNAIII

First, the pet3d-TβΔcyt was digested with XbaI, followed by filling the sticky ends with Klenow polymerase to create blunt ends. Then, the linearized vector was digested with BamHI to release the TβΔcyt. Next, the pcDNAIII vector was digested with HindIII followed by Klenow to create blunt ends. Then, pcDNAIII was digested with BamHI. Now the TβΔcyt was ligated into pcDNAIII and transformed into *E. coli* C600, where the quality of the clones was analyzed by PCR screening and sequencing.

5.1.3 Recombinant PCR

First, the thymidine kinase was cut from the FOV-7-TK vector using NcoI and NruI. The TβΔcyt fragment was freed from the pcDNAIII using NcoI and BamHI. The first PCR used the thymidine kinase fragment and the primers TTK1 and TTK2 (view IV2.4.2), resulting in a fragment that covered the TβΔcyt stop codon destroying it, creating a linker between the end of TβΔcyt and the TK start, leading up to the end of the TK. This fragment was of the length of 1168 bp, including the 1127bp of the TK and 27bp of the newly created linker region. The second PCR, using T7 and TTK3, amplified the TβΔcyt destroying the stop-codon and creating the same linker region as well. In a third step, the recombinant PCR was performed as hot start low stringency PCR with the primer T7 and TTK2:

96°C 6min→ addition of Pfu→ 95°C 45s→ 48°C 1min→ 72°C 4min→ 72°C 10min

This recombinant PCR resulted in the TβΔcyt-TK fragment, 1844bp in length:

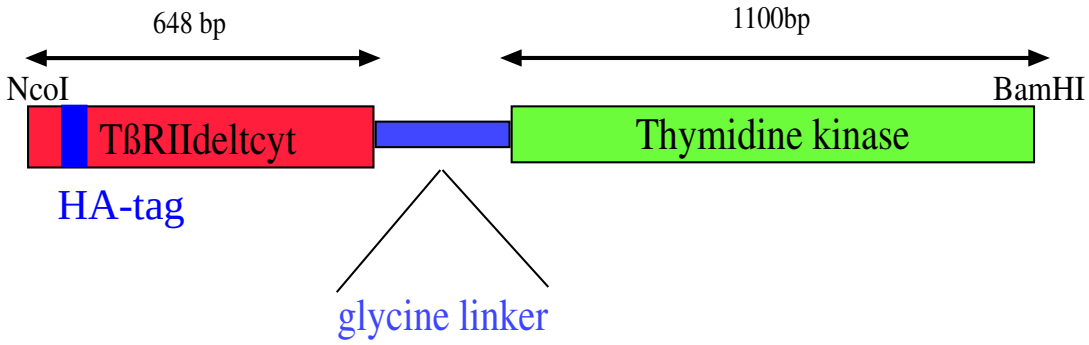


Figure 43: TβTK construct as result of the recombinant PCR. The construct is flanked by unique NcoI and BamHI sites. The extracellular TβRII-deltcyt has a size of 648bp, carries a HA tag and is glycosylated in the cell. The middle consist of a linker region, followed by the thymidine kinase on the intracellular side (1100bp).

5.1.4 Cloning of TβΔcyt-TK in pcDNAIII and EGIRT

To insert the TβΔcyt-TK construct into the pcDNAIII vector, it proved to require another PCR step. As other unique restriction sites in the TβΔcyt-TK construct were absent, the former HindIII site that had been destroyed using blunt-end ligation had to be restored by PCR with the primer TTK4 (view IV2.4.2.) Both pcDNAIII vector and TβΔcyt-TK were digested with BamHI and HindIII, followed by ligation, transformation and sequencing of the positive clones. Then, the TβΔcyt-TK was digested with NcoI/BamHI and was inserted into the retroviral EGIRT vector.

Sequence of the TβΔcyt-TK in pcDNAIII:

T7 primer start

TAATACGACTCACTATAGGGAGACCCAAGCTCTAGAAATAATTTTGTTTAACTTT

NcoI

AAGAAGGAGATATACCATGGgtcgggggctgctcaggggcctgtggccgctgcacatcgtcctgtggacgcgtat

HA-tag

cgccagcacgatcccaccg**TACCCATACGACGTCCCAGACTACGCC**cacgttcagaagtcggttaataa

cgacatgatagtcactgacaacaacggtgcagtcaagtttccacaactgtgtaaattttgtgatgtgagattttccacctgtgacaaccaga

aatcctgcatgagcaactgcagcatcacctccatctgtgagaagccacaggaagtctgtgtggctgtatggagaaagaatgacgagaa

cataacactagagacagtttgccatgaccccaagctcccctaccatgactttattctggaagatgctgcttctccaaagtgcattatgaag

gaaaaaaaaaagcctggtgagactttcttcatgtgttcctgtagctctgatgagtgcaatgacaacatcatcttctcagaagaatataacac

cagcaatcctgacttgttgctagtcatatttcaagtgacaggcatcagcctcctgccaccactgggagttgccatatctgtcatcatcatctt

TTK1 primer

ctactgctaccgcgttaaccggcagcagaagctgagt**GGAGGTGGCGGGGGAGGT**gcttcgtacccctgccatca

TTK3 primer **linker**

acacgcgtctgcgttcgaccaggctgcgcgttctcgcggccataacaaccgacgtacggcgttgcgccctcgccggcaacaaaaag

ccacggaagtccgcctggagcagaaaatgcccacgctactgcgggtttatatagacggtccccacgggatggggaaaaccaccacc

acgcaactgctggtggccctgggttcgcgcgacgatatcgtctacgtacccgagccgatgacttactggcgggtgttgggggcttccg

agacaatcgcgaacatctacaccacacaacaccgcctcgaccagggtgagatatcggccggggacgcggcggtggtaatgacaag

cgcccagataacaatgggcatgccttatgccgtgaccgacgccgttctggctcctcatatcgggggggaggctgggagctcacatgc

cccgcccccggccctcaccctcatcttcgaccgccatcccatcgccgccctcctgtgctacccggccgcgcgataccttatgggcagc

atgacccccaggccgtgctggcgttcgtggccctcatcccgccgaccttgcccggcacaaacatcgtgttgggggcccttccggag

gacagacacatcgaccgcctggccaaacgccagcgccccggcgagcggcttgacctggctatgctggccgcgattcgccgcgtttat

gggctgcttgccaatacggtgcggtatctgcagggcggcgggtcgtggcgggaggattggggacagctttcgggggcggccgtgc

cgccccagggtgccgagccccagagcaacgcgggcccacgaccccatatcggggacacgttatttaccctgtttcgggcccccgag

ttgctggcccccaacggcgacctgtataacgtgtttgcctgggctttggacgtcttggccaaacgcctccgtcccatgcatgtctttatcct

ggattacgaccaatcgcccgccggctgccgggacgccctgctgcaacttacctccgggatggtccagacccacgtcaccaccccag

BamHI

gctccataccgacgatctgcgacctggcgcgcacgtttgcccgggagatgggggaggctaactgaGGATCCACTAGTA

TTK2 primer

ACGGCCGCCTAATCGAT

5.2 Expression of TβΔcyt-TK in transfected cells

5.2.1 Expression in 293T cells

To verify that the hybrid construct is indeed expressed by transfected cells, 293T cells were transfected with the construct and the expression of TβΔcyt-TK was analyzed in WB. As it is shown in Figure 44, the 293T cells express the contruct as detected by α-HA western blot at a size of 84kD.

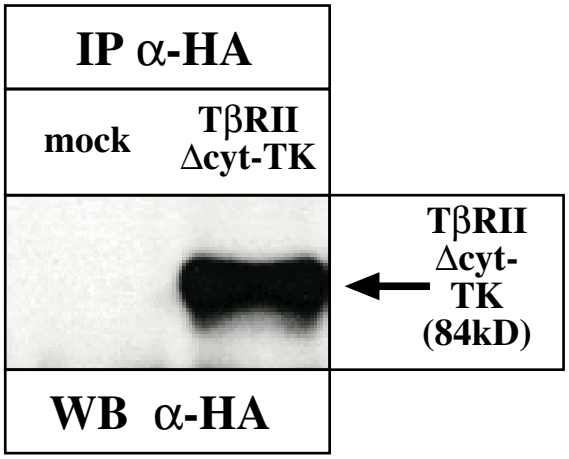


Figure 44: Expression of TβΔcyt-TK in 293T cells. 293T cells were transfected following the $CaPO_4$ transfection protocol (materials & methods, IV3.3.2.) As mock control, CMV-GFP was utilized. After 3 days of transfection, the cells were lysed in multiple freeze-thaw-cycles in lysis buffer, and subjected to a preclear with PAS. The TβΔcyt-TK was precipitated using α-HA antibody. The samples were run on a 12% SDS gel, blotted, and detected with α-HA antibody.

5.2.2 Expression in CTLL-2 and primary T cells

It was also attempted to express the TβΔcyt-TK construct in the CTLL-2 cytotoxic T cell line and primary splenocytes. However, neither CTLL-2 nor primary T cells were able to be transfected by $CaPO_4$, DEAE-Dextran or various liposomal based (Lipofectamine and numerous others) transfection techniques. Retroviral infection also failed due to unknown reasons. Therefore, it remains to be proven that the TβΔcyt-TK construct indeed confers resistance to TGF-β1 and leaves the possibility to eliminate the transformed T cells after exerting their lytic function.

III Discussion

As described in the results section, the aim of this study was to analyse TGF-β1 action on T cells on cellular and molecular levels. Of major importance hereby was to dissect the influence of different experimental conditions, as we thought that divergent stimulation, cellular context and thus availability of cytokines may influence the sensitivity of T cells to TGF-β1. Therefore, we analysed both total murine spleen cells, enriched for T cells, and purified CD8+ T cells. Both types of cultures were either stimulated with IL-2 alone without TCR-activation, or stimulated polyclonally via TCR crosslinking (αCD3) plus IL-2, or by addition of lectin (ConA) plus IL-2. The utilized TGF-β1 concentration was hereby kept constant at 240pM, and treatment duration was restricted to 5 days (cytotoxicity assay) and 3 days (other assays). All data are summarized in Table 2.

	Proliferation		**Cyto-toxicity**	**Smad2-Phosphorylation**		**JNK-phosphorylation**	
	total	***CD8+***	***total***	***total***	***CD8+***	***total***	***CD8+***
IL-2	sensitive	sensitive	sensitive	none	none	none	n.d.
αCD3/IL-2	resistant	sensitive	n.d.	sustained	none	sustained	none
ConA/IL-2	resistant	slightly sensitive	sensitive	sustained	weak sustained	sustained	n.d.

Table 2: Summary of effects of TGF-β on differentially stimulated total spleen versus purified CD8+ T cells. All results depicted were obtained including TGF-β1 in the culture conditions. "Resistant" cells did not exhibit TGF-β1 mediated growth inhibition, whereas "sensitive" cells were growth inhibited by TGF-β1. "Sustained" phosphorylation is defined as a phosphorylation, that is induced by TGF-b1 in culture but persists even in the absence of TGF-b1 for more than 3h (JNK) or 6h (Smad2). "None" phosphorylation referres to cells that were unable to phosphorylate JNK or Smad2 after 15min of TGF-β1 stimulation.

To simplify further explanations, two distinct T cell phenotypes are defined based on the results of the differentially stimulated primary splenocytes: The "resistant" phenotype shows unresponsiveness to TGF-β1 mediated growth inhibition (Figure 18), sustained Smad2 phosphorylation (Figure 25) and constitutive JNK phosphorylation (Figure 29). The "sensitive" cells are growth inhibited by TGF -β1 (Figure 18), do neither show sustained

Smad2 phosphorylation nor Smad2 phosphorylation induced by 15min of ligand addition, (Figure 25) and do not show JNK phosphorylation (Figure 29).

1 Characteristics of total splenocyte cultures versus purified CD8+ T cell cultures

The primary cell cultures were initially stimulated with a combination of three potent activators of T cells: αCD3, ConA and IL-2. The αCD3 antibody in combination with IL-2 is a sufficient stimulus to generate lymphocyte expansion. ConA is a widely employed mitogen that interacts nonspecifically with surface glycoproteins and is capable of activating T cells through the T cell receptor and likely through other glycosylated surface receptors [Pani G #236]. At high doses, IL-2 activates primary T lymphocytes and enables CD8+ T cells to express lytic potential. Primary cells normally require 3 divergent signals for proliferation and CTL gene expression: First they are stimulated through the T cell receptor, second by IL-2 through the IL-2R, and third through further costimulatory signals such as CD28 [Lenschow DJ #174; Kane LP #144]. Cells stimulated only by IL-2 are lacking activation via TCR/CD28 compared to ConA/IL-2 stimulation, and αCD3/IL-2 stimulation lacks CD28 and other costimulations. Looking at purified cultures versus total splenocytes, the picture is even more complex. Whereas in the total splenocyte pool cells such as B-cells, macrophages and Th-cells may provide costimulation via cell-cell-contact and help via cytokine production, in the purified CD8+ cell pool these effects are excluded. This might be the reason for differences observed in αCD3/IL-2 stimulated CD8+ cells versus total splenocytes. Here, costimulation and cytokines such as IFNγ, known to be abundantly produced by Th1 cells, are missing. However, in CD8+ purified cultures, ConA compensates partially for non-CD8+ T cells, as ConA crosslinks glycosylated surface molecules other than the TCR and thus mimicks costimulation via CD28, CD40L, CD2 or CD45 [Holter W #119; Lenschow DJ #174; Justement LB;# 141; Grewal IS #99]. This appears in Figure 18B and Figure 25B (lanes 11 and 12), where ConA/IL-2 stimulated CD8+ T cells adopt the the phenotype of total splenocyte cultures. Additional treatment with TGF-β1 might cause direct or indirect effects on T cells. It is known, for example, that the effect of TGF-β1 on T cells is partially mediated via APC [Takeuchi M #306; King C #153], whereas others report that TGF-β1 suppresses T cell function in the absence of APC [Ludviksson BR #192]. In general, it was proven that CD28 costimulation has an influence on the reactivity of T cells to TGF-β. McKarns *et al*. showed that in activated splenocytes CD28 costimulation alters the effects of TGF-β1 on IL-2

protein expression [McKarns SC #209]. CD28 costimulation may also indirectly modulate the effect of TGF-β1 in response to CD3 antibodies by enhancing additional cytokine production [Boise LH #17]. Whereas the action of TGF-β is modulated by CD28 costimulation, TGF-β1 itself appeares to have no influence on the expression levels of costimulatory molecules such as CD28 or B7-1 [Ludviksson BR #192]. Concluding from the fact that purified CD8+ T cells may adopt the "resistant" phenotype either upon cocultivation with non -CTL cells OR upon stimulation with ConA, we suggest a direct mode of TGF-β1 action on T cells.

2 Proliferation

In the literature, the most controversial results concerning TGF-β effects on T cells is proliferation. Lee *et al*. suggest that TGF-β1 costimulates αCD3-induced splenic T cell proliferation independent of IL-2 [Lee HM #171]. Consistent with these results, Zhang et al report that IL-2 and TGF-β synergize to yield higher cell numbers [Zhang X #365], in part by the blockage of apoptosis by TGF-β, which is also described by Cerwenka et al. [Cerwenka A #30]. Other reports stress the inhibitory properties of TGF-β on T cells *in vitro* and *in vivo* [Bright JJ #20; Vanky F #319; Bright JJ #21; Nagy N #224; Lucas PJ #191; Boussiotis VA #19], which is also confirmed by TGF-β1 knockout mice, which exhibit multifocal inflammatory diseased due to the lack of T cell growth inhibition [Christ M #47].

2.1 Proliferation of total splenocyte cultures

Varying the culture conditions of splenocytes and purified CD8+ T cells (CTL) we found that total splenocytes stimulated with IL-2 alone are sensitive to TGF-β1 mediated growth inhibition (Figure 18A). In contrast, ConA/IL-2 and αCD3/IL-2 stimulated total cells are resistant to TGF-β1 mediated growth inhibition (Figure 18A). As TGF-β was also reported to influence apoptosis, the cells were also stained with trypan blue to detect cells that are not viable. However, in none of the cultures significant amounts of dead cells were observed, suggesting that the observed differences of the cultures in total cell numbers are caused by regulation of proliferation instead of regulation of cell death. To evaluate these findings, it is important to consider the percentage of T cells in these cultures, as it might seem possible that the differences between cytokine only (IL-2) and cytokine + TCR (IL-2 + ConA/αCD3) cultures may be based upon different amounts of T cells. As shown in Figure 19, the percentage of CD8+ T cells varies in these cultures from 30 to 40 percent and is thus not considered as significant. Most important, the CD8+ cell percentage between the resistant

αCD3/IL-2 and the sensitive IL-2 stimulated cultures is equally high (30%, Figure 19A). Although the CD8+ content in IL-2 stimulated cells drops slightly upon long-term culture with TGF-β1, whereas the percentage in ConA/IL-2 and αCD3/IL-2 stimulated cells remains contant, this reduction of 5% is unlikely to determine sensitivity versus insensitivity to TGF-β1 mediated growth inhibition (Figure 19). This is in contrast to the findings of Lucas *et al*., where transgenic T cells were rendered insentitive to TGF-β1 by a DN TβRII [Lucas PJ #191]. Here, mainly the CD8+ T cells were shown to proliferate abnormally, suggesting that TGF-β1 exerts its control predominantely on CD8+ cells. Our data, however, show that CD8+ cells are not stronger inhibited by TGF-β1 as other splenocytes. Differences in TGF-β1 sensitivity are based upon divergent stimulation instead. This is in agreement with findings where TGF-β did not affect proliferation of neither CD4+ T cells [Zhang X #365] nor CD8+ T cells [Lee HM #171]. However, later Lee *et al*. report stimulation of CD8+ T cells and reduced numbers of CD4+ T cells upon TGF-β costimulation of splenic T cells [Lee HM #172].

Bright *et al*. analyzed, comparable to our study, the effect of TGF-β2 on the proliferation of ConA-stimulated splenocytes in the SJL/J mouse strain [Bright JJ #21]. In contrast to our findings, they report that TGF-β abrogates signaling via the Jak-Stat pathway, leading to decreased proliferation and increased apoptosis. One explanation concerning the contradictory results could be the fact that they used TGF-β2 (versus TGF-β1) and SJL/J mice (versus C57BL/6), as from knockout studies it is known that TGF-β1 and -β2 have different effects [Christ M #47; Sanford LP #272], and it is also known that mice strains react differently to immmunological challenge (compare Th2 bias in Balb/c mice and Th1 bias in C57BL/6 mice). More important, however, is the fact that Bright *et al*. did not supplement the cultures with exogeneous IL-2. If no IL-2 is added, all of our cultures are sensitive to TGF-β1. The findings that TGF-β impairs the signal transduction of the IL-2Rβ [Bright JJ #20], however, does not explain these findings, as exogeneous IL-2 is unlikely to overcome defects based on disturbed signal transduction. The question why exogeneous IL-2 is able to abrogate TGF-β mediated growth inhibition remains unsolved, and our findings here are in contrast to other findings, where exogeneous administration of IL-2 did not reverse the suppressive effects of TGF-b1 [Ludviksson BR #192]. We did not follow the findings obtained without addition of exogeneous IL-2, as purified CD8+ T cells are unable to survive without exogeneous IL-2, particularly as our focus lay on the analysis of CTL with respect to tumor defence. We thus

had to add IL-2 in any case, which we did also with the total splenocyte cultures for direct comparison.

2.2 Proliferation of CD8+ CTL

In contrast to total splenocyte cultures, purified CD8+ T cell cultures do not contain accessory cells such as macrophages, B cells or CD4+ T cells that would be able to provide costimulation via cytokine secretion or ligation of costimulatory molecules such as CD28. This is the important difference between the two types of cultures when considering the results obtained from the purified cells. In contrast to total splenocytes, TGF-β1 downregulates proliferative responses in purified CD8+ cell cultures stimulated with both IL-2 and αCD3/IL-2 (Figure 18B), whereas CTL stimulated with ConA/IL-2 showed a much weaker inhibition. This suggests, that in CTL cultures, ConA compensates partially for non-CD8+ T cells, as ConA/IL-2 stimulated cells show almost no reduction in proliferation rate. One possible mechanism is CD28 costimulation, as it was shown that in activated splenocytes CD28 costimulation alters the effects of TGF-β1 on IL-2 protein expression [McKarns SC #209]. CD28 costimulation may also indirectly modulate the effect of TGF-β1 in response to CD3 antibodies by enhancing additional cytokine production [Boise LH #17], whereas TGF-β1 itself appeares to have no influence on the expression levels of costimulatory molecules such as CD28 or B7-1 [Ludviksson BR #192]. The mechanisms by which CD28 possibly modulates TGF-β1 sensitivity are discussed in chapter 6.4. As described for the total splenocytes, we also prove that in purified cultures the differences in TGF-β1 sensitivity are not due to a selective outgrowth of non-CTLs. In cultures where TGF-β1 leads to inhibition of cell growth, CD8+ CTL cell numbers do not differ from those measured in resistant cultures (Figure 19B).

Focussing on the CD8+ cells our data is in agreement with Lucas *et al.* [Lucas PJ #191]. The TGF-β1 resistant, hyperproliferating CD8+ T cells Lucas *et al.* analysed, were unchallenged, and hypoproliferation was observed *in vivo* as splenomegaly. This suggests that CTL without TCR activation and costimulation, as analysed in IL-2 and αCD3/IL-2 stimulated T cells (Figure 18B), are growth inhibited, and does not exclude resistance to TGF-β1 in fully activated CTLs (Figure 18B).

2.3 Direct TGF-β interaction versus indirect interaction

Whether the effect of TGF-β on T cells is mediated directly or indirectly via APC and non-T cells is controversely discussed in the literature. Some studies report that the effect of TGF-β1 on T cells is partially mediated via APC [Takeuchi M #306; King C #153], whereas others report that TGF-β1 suppresses T cell function in the absence of APC [Ludviksson BR #192]. Considering our experiments, one can exclude non-T cell-effects in purified CTL cultures (mimicked, however, by ConA), whereas involvement of non-T cells is certainly present in total splenocyte cultures. Concluding from our results, we suggest that the inhibitory action of TGF-β1 on CTLs is direct, as purified CTLs are inhibited in the absence of other cells (Figure 18B). Resistance to TGF-β1, however, is mediated indirectly, as for resistance to growth inhibition (in addition to TCR ligation) either the presence of non-T cells or crosslinking of surface receptors by ConA is a prerequisite (Figure 18A and B).

Taken together, the proliferation data suggests that upon the presence of sufficient IL-2, correct TCR activation in concert with appropriate costimulation, T cells in general and CD8+ cytotoxic cells in particular are resistant to TGF-β1 mediated growth inhibition. If IL-2 is insufficient, no TCR stimulus is provided (IL-2 stimulation only) and/or costimulation is absent (CD8+ αCD3/IL-2 stimulated), the cells are growth inhibited by TGF-β1. As explained earlier (view discussion section 2.1), TGF-β1 in this case is not regulating proliferation via inhibiting IL-2. Therefore, inhibition of proliferation may be due to a direct effect of TGF-β1 to impair cell cycle progression. For example, TGF-β1 blocks T lymphocyte progression through the G1/S phase checkpoint of the cell cycle [Wahl SM;# 324], presumably by modulating cyclin-dependent kinase activity [Saltis J;# 271].

2.4 Effects of TGF-β on T cell differentiation

Although the effect of TGF-β1 on differentiation of T cells was not the major aspect of our study, it is nevertheless an important feature. In our cultures, with the exception of the percentage of CD8+ cells in the cultures, we did not analyse variations of T cell subpopulations. Nevertheless, it cannot be excluded that differentiation occurred, and therefore the influence of TGF-β on T cell differentiation is discussed briefly.

During T cell development, a key step involves the positive selection of cells that recognize antigen presented by the MHC molecules. The work of Takagi *et al*. suggests the transcription factor Schnurri-2 (Shn-2) to play a role in this important maturation process [Takagi T #303]. Drosophila Shn acts as a cofactor of Smad homolog, and Vertebrate Shn 1-3 are thought to

act as nuclear targets in signal transduction of the TGF-β family proteins. Several studies have been undertaken concerning TGF-β determining the generation of Th1 versus Th2 and Tc1 versus Tc2 cells (view introduction I2.2). Erard *et al.* propose, that, depending on the presence or absence of IL-4, TGF-β either inhibits or induces the generation of type 1 CD8+ T cells [Erard F #78].

Studies from other groups confirm differences in TGF-β1 effects dependent on the type and differentiation state of T cells. Lúdviksson *et al.*, for example, report that TGF-β1 inhibits Th1 and Th2 cells when added in the priming phase of the immune response. In contrast, when added to established memory T cells, TGF-β1 did solely inhibit Th1 cells but not Th2 cells [Ludviksson BR #192]. TGF-β1 is also known to downregulate Th2 development of naïve CD4+ cells and their GATA-3 expression, as well as it diminished IL-4 induced STAT6 activation [Gorelik L #95; Heath VL #110]. Additionally, in a more recent study Gorelik *et al.* describes the mechanism of TGF-β-induced inhibition of Th1 cells [Gorelik L #94]. Interestingly, they are coupling the *in vitro* study to an *in vivo* study, explaining the inability of Th2 biassed Balb/c mice to mount an immune response to Leishmania-infection to the TGF-β mediated inhibition of Th1 responses. These studies provide an insight into the complex field of T cell differentiation and the involvement of TGF-β herein, being of major importance in the field of allergy and infectious diseases.

3 Lytic activity

Specific lysis of target cells by CTL was shown to be negatively influenced by TGF-β1 in multiple experimental systems including tumor-specific human CTL and murine splenocytes [Vanky F #319; Pardoux C #238]. However, it is also reported that TGF-β costimulated CD8+ splenocytes exhibit cytolytic activity [Lee HM #172] if the assay is performed in the absence of TGF-β. Therefore, to analyze the lytic potential of "resistant" versus "sensitive" (with respect to proliferation) T cells, we assayed differentially stimulated total splenocyte cultures for lytic function (Figure 20 and Table 2). In IL-2 stimulated cultures we measured abrogation of lytic activity upon TGF-β1 addition (Figure 20A). These cells were shown to be growth inhibited by TGF-β1 (Figure 18A) and resembled a non-activated cell morphology. Surprisingly, however, ConA/IL-2 stimulated cells also lost their lytic activity upon long-term exposure to TGF-β1 (Figure 20B). This is consistent with previous findings, where lytic activity and proliferation of CTL do not always have to occur simultaneously [Rollinghoff M #266].

However, several reports indicate that cytolytic effector function fails to develop if proliferation is inhibited [Cronin DC, 2nd #55]. In contrast, when ConA-stimulated T cells were also exposed to various lymphokine containing supernatants, the extent of proliferation did not correlate with the magnitude of the cytolytic response generated. It was concluded that although IL-2 could cause replication of CTL precursors, other (unknown) factors were required for differentiation into CTL [Wagner H #323]. Nevertheless, in the case of TCR and costimulation in concert with IL-2, all leading to proliferation, cytolytic T cells are prone to arise [Cronin DC, 2nd #55]. In contrast, if the cells are inhibited by cell cycle inhibitors, they fail to develop lytic activity [Cronin DC, 2nd #55]. Most astonishing, however, is that in our primary cell system, if ConA/IL-2 stimulated cells are cultured long-term in the presence of TGF-β1, they proliferate normally (Figure 18), but do not express lytic activity (Figure 20). This suggests that neither stimulation of the TCR nor proliferation is sufficient to induce cytolytic activity and that another pathway has to be activated. A possible mechanism involved hereby is the inhibition of Granzyme B mRNA levels by TGF-β [Ranges GE #255; Inge TH #128] or interference with the expression of pore-forming protein [Smyth MJ #289]. TGF-β1, as concluded from our results, is likely to inhibit this pathway in ConA/IL-2 stimulated splenocytes (Figure 20A). As for the sensitive IL-2 cells, TGF-β1 might inhibit proliferation and concomitant the lytic activity, or proliferation and cytotoxicity separately. This remains indistinguishable.

Nevertheless, the loss of lytic potential of CTLs upon long-term exposure to TGF-β1 is a phenomenon of great importance, in particular concerning elimination of tumor cells. As it is well known, numerous types of tumors secrete TGF-β in a late stage of tumor progression, after aquiring resistance to TGF-β themselves [Inge TH #127; Knaus PI #155; Hata A #108; Nagy N #224; Cook G #52; Gold LI;# 93; Donovan J #71] (and others). This results in loss of lytic activity in tumor infiltrating lymphocytes, as is described by Vánky et al. The analyzed carcinoma samples produced 0.03-0.4 ng/ml active TGF-β per million cells, including latent forms of TGF-β the concentrations were rising up to 1 ng/ml [Vanky F #319]. Considering the dose-response curve shown in Figure 16, where 1ng/ml equals 30pM, even the lowest TGF-β doses produced by the tumor cells are in the inhibitory range of sensitive cells. In the study of Vánky et al, all autologous lympohocytes showed abrogation of lytic activity when cultured in the vicinity of the TGF-β producing tumor cells. This effect is also thought to contribute to tumor immunotherapy, as it will be discussed in the last chapter of the discussion. However, TGF-β plays a role in T cell differentiation and apoptosis as well. These facts stress the importance to closer investigate the effects of TGF-β on naïve versus activated

versus memory T cells. This knowledge is needed to weigh the advantages of rendering T cells resistant to TGF-β in the course of tumor therapy and the disadvantages concerning T cell apoptosis, proliferation and differentiation, effects of TGF-β that are not fully understood yet.

4 Surface expression of receptors

TGF-β receptors are found on all lymphoid cells [McCartney-Francis NL #208], and particularly resting and naïve T cells constitutively express the TβRII [Cottrez F #54]. However, whereas upregulation of TGF-β receptors is reported in one system [McCartney-Francis NL #208], and increased sensitivity of activated T lymphocytes due to upregulated TGF-β receptors has been demonstrated [Wahl SM;# 324], others report that TβRII was downregulated after activation of T cells with αCD3/αCD28, this, however, based upon the analysis of RT-PCR. Most surprisingly, however, is the fact that upon analyzing the expression of TβRII using cell surface biotinylation, we found that in none of the cultures, regardless of sensitive or insensitive phenotype, the type II receptor was downregulated upon long-term exposure to TGF-β1 (Figure 21). Moreover, IL-2, αCD3/IL-2 and ConA/IL-2 stimulated cells displayed comparable amounts of the receptor, whether they had been cultured in the presence or absence of TGF-β1 or not (Figure 21). Surprisingly, when analyzing the ability of the type II receptor to bind radioactive labelled ligand (Figure 38), a different outcome is observed. Wheras in resting splenocytes both TβRI and TβRII are present in considerable amounts and bind to the ligand, activated splenocytes reveal differential surface expression of their TGF-β receptors. ConA/IL-2 stimulated splenocytes cultured for 4 days in the absence of TGF-β1, show unaltered TβRII expression compared to resting cells at day 0. However, the type I receptor seems not to be present any more, or, at least, is unable to bind the ligand. Most astonishing, when the cells are cultured in the presence of TGF-β1, neither TβRI nor TβRII seems to be present – or is unable to bind the ligand. As shown using biotinylation (Figure 21), in this experimental setup the type II receptor can always be detected. The discrepancy between these two methods cannot be completey solved. Sensitivity problems are quite unlikely, as the radioactive crosslink is a very sensitive way to measure ligand binding. One theory might be, that possibly the receptors are expressed on the surface, but are unable to bind the ligand any more. This could – as a hypothesis – be possible by masking the binding site of the type II receptor by an unknown cell surface protein upregulated as a response to TGF-β signaling in ConA-

stimulated lymphocytes. If the receptors are present and bind the ligand, the blockage of the TGF-β1 signal transduction leading to the resistant T cell phenotype has to be further downstream in the signal transduction. (This possibility will be discussed in the discussion, section 6). However, if we presume loss of binding capacity to occur, the question arises, which receptor in this case causes the sustained Smad2/3 phosphorylation (Figure 22). This question will be discussed in the following chapter.

Another important point to consider is the possibility, that alterations in the TβRII may abolish TGF-β mediated growth inhibition, but leave other TGF-β mediated effects intact. For example, Lu *et al*. showed that while phosphorylation of TβRII at threonine 315 is dispensable for extracellular matrix protein formation, it is essential for growth inhibition by TGF-β [Lu SL #190]. This raises the possibility, that in resistant T cells which bear the TβRII at their cell surface, the pathway leading to growth inhibition is inhibited, while other actions remain functional.

5 TGF-β effects on Smad proteins

5.1 Alteration of Smad2 phosphorylation

As the unresponsiveness of total splenocytes stimulated with αCD3/IL-2 and ConA/IL-2 was not caused by downregulation of the high-affinity receptor TβRII, but might be mediated by subsequent molecules of the TGF-β signal transduction cascade (Figure 1), we analysed phosphorylation of Smad2. As shown in Figure 22, total splenocytes stimulated with IL-2 cultured in the absence of TGF-β1 phosphorylate Smad2 upon 15 minutes of TGF-β1 stimulation. However, upon long-term exposure to TGF-β1, Smad2 can not be phosphorylated upon TGF-β1 addition, even though it is still present in the cell. This is astonishing, as these cells were shown to be growth inhibited by TGF-β1 (Figure 18) and display the type II receptor at their cell surface (Figure 21). So the fact is, there is no Smad2 phsophorylation, although the cell is growth inhibited. One possibiliy to explain this phenomenon is described by Lúdviksson *et al*. They report that if CD4+ cells are primed in the presence of TGF-β1, they exhibit reduced secondary αCD3/αCD28-induced immune responses even when TGF-β1 was absent during the secondary response [Ludviksson BR #192]. However, Lee *et al*. observe reconstitution of lytic activity of CD8+ cells stimulated in the presence of TGF-β, when these cells were cultured for 2 days without TGF-β [Lee HM #172]. In contrast to these findings, our cells are neither pure CD4+ cells nor pure CD8+

cells, and regulation of cytotoxicity may not follow regulation of proliferation, as described earlier (discussion, section 3). Nevertheless, one cannot exclude the possibility of a permanent imprinting effect by TGF-β1 in the early phase of T cell stimulation, that might last throughout the T cell life independently of Smad phosphorylation. Besides a long lasting imprinting effect, one can speculate that there exists another pathway besides the Smad pathway, by which TGF-β is able to exert cell cycle regulation. This might be possible, as TGF-β is known to activate the Ras-Erk-pathway [Reimann T #259; Axmann A #11] or the JNK (Figure 8), all of which are implicated in T cell funtion as well [Su B #295; DeSilva DR #66] and might impair T cell functions independent of the Smad pathway.

Surprisingly, when ConA was added to IL-2 stimulated total splenocyte cultures (Figure 22), a sustained Smad2 phosphorylation emerged - independent of the 15 minute TGF-β1 addition – in those cells that were cultured in the presence of long-term TGF-β1. Implying protein synthesis, this effect was observed after more than 6h of ConA stimulation (Figure 22). However, normal ligand-induced Smad2 phosphorylation was never observed upon long-term exposure to TGF-β1. This sustained Smad2 phosphorylation coud not be provoked by ConA stimulation alone, in the absence of TGF-β1 (Figure 23), suggesting that the TGF-β1 pathway and another so far unknown pathway have to be active to obtain the sustained phosphorylation. Most notably, the antibody used in WB was directed against the C-terminal SSXS motif (Materials&Methods, section IV2.3.6), and thus the sustained phosphorylation and ligand-induced phosphorylation took place at the identical phosphorylation site, normally activating the Smad pathway. The stability of this sustained Smad2 phosphorylation was greater than 6h (Figure 24), and is thus unlikely to be evoked by residual TGF-β1 binding to the receptors after washing the cells and replacing the media. As stimulation of total splenocytes with IL-2 leads to TGF-β1 responsivity, whereas treatment with ConA/IL-2 results in resistance to growth inhibition, we systematically analyzed the differentially stimulated CD8+ T cell cultures and total splenocyte cultures for Smad2-phosphorylation. Long-term treatment with TGF-β1 results in loss of Smad2 phosphorylation in both total splenocytes and CTL activated with IL-2 alone (Figure 25A and B, lanes 1-4), as well as in CD8+ T cells activated with anti-CD3/IL-2 (Figure 25B, lanes 5-8). Sustained phosphorylation of Smad2 is observed upon long-term exposure to TGF-β1 in total splenocytes activated with αCD3/IL-2 (Figure 25A, lanes 5-8) and ConA/IL-2 (Figure 25A, lanes 9-12). Weaker but sustained phosphorylation is found in CTL upon activation with ConA/IL-2 (Figure 25B, lanes 9-12). Comparing the data from Smad2 phosphorylation experiments with the results of the proliferation assays, there is a strong correlation between

sustained Smad2 phosphorylation and proliferation, as summarized in Table 2. Total splenocytes or CTL that are not able to properly phosphorylate Smad2 after TGF-β1 treatment and do not show sustained Smad2 phosphorylation, reveal growth inhibition. On the contrary, showing strong sustained Smad2 phosphorylation, they remain resistant to growth inhibition. CTL stimulated with ConA/IL-2 show this phenomenon in an intermediate manner, as they are weakly growth inhibited, and sustained phosphorylation of Smad2 is moderate. In CTL cultures it is very unlikely that the weak sustained phosphorylation observed in the ConA/IL-2 stimulated cells is due to residual non-CD8+ T cells. If this was the case, then in the αCD3/IL-2 stimulated CD8+ T cells this phosphorylation would be apparent as well (Figure 25B, lanes 5-8).

Sustained phosphorylation is not an inherent property of certain subtypes of T cells only present in a total splenocyte culture, a fact which is shown in Figure 25C. If CD8+ cells are first cultured in the presence of TGF-β1 and remain in close contact with the total splenocyte population, they do show the sustained phosphorylation when separated on day 3. We thus conclude, that the sustained phsophorylation is characteristic for CD8+ T cells (and others, as CD8+ depleted cells show sustained phosphorylation as well) that are exposed to TGF-β1 and received a defined set of specific stimuli from other cells.

The mechanism by which TGF-β1 accomplishes either abrogation of Smad2-activation or sustained phosphorylation depending on activation and cellular context remains unclear. One could postulate that by action of an unknown pathway, ubiquitinylation and degradation of Smad2, either by Smad ubiquitinylation regulatory factor-2 (Smurf2) [Zhang Y #366] or by constant degradation in the nucleus [Lo RS #187] might be inhibited. Another possible mechanism is that the second presumably involved pathway phosphorylates Smad2 irreversibly at the SSXS, presupposed that the TGF-β pathway is activated. Alternatively, a divergent modulation of Smad2, possibly phosphorylation at a site different from the SSXS motif, may be the cause. This could be accomplished by steric hindrance or masking ubiquitination or Smurf binding sites, and may thus hinder Smad2, phosphorylated by the TβRI, to become degraded. For example, Smad3 was shown to be rapidly activated by JNK outside its SSXS-motif [Brown JD #22; Engel ME #77]. Protein kinase C phosphorylates Smad3 at serines 37 and 70, and Smad2 at serines 47 and 110 [Yakymovych I #353]. This way, PKC blocks DNA binding of Smad3, but not of Smad2, as Smad2 does not bind to DNA directly.

Several other proteins are known to interact with and inhibit the TGF-β signal transduction. For example, Smad nuclear interacting protein-1 (SNIP1) suppresses p300-dependent TGF-β

signal transduction by binding to CBP/p300. Additionally, SNIP1 was found to interact with the MH2 domain of Smad4 [Kim RH #152; Kim RH #151]. E1A, an adenoviral oncoprotein, is described to inhibit TGF-β induced transactivation by binding to CBP/p300 as well [Nishihara A #227]. Involvement of these proteins remains to be analyzed in order to identify the mechanism by which Smad signaling and/or growth inhibition is abrogated in T cells.

5.2 Alteration of Smad3 phosphorylation

Besides Smad2, Smad3 is the other receptor regulated Smad protein present in hematopoietic cells, and was shown to play a role in transcriptional activation [Zhang Y #367; Biggs JR #13]. In parallel to Smad2, Smad3 mutant mice die due to a primary defect in immune function [Yang X #360]. However, the function of Smad2 and Smad3 in regulating immune responses does not seem to be identical, as in single knockout mice Smad2 and Smad3 cannot replace each other. Additionally, Smad3 knockouts show formation of bacterial abscesses adjacent to mucosal surfaces, suggesting downregulation of certain immune responses upon insensitivity to TGF-β.

Therefore, we analyzed if Smad3 showed sustained phosphorylation in the same manner as Smad2. Figure 26 shows differentially stimulated purified CD8+ T cells and total splenocytes. Confirming our results with Smad2 (compare to Figure 25), αCD3/IL-2 stimulated CTLs do not express sustained Smad3 phosphorylation, whereas ConA/IL-2 stimulated CTLs and αCD3/IL-2 stimulated total splenocytes do show sustained Smad3 phosphorylation upon long-term exposure to TGF-β1. Thus, divergent functions of Smad2 and Smad3 in immune regulation seem to be independent of the ability of Smad2 and Smad3 to express sustained phosphorylation of the SSXS motif in the course of long-term TGF-β1 exposure.

5.3 Localization of Smad proteins in different cellular compartments

To further analyze the phenomenon that T cells are not growth inhibited by TGF-β1 while showing sustained Smad2 phosphorylation, we wanted to determine whether the unresponsiveness to TGF-β1 was caused by retention of Smads in the nucleus. As in earlier experiments total cell lysates were used for detecting Smad2/3 phosphorylation. This does not allow to distinguish between cytoplasmatic and nuclear Smads. To exclude that resistance to growth inhibition was not caused by impairment of TGF-β signal transduction due to the lack of Smads in the cytoplasm, we determined the localization of phosphorylated Smad2, Smad3 and Smad4 in the cell.

We found that in splenocytes stimulated with αCD3/IL-2 and exposed to long-term TGF-β1, P-Smad2 is both in the nucleus and the cytoplasm (Figure 27A, lanes 2 and 4). In lane 2, no total Smad is detected (lower panel), whereas P-Smad2 is present (upper panel). This is explained by the higher sensitivity of the α-P-Smad antisera. Lacking any TGF-β stimulus, very little Smad2 is detected in the nucleus (Figure 27A, lane 1). We also determined the cellular distribution of Smad3 and Smad4. Total splenocytes stimulated with αCD3/IL-2 show sustained phosphorylation of Smad3 after long-term TGF-β1 treatment (Figure 27C, lanes 2 and 4). Comparable to P-Smad2, P-Smad3 is present in cytoplasm and nucleus (compare Figure 27A and Figure 27C). There is only a minor fraction of unphosphorylated Smad3 present in the nucleus before TGF-β1 stimulation (Figure 27C, lane 1), as it was observed with Smad2 (Figure 27A, lane 1). In contrast, Smad4 is present in the nucleus independent of TGF-β1 stimulation. This is in agreement with the model of continuous nucleocytoplasmic shuttling of Smad4 [Pierreux CE #247]. However, in the cytoplasmic fraction Smad4 is represented by a double band, independent of TGF-β stimulation, suggesting two different types of Smad4 in the cytoplasm. This observation however could not be confirmed in the available literature. However, in *Xenopus* different splice variants exist, where Smad4a is located preferentially in the cytoplasm, whereas Smad4b is found in the nucleus [Howell M #123; Masuyama N #204]. Another likely possibility is the presence of a Smad-unrelated cytoplasmatic cross-reacting protein. Concluding from these data, unavailability of Smad2/3 for activation by the TGF-β receptors is not the reason for growth resistance to TGF-β1.

6 T cells, TGF-β and the MAP kinases

As described above, differential stimulation of T cells evoked either resistant or sensitive T cell phenotypes with respect to TGF-β growth inhibition. These phenotypes were shown to be connected with sustained versus no Smad2/3 phosphorylation, respectively. Next, we wanted to analyze the underlying signaling pathways. As MAP kinase pathways have been implicated in T cell signal transduction, and were shown to interfere with the TGF-β signal transduction pathway, we focused on the MEK/Erk and the JNK kinase pathways.

6.1 Involvement of the MEK-1/Erk pathway

As it was shown earlier [Su B #295; Reimann T #259; Axmann A #11], MEK-1 is involved in T cell activation, and TGF-β itself is able to induce MEK-1 activation. Thus we inhibited MEK-1 kinase using PD98059. As shown in Figure 28, application of MEK-1 inhibitor to αCD3/IL-2 stimulated total splenocytes renders these cells sensitive to TGF-β1 mediated growth inhibition, in contrast to cells with intact MEK-1 pathways. To elucidate whether the conversion of TGF-β1 sensitivity in total splenocytes was caused by PD98059 favouring the development of CD8+ T cells, we analyzed the populations by fluorescent staining, however, we did not observe increased CD8+ populations in total splenocyte cultures treated with PD98059.

Inhibition of MEK-1 also converted the Smad2 phosphorylation pattern of total splenocytes stimulated with αCD3/IL-2 (Figure 29A). With intact MEK-1 pathway, these cells show strong sustained Smad2 phosphorylation following long-term TGF-β1 application, while purified CD8+ T cells are unable to phosphorylate Smad2 under the same conditions. If MEK-1 is blocked, the sustained Smad2 phosphorylation is completely abrogated (compare Figure 29A, lanes 7 and 8 to Figure 29A, lanes 3 and 4).

In contrast, CD8+ T cells treated with the same cultural conditions did not alter their phenotype compared to cells with intact MEK-1 pathway (Figure 30). In contrast to total splenocytes, however, even the cells that did not receive TGF-β1 showed reduced total cell numbers (compare Figure 29to Figure 30). This is in agreement with other reports [Ip YT #130; Kane LP #144], where it was shown that T cells from Erk-deficient mice were severely impaired in their ability to proliferate *in vitro* after TCR stimulation [Pages DD #235]. These data adds proof to the earlier mentioned explanation, that in total splenocytes stimulated with αCD3/IL-2 - compared to CD8+ T cells – an additional stimulatory pathway is activated, that renders the cells resistant to TGF-β1 mediated growth inhibition, and protects against growth inhibition induced by abrogation of the MEK-1 pathway. Interestingly, Biggs *et al*. observed an influence of MEK-1 on the Smad pathway as well. PD98059 blocks phosphorylation of Smad3 in leukemic cells stimulated with phorbol 12-myristate13-acetate (PMA), suggesting the need of direct phosphorylation of the Smads by Erk for transcriptional activation [Biggs JR #13]. It was reported by Schiött *et al*. [Schiött A #275], that TGF-β1 decreases phosphorylation of Erk. This implies that TGF-β1 and the MEK-1/Erk pathway have

opposing effects. This is also true in our system, apparent in the fact that inactivation of MEK-1 renders formerly TGF-β1 resistant cells responsive.

These data suggest that the MEK-1 pathway induces sustained Smad2 phosphorylation at the conserved SSXS motif upon long-term exposure to TGF-β1. In contrast, the short-term effect of TGF-β1 is independent of MEK-1 as Smad2 phosphorylation remains intact in splenocytes cultured with PD98059 but without TGF-β1 (Figure 29A, lanes 5 and 6). Taken together, these data imply that MEK-1 confers resistance to TGF-β1 mediated growth inhibition.

6.2 Involvement of the JNK pathway

In addition to the MEK-1 pathway, the JNK pathway has been suggested as possible crosstalk to the TGF-β pathway. For example, Smad3 was shown to be rapidly activated by JNK outside its SSXS-motif [Brown JD #22; Engel ME #77], whereas others observe inhibition of the Smad-pathway by JNK either by correlation with Smad7 expression [Mazars A #207] or by interaction with the corepressor TGIF [Pessah M #244]. On the other hand, TGF-β initiates a signaling cascade leading to the activation of JNK [Atfi A #10], and the expression of dominant-negative forms of JNK or an upstream mediator, MKK4, results in loss of TGF-β stimulated fibronectin synthesis [Hocevar BA #116].

In T lymphocytes JNKs are synergistically activated by costimulation of TCR and CD28 [Su B #295; Jacinto E #137]. Considering our results as shown in Figure 31, this is confirmed. Here, total splenocytes stimulated with high doses of IL-2 under omission of TCR and costimulation signals do not show JNK phosphorylation in comparison to ConA/IL-2 stimulated cells (Figure 31A). However, αCD3/IL-2 stimulation reveals somewhat different results, suggesting TGF-β1 to play a major role in JNK phosphorylation: Total splenocytes do not phosphorylate JNK unless they are activated by TGF-β1 long-term (Figure 31A). In contrast, purified CD8+ cells stimulated with αCD3/IL-2 and lacking costimulatory signals do show constitutive JNK phosphorylation until they are cultured in the presence of long-term TGF-β1 (Figure 31B). In all cases the effects mediated by TGF-β1 are most likely to involve transcriptional processes, as shorter periods of TGF-β1 (15min) do not suffice to induce this effect (Figure 31A, lanes 6 and 10, Figure 31B, lane 6). How TGF-β1 induces JNK phosphorylation in total cells and diminishes JNK phosphorylation in CTLs on the molecular level is not understood. As this TGF-β effect requires transcription and is distinct in different cell types, JNK phosphorylation is likely to involve the action of a coactivator or corepressor of TGF-β mediated gene regulation.

Interestingly, upon blocking MEK-1 in αCD3/IL-2 stimulated total splenocytes, the JNK expression pattern is altered. In cultures without TGF-β1 exposure, intact MEK-1 results in no JNK phosphorylation (Figure 31A), whereas blocked MEK-1 leads to JNK phosphorylation (Figure 31C). This phenotype is identical to purified CTLs. As no direct way is known by which MEK-1 phosphorylates JNK, we propose that MEK-1 - by unknown molecular pathways - regulates TGF-β1 responsivity and concomitant JNK phosphorylation. We also adressed the question, whether TGF-β1 regulates JNK phosphorylation, or JNK phosphorylation regulates TGF-β1 sensitivity. However, as we failed to transfect primary T cells, we had to rely on inhibitors. Unfortunately, the dependance of T cells on an intact JNK pathway is crucial, and T cells exposed to JNK inhibitors did not proliferate with any kind of stimulation. Thus, the nature of coregulation between TGF-β sensitivity and JNK phosphorylation could not be explained in detail.

How the JNK expression pattern is related to cellular proliferation is another question of interest. As it is described by Sabapathy *et al.*, mice lacking the JNK2 gene were defective in peripheral T cell activation, a defect that is restored upon adding exogenous IL-2 [Sabapathy K #268]. In addition, it is known that the importance of JNK in T cell activation is based upon IL-2 induction and the transcription factor AP-1, that is induced by JNK as well [Su B #295]. Although in our cells absence of any JNK phosphorylation correlated with either lower proliferation rates in IL-2 only stimulated cells or growth inhibition in αCD3/IL-2 stimulated cells, there are differences to the work of Sabapathy *et al*. In our cultures, there is always exogeneous IL-2 present, and the cells still are growth inhibited, whereas Sabapathy *et al.* describe restauration of proliferation by exogeneous IL-2. Thus, regulation of proliferation in this case may not be based upon the proposed role of JNK in IL-2 regulation, but on the ability of JNK to induce the transcription factor AP-1.

6.3 Possible connections of JNK and TGF-β signal transduction

As described above, JNK and TGF-β pathways are likely to influence each other. The two pathways may converge at the level of JNK directly, or, much likely, at the level of JNK targets. Smad3, for example, is able to enhance the reponse of both Jun and Fos to TGF-β [Zhang Y #367]. Jun and Fos, constituting the transcription factor AP-1, both bind to either Smad3 and 4 or Smad3, respectively [Itoh S #133; Padgett RW #234]. Conversely, JNK is reported to be activated by TGF-β in a Smad independent manner, and, most interestingly, phosphorylates Smad3 outside its SSXS motif [Engel ME #77], and MEKK-1, an upstram MAPK kinase kinase of JNK, is able to activate Smad2 induced transcription in endothelial

cells [Brown JD #22]. This described effect, surprisingly, does not require the presence of the SSXS motif. However, JNK is unlikely to be the agent causing the sustained Smad2 phosphorylation we observed, as this takes place at the SSXS motif. Possibly, phosphorylation at other sites leads to conformational changes, ultimatively resulting in sustained Smad2 phosphorylation and mediating TGF-β1 opposing effects.

Recently, the JNK pathway has been implicated in inhibiting TGF-β through the nuclear transcriptional corepressor TG-interacting factor (TGIF). Pessah *et al.* report, that the activation of the JNK cascade blocked the ability of Smad2 to mediate TGF-β dependent activation of FAST proteins [Pessah M #244]. This inhibitory activity was mediated through the transcription factor c-Jun, which enhances the association of Smad2 with TGIF. Thereby, c-Jun interferes with the assembly of Smad2 and the coactivator p300 in response to TGF-β signaling. This is a very intersting finding, moreover, as the activation of JNK does not interfere with the phosphorylation of Smad2 by the type I receptor and its subsequent heterooligomerization with Smad4. In addition, stimulation with TGF-β induces the association of c-Jun with TGIF, and JNK is reported to be rapidly activated by TGF-β in a Smad-independent manner [Engel ME #77]. This mechanism of blocking TGF-β action is very likely to play a role in the data we obtained. In the presence of TGF-β, activation of JNK is a prerequisite to render the T cells resistant to TGF-β1 mediated growth inibition. To further analyze this hypothesis, it would be necessary to measure transcriptional activity induced by TGF-β, for example by using luciferase assays, including experiments with constitutive active forms of JNK, or dominant negative forms of JNK. Unfortunately, however, the primary splenocytes could not be transfected. In a further study, Pessah *et al*. [Pessah M #243] suggests Ski constituting a corepressor being bound by c-Jun, widening the possibilies for JNK-induced TGF-β repression.

Besides AP-1, other JNK subtrates include Activating transcription factor (ATF2), Elk1, and nuclear factor of activated T cells (NFAT4) [Minden A #210; Ip YT #130]. Of these JNK targets, several are known to bind to Smads. ATF2 for example can bind to the MH1 domain of Smad3 and 4. ATF-2 is also phosphorylated by TGF-β-activated kinase-1 (TAK-1), a member of the mitogen-activated protein kinase kinase kinase, and the activity of ATF-2 is enhanced by JNK [Sano Y #273]. In addition, TAK-1 is reported to signal via JNK [Shirakabe K #283]. There is also evidence for a role of the JNK cascade in Smad7 mediated apoptosis [Mazars A #207]. Expression of Smad7 resulted in strong and sustained activation of JNK, while the inhibitory function of Smad7 proved to be dependent on JNK.

6.4 CD28 regulating JNK

The observed differences between resistant and sensitive T cells are most likely due to divergent costimulation, as described earlier. Hereby, a mechanism relying on cell-cell-contact is proposed, as supernatant from total splenocyte cultures did not have this effect. One important stimulus absent in purified CTL populations is costimulation via CD28. Until recently, JNK activity was believed to correlate strongly with CD28 costimulation, as activation of JNK in Jurkat cells was observed with TCR-CD28, but not with TCR ligation alone [Su B #295]. Ligation of the TCR leads to induction of JNK gene expression, while phosphorylation of JNK also requires CD28 mediated costimulation [Weiss L #336]. Most interesting, an inhibitor of proliferation was decribed in T cells [Tzachanis D #315] that associated with Smad2 and Smad4. Tob was expressed in unstimulated lymphocytes, and was found to be downregulated during activation. This downregulation was associated with CD28 costimulation.

However, the precise role of JNK activation is unclear, as shown in data provided by Rivas *et al*., where CD28 is not required for JNK activation in T cells, implicating another pathway activating JNK [Rivas FV #261]. Moreover, Dong *et al*. reported that, using three different model systems, JNK was not necessary for T cell activation [Dong C #70]. As JNK is also implicated in cross-talk to the TGF-β pathway, we first thought CD28 to be the relevant molecule to determine TGF-β resistance when activated and TGF-β sensitivity when inactive. We thus attempted to convert the sensitive phenotype towards a resistant phenotype employing soluble activating αCD28 antibody. As shown in Figure 32, Figure 33 and Figure 34, activation of CD28 does not revert the sensitive phenotype. It neither renders CD8+ T cells (Figure 33) nor IL-2 stimulated total splenocytes (Figure 34) resistant to TGF-β1 mediated growth inhibition, nor induces sustained Smad2 phosphorylation in αCD3/IL-2 stimulated CTLs (Figure 32). This suggests, that either CD28 needs another activated pathway to confer resistance to TGF-β, or other (known or unknown) costimulatory molecules determine the cellular phenotype. Although McKarns *et al*. describe that CD28 costimulation partly diminishes the inhibitory effects of TGF-β1 on activated splenocytes [McKarns SC #209], in their experimental setup this is validated only for TGF-β1 inhibiting IL-2 production. As we added exogeneous IL-2 to the cultures, inhibition of endogeneous IL-2 production cannot account here for growth inhibition anyway. An other costimulatory pathway that could act as the resistance conferring mechanism, the CD40/CD40L, is thought to activate the JNK cascade as well. However, this is shown only in human B cells [Sakata N

#270]. In addition, ConA as a lectin is known to mimick not only CD28 costimulation, but also costimulation via CD40L [Holter W #119; Lenschow DJ #174; Justement LB;# 141; Grewal IS #99].

IFNγ is suggested as an important inhibitor of TGF-β1 signaling upon upregulating Smad7 expression [Ulloa L #317]. In contrast, NF-κB, an important regulator of cellular responses initiated by proinflammatory cytokines such as IFNγ, is reported to induce repression of TGF-β induced activation of the Smad7 promoter [Nagarajan RP #222]. Confirming the results of Ulloa *et al*., Bitzer *et al.* showed that the RelA subunit of NFκB up-regulates Smad7 synthesis, resulting in inhibition of TGF-β induced Smad signaling [Bitzer M #14]. Considering our study, an important observation is that the JNK cascade cooperates with the Smad signaling to induce Smad7 transcription through the AP-1 element [Uchida K #316]. Vice versa, it was reported by Schiött *et al*. [Schiött A #275], that PD98059 in concert with TGF-β1 abrogates IFNγ production in rat T cells. Therefore, we added exogeneous IFNγ to the TGF-β1 sensitive T cells and analyzed proliferation and Smad phosphorylation. However, we did not observe alterations in the TGF-β1 reactivity of these cells. In fact, CD8+ T cells showed higher total cell numbers in response to IFNγ, but upon long-term culture with TGF-β1, the cells remained sensitive to growth inhibition (Figure 33). Total splenocytes did neither show elevated cell numbers nor reversion of the sensitive phenotype (Figure 34). Concerning Smad2 phosphorylation, the CD8+ T cells did not express sustained Smad2 phosphorylation upon long-term exposure to IFNγ and TGF-β1 (Figure 32). As it is suggested that the JNK cascade cooperates with the Smad signaling to induce Smad7 transcription through the AP-1 element [Uchida K #316], we added simultaneously IFNγ and αCD28 to IL-2 stimulated total splenocytes, still, the sensitive phenotype could not be averted (Figure 34).

There is also evidence for a role of the JNK cascade in Smad7 mediated apoptosis [Mazars A #207]. Expression of Smad7 resulted in strong and sustained activation of JNK, while the inhibitory function of Smad7 proved to be dependent on JNK. In addition, it was shown for PC12 rat pheochromocytoma cells that the BMP-induced neurite outgrowth, which depends on the activation of the TGF-β activated kinase-1 (TAK1)-p38 kinase pathway, is inhibited by Smad7 [Yanagisawa M #358]. TAK1 itself is reported to signal via JNK [Shirakabe K #283]. Smad7, however, is unlikely to be involved in the lack of TGF-β1 responsiveness here. Smad7 binds the activated type I receptor, preventing the access and phosphorylation of receptor-regulated Smads. We observed, that cells growth inhibited by TGF-β1 did not show Smad2/3 phosphorylation, whereas the cells not growth inhibited by TGF-β1 showed

sustained Smad2/3 phosphorylation. The proposed model of Smad7-induced inhibition would imply contrasting effects and thus cannot account to these cellular phenotypes.
Concluding from these data, it is unlikely that solely the activation of either CD28 or the JAK/STAT pathway via IFNγ is sufficient to induce a resistant phenotype in primary splenocytes. Additionally, combining the two pathways was not effective in this respect as well. However, as we did not check Smad7 levels in these cultures, it cannot be excluded that Smad7 is involved in conferring resistance to TGF-β1 mediated growth inhbition. In further experiments it was verified that supernatant obtained from resistant cells did not render sensitive cells insensitive, therefore, we suggest a mechanism involving cell-cell-contact. Cell contact could, in this case, be the sole reason for TGF-β resistance, or acts in combination with soluble factors such as IFNγ.

7 Cell lines

Immortalized immune cell lines as model systems for *in vivo* situations face certain difficulties, as it is unknown whether the immortalized state of these cell lines reflects the state of primary activation. Primary lymphocytes must remain sensitive to a variety of signals so that inducible genes can be precisely controlled by subtly different stimuli. In contrast, cell lines are locked into invariably active signal transduction pathways, and thus many differences may arise. For example, differences were reported regarding the regulation of the IL-2 promoter between Jurkat cells and primary T cells, as in transfection experiments several mutations proved more inhibitory for Jurkats than for primary cells [Hughes CCW #124]. This may reflect the removal from normal growth and transcriptional control mechanisms which is typical for immortalized cell lines. However, as primary splenocytes were found unable to be transfected or retrovirally infected in the course of the study, various established T cell lines of human and mouse origin were analyzed with respect to TGF-β1 sensitivity.
In Figure 35, representative results are shown with respect to sensitivity of various cell lines to TGF-β1 mediated growth sensitivity. Neither the human T cell lymphoma line Jurkat nor the murine T cell lymphoma EL-4 was significantly growth inhbited by TGF-β1. However, Jurkats were shown to express the TβRII at their surface using biotinylation (Figure 37), and EL-4 were demonstrated to bind radioactive TGF-β1 to TβRI and TβRII in crosslinking experiment (Figure 38). Therefore, insensitivity of these cell lines has to be based on further downstream elemants of the signaling cascade or on inactivating mutations within the receptor. Figure 39 and Figure 41 indicate the nature of the TGF-β signaling blockage, as

Jurkat cells show sustained Smad2 phosphorylation regardless of ConA/IL-2 stimulation when treated with TGF-β1 (Figure 39). Additionally, Jurkat cells show constitutive activation of JNK, independent of TCR stimulation and TGF-β1 exposure (Figure 41). This reminds of the primary splenocytes, where resistance to growth inhibition was connected with sustained Smad2 phosphorylation (Figure 18 and Figure 25) and activation of JNK (Figure 31). Therefore, we suggest that Jurkat cells reflect a permanently activated state which enables them to overcome TGF-β1 mediated growth inhibition.

Recent studies describe specific phosphorylation of Smad2 in Jurkat cells following TCR ligation by αCD3 [Mamura M #199], whereas thus phosphorylated Smad2 failed to interact with Smad4. In contrast to these findings, we did neither observe Smad2 phosphorylation of Jurkat cells (Figure 39) nor primary cells (Figure 25) upon αCD3-treatment without addition of TGF-β1. These differential findings cannot be accounted to phosphorylation of other sites in the Smad2 molecule, as in all these studies αP-Smad2 antibodies specific for the C-terminal phosphorylated peptide were used (Figure 25 upper panel, [Mamura M #199]). Concerning functional interaction of phosphorylated Smad2 with Smad4, we cannot rule out the possibility that this interaction is not disturbed in the primary cells as well, as we did not analyze that. Interestingly, Mamura *et al*. inhibited MEK-1 using PD98059 in Jurkat cells as well. This treatment suppressed the αCD3 induced Smad phosphorylation, comparable to the sustained Smad2 phosphorylation in primary cells (Figure 29). This implies a connection between these two observations, although the initial finding of Mamura *et al*., Smad2 activation upon TCR ligation, could not be confirmed.

CTLL-2 cells were the only cell line analyzed that was found to be growth inhibited by TGF-β1 (Figure 36), consistent with the results reported by Inge *et al*. [Inge TH #128]. However, the magnitude of the inhibition was not dependent on the activation state as reported by Chung *et al.*, where TGF-β induced cell death was more enhanced in a CTLL-2 subline which reflected a more activated state that the founder cell line [Chung EJ #48]. However, the study of Chung *et al.* focused on apoptosis, and as we did measure proliferation, not apoptosis in the T cell lines. Although unlikely, it is not impossible that differences in results arise comparing apoptosis and proliferation, as cells may proliferate more and show increased apoptosis at the same time. Different methods of analyzing proliferation may perhaps also lead to divergent results. The majority of studies employs the ^{3}H-thymidine incorporation assay, wheras in our studies the MTT-based CellTiter colorimetric assay (Promega) was used (view chapter "Materials & Methods"). While thymidine incorporation directly measures cell division, the colorimetric MTT assay determines total cell numbers. However, as the cell numbers on day 0

were standardized as well, differences in the outcome of experiments are extremely unlikely to be caused by the usage of MTT versus thymidine incorporation assay. Most important, our results show (Figure 36) that, in contrast to primary cells, CTLL-2 cells are inhibited by TGF-β1 regardless of stimulation mode. Consistently, CTLL-2 cells do not show sustained Smad2 phosphorylation in response to TGF-β1 when stimulated with ConA/IL-2 (Figure 40). Thus we propose that CTLL-2, in the course of immortalization, have lost the pathways triggering sustained Smad2 phosphorylation and conferring resistance to TGF-β1 mediated growth inhibition.

Further cell lines analyzed include MD45 (murine hybridoma obtained by fusion of CTL and lymphoma cells), P815 (murine mastocytoma) and A301 (human lymphoma). All of these were also resistant to TGF-β1 mediated growth inhibition. While unresponsiveness of A301 cells is supposed to be caused by lacking the type II receptor at the surface (Figure 37), resistance of MD45 cells seems to be based upon constitutive JNK activation (Figure 42), as MD45 express the type II receptor at the surface (Figure 37). P815 cells reveal conflicting results, as the type II receptor is found unable to be detected in biotinylation experiments (Figure 37), whereas in crosslinking experiments, radioactive TGF-β1 binds significantly to the type I receptor, only in trace amounts to the type II receptor (Figure 38). In the course of the study, this question could not be adressed.

8 Implications for tumor therapy and future aims

Epithelial and hematopoietic cells have a high turnover, and their progenitor cells divide continuously. This causes these cells to be primary targets for genetic and epigenetic changes that lead to cell transformation and tumorigenesis. The consequent changes in cell behaviour and cellular responsiveness result not only from genetic alterations, for example activation of oncogenes or inactivation of tumor suppressor genes. They result also from changes in the production or responsiveness to growth and differentiation factors. TGF-β hereby acts as a key determinant of carcinoma cell behaviour. The autocrine and paracrine effects of TGF-β on the tumor cells themselves or the environment of the tumor including, for example, tumor infiltrating lymphocytes, exert both negative and positive effects on cancer development. Additionally, the effects of TGF-β on the immune system are not only inhibitory, and it is known that transgenic mice overexpressing TGF-β1 also succumb to infectious diseases as a result of chronic inflammation [Wahl SM;# 325]. This is important to reconsider, when undertaking approaches for tumor immunotherapy [Chattopadhyay U;# 37; Akhurst RJ #2;

Derynck R #63; Gorelik L #96]. Wheras TGF-β induces apoptosis in early stage tumors that have not yet lost the ability to respond to TGF-β and thus acts as an inhibitor of tumorigenesis, other features of TGF-β are tumor promoting. TGF-β is able to induce angiogenesis and expression of vascular endothelial growth factor (VEGF) which directly acts on endothelial cells to stimulate cell proliferation and migration, and TGF-β attracts monocytes, which then in turn release angiogenic cytokines [Derynck R #63]. A major effect is, that TGF-β mediates tumor progression through escape from immunosurveillance, as it inhibits the functions of lymphocytes. Elevated production of TGF-β by tumors as described e.g. by Vánky *et al.* and Nagy *et al*. [Vanky F #319; Nagy N #224], has been implicated in the failure of immunotherapy. One example is injection of IL-2 expanded lymphokine activated killer cells (LAK) into the tumor site of gliobastoma [Jachimczak P;# 136]. Successful therapeutic approaches used oligonucleotides encoding antisense TGF-β2 that enhanced immunity in malignant glioma cells *in vitro* [Jachimczak P;# 136]. Other approaches aimed at the provision of tumor antigen-specific T cells, as tumors downregulate their MHC expression and thus cannot be recognized by specific T cells. ErbB2 has come into focus in this respect (view introduction section I2.2.4), when erbB2 specific chimeric single-chain antibodies coupled to CD3ζ chains were used in tumor immunotherapy [Moritz D #215; Stancovski I #293; Altenschmidt U #6]. However, it is also reported [Altenschmidt U #6] that the provision of tumor cell specific T lymphocytes is not sufficient, as tumors secrete TGF-β and thereby inhibit the cytotoxic activity of the T cells. Our initial aim was therefore to construct T cells designed to express a dominant-negative TβRII (DNTβRIIΔcyt: Marion Lutz and Petra Knaus) at their cell surface (view results, section II5). However, rendering T cells unresponsive to TGF-β might itself lead to transformation of T cells and result in the generation of lymphomas. The idea was now, to create a chimeric construct of this DNTβRIIΔcyt, linked to the Herpes simplex virus thymidine kinase (HSV-TK) constituting a suicide gene. The TβRIIΔcyt-TK construct (Figure 43) would therefore, in concert with the ScFv construct, provide the possibility to eliminate the TGF-β resistant T cells after clearance of tumor cells. However, whereas the construct was found to be expressed transiently in 293T cells (Figure 44), we were not able to stably transduce primary murine splenocytes with this retroviral construct in the course of this study. This remains a major future aim, in order to verify that the transduced primary T cells are specific for erbB2, resistant to TGF-β and are able to be eliminated upon activation of the suicide gene.

Concerning tumor therapy in general, it would be desireable in future studies to retain the TGF-β-mediated stimulation of apoptosis in tumor cells, but to inhibit the induction of invasion, metastatic spread, angiogenesis and immunosuppression. Indispensable therefore is the aim to dissect specific pathways which accomplish these divergent effects of TGF-β. In this study, we were able to shed light on the mechanism by which TGF-β1 acts on immune cells, in particular, cytotoxic T cells. Taken together, the data suggest that both MAP-kinase pathways, MEK-1 and JNK, have to be active to render T cells resistant to TGF-β1 mediated growth inhibition (Table 2). This resistance correlates with sustained Smad2 phosphorylation and activation of JNK upon long-term exposure to TGF-β1. How this scenario might take place in an *in vivo* situation is depicted in Figure 45. CTL activated in close proximity to bystander cells such as CD4+ T cells, B cells or macrophages exhibit Smad2 and JNK phosphorylation and are unaffected by TGF-β1 mediated growth inhibition. However, if CTL migrate to a tumor site and are exposed to long-term TGF-β secreted by the tumor, the cells are unable to proliferate and their lytic potential is abrogated.

However, as the exact molecular mechanisms are not yet fully identified, future studies might elucidate the involvement of kinases, JNK, MEK-1 or PKC, phosphorylating Smad2 outside the SSXS or directly at the SSXS. Transcriptional inhibitors might be detected that inhibit Smad2 signaling such as E1A, SNIP or NFκB, or the upregulation of Smad7 might be proven. By this, hopefully TGF-β signaling in T cells will be wholly understood, enabling to work on therapies of fatal diseases such as cancer and autoimmunity.

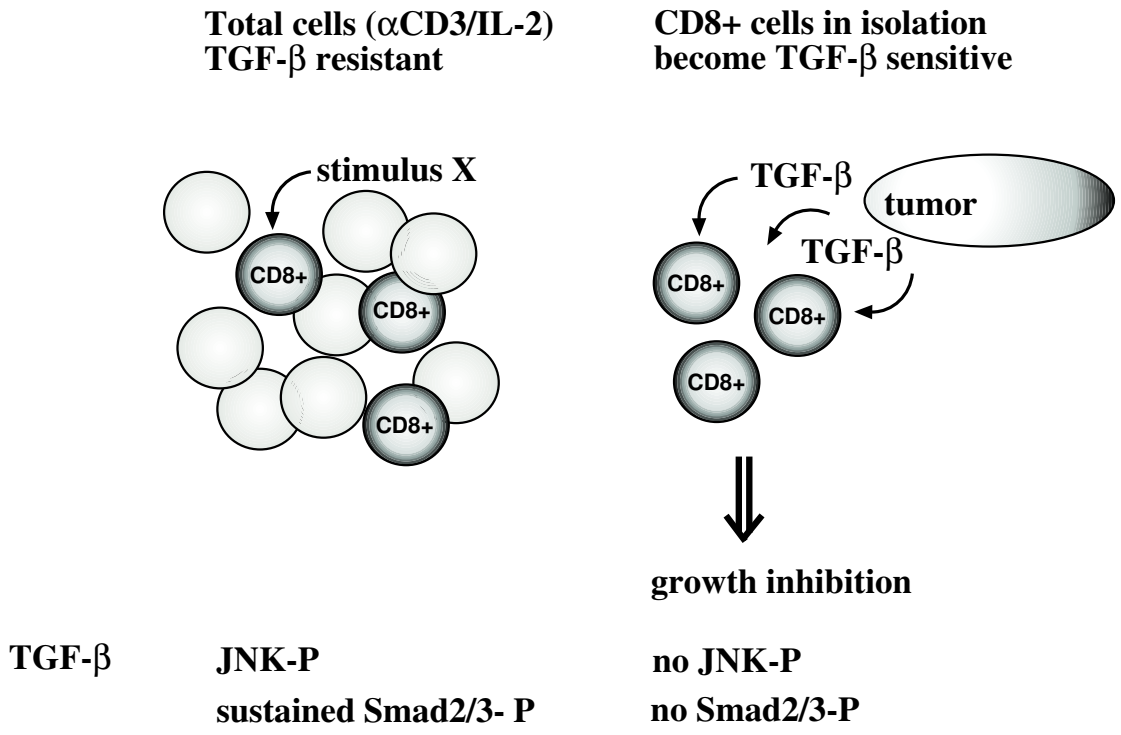


Figure 45: Proposed *in-vivo*-situation of cytotoxic T cells. If CTL are in close neighborhood to other cells such as CD4+ T cells, B cells or macrophages, they show sustained Smad2/3 phosphorylation and JNK phosphorylation. These cells are not growth inhibited by TGF-β. In contrast, if the CTL lack contact to other immune cells, and receive TGF-β from tumor cells, they are not able to phosphorylate JNK and Smad2/3. These cells are growth inhibited and unable to lyse the tumor cells.

Therefore, the proposed model for the observed differential effects of TGF-β1 on T cells is the following:

1) Upon long-term exposure to TGF-β1, the lytic activity of CTLs is abrogated.
2) The TGF-β receptors, MEK-1 and costimulatory molecules synergize to result in sustained Smad2/3 phosphorylation.
3) Sustained Smad2 phosphorylation ultimatively leads to resistance to TGF-β1 mediated growth inhibition.
4) Resistance to growth inhibition is likely to be mediated by JNK, as c-Jun impairs Smad2 mediated transcription [Pessah M #244].

IV Materials and Methods

1 Abbreviations

α-	anti-
2-ME	Beta-mercapto-ethanole
aa	Aminoacid
Ab	Antibody
Amp	Ampicillin
APS	Ammoniumpersulphate
BMP	Bone morphogenetic protein
bp	Base pairs
BSA	Bovine serum albumine
Da	Dalton
DEAE	Diethyleminoethyle
DMEM	Dulbecco's modified eagle medium
DMSO	Di-methyl sulfoxide
DN	Dominant negative
DNA	Desoxyribonucleic acid
DNase	Desoxyribonuclease
dNTP	2'-desoxy-nucleoside-5'-triphosphate
ds	Double stranded
DSS	Disuccinimidyl suberate
E.coli	*Escherichia coli*
ECL	Enhanced chemoluminiscence
EGF	Epidermal growth factor
EMEM	Eagles minimal essential medium
EtBr	Ethidium bromide
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
HA	Hemagglutinine
HBS	Hepes buffered saline

HGF	Hepatocyte growth factor
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
HRP	Horse radish peroxidase
IL	Interleukin
JNK	c-Jun N-terminal kinase
MAD	Mothers against decapentaplegic
MH	Mad homology
MW	Molecular Weight
NaPyr	Sodium pyruvate
NEAA	Non-essential amino acids
OD	Optical densitiy
P-	Phospho-
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
Pen	Penicilline
PI	Protease inhibitor
PKC	Protein Kinase C
PMSF	Phenylmethylsulfonyl fluoride
Pox	Peroxidase-conjugated
RBC	Red blood cell
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rounds per minute
RT	Room temperature
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyle sulphate
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
Strep	Streptomycine

TBS	Tris buffered saline
TEMED	N,N,N',N' - tetramethylethylenediamine
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Tris	Tris-(hydroxymethyle)-aminomethane
TβRI/II	TGF-β type I/II receptor
U	Unit
UV	Ultraviolet
WB	Western blot
wt	Wild type

Table 3: Abbreviations

2 Materials & Reagents

2.1 Hardware

	Used in	**Company**
Beckmann J2-21	Centrifuge (cooling)	Beckmann
Caompact 35	X-ray film developing machine	Protec
Centrifuge 5417C	microcentrifuge	Eppendorf
Cetus DNA Thermal cycler	PCR-cycler	Perkin Elmer
ELISA-reader	MR5000	Dynatech
Epics XL	Flow cytometer	Coulter
FB 12	luminometer	Bethold
Gamma 4000	Gamma counter	Beckmann
Heraeus 6000	incubator	Heraeus
HLB 2472	hood	Heraeus
Kontron Uvicon 930	spectrometer	Kontron
Leitz DM IL	microscope	Leica

	Used in	Company
Mini-Protean II	PAGE electrophoresis chamber	BioRad
Mini-V 8.10	Blot system	BioRad
Phosphoimager		Molecular dynamics
Plate centrifuge	centrifuge	Hettich
Power supply	electrophoresis	Biotech Fischer
Rotanta	centrifuge	Hettich
Sepatech Megafuge 1.0	centrifuge	Heraeus
Slab dryer 483	Gel dryer	BioRad

Table 4: Hardware

2.2 Plasticware

	Used in	Company
0,2µm syringe filter	Sterile filtration	Roth
0,45µm syringe filter	Retroviral infection	Roth
Cell strainer	T cell preparation	BD/Falcon,(Heidelberg, Germany)
FACS tubes	FACS, Luciferase test	Hartenstein (Würzburg)
Konika X-ray films	WB, BxL	Hartenstein
Nitrocellulose BA85 0.4µm	WB	Schleicher&Schüll
Nylon wool columns	T cell preparation	WAK-Chemie (Bad Homburg, Germany)
Sephadex-G-25 column	Iodination	Pharmacia

Table 5: plasticware

All further used plasticware for cell culture was obtained by Greiner and Falcon if not indicated otherwise

2.3 Chemicals

The basic chemicals were obtained at highest purity from Merck, Roth, Serva and Sigma, and were dissolved in deionized H_2O.

2.3.1 Inhibitors

	Type of reagent	**Stock concentration**	**Final concentration**	**company**
MEK-1 inhibitor (PD98059)	Organic compound	50mM	50µM	Cell Signaling (Beverly, MA)
PMSF	Protease inhibitor	0.1M	1mM	Roche, Mannheim, Germany
Protease inhibitor mix	Protease inhibitor	25x	1x	Complete™, Roche

Table 6: Inhibitors

2.3.2 Cell culture reagents

	effective concentration	**company**
2-ME	5µM	Roth
ConA	2µg/ml	Sigma (Taufkirchen)
DMEM		Life Technologies Eggenstein,Germany
EMEM		BioWhittaker
FCS	5-10%	Life Technologies
HEPES	various	Roth
L-Glutamine	1mM	Biochrom
NaPyr	1:100	Biochrom
NEAA	1:100	Biochrom

	effective concentration	**company**
Penicilline	1U/ml	Biochrom
RPMI		Life Technologies
Streptomycine	1U/ml	Biochrom
Trypan blue	1:4 in PBS	Biochrom
Trypsine	2x	Biochrom
T-Stim™	10%	Becton Dickinson

Table 7: Cell culture reagents

2.3.3 Kits

CELLection ™ Mouse CD8	CD8 T cell purification	Dynal, Hamburg
CellTiter96® AQ_{ueous} One Solution Cell Proliferation Assay	Proliferation assay	Promega
CytoTox96 Non-Radioactive Cytotoxicity assay	Cytotoxicity assay	Promega
Geneclean	Elution of DNA fragments from agarose gels	Dianova

Table 8: Kits

2.3.4 Cytokines & Growth factors

	concentration	**company**
IFN-γ (murine recombinant)	5ng/ml	Cell Concepts, (Umkirch)
IL-2	500ng/ml	Prof. Sebald
TGF-β1	240pM	R&D Systems (Minneapolis, MN)

Table 9: Cytokines and growth factors

2.3.5 Other reagents

	Used in	**Company**	**concentration**
β-mercaptoethanol	Cell culture	Sigma	5µM
BSA	WB, FACS,…	Sigma	various
C14-rainbow marker	Crosslink	Amersham	various
Chloroquine	Transfection	Sigma	
DMSO	variable	Roth	variable
DNase I	CD8+ cell preparation	Roche	120U/ml
dNTP	PCR	Pharmacia	10pmol
DSS	Cross link	Pierce	0.1mg/ml
EZ-Link Sulfo-NHS-LC-Biotin	Biotinylation	Pierce	0,5mg/ml
H_2O_2	Western Blot	Merck	variable
Lipofectamine	transfection	Life technologies	variable
Luminol	Western blot	Sigma	1.25mM
Lysozyme	DNA preparation	Roth	5mg/ml
NaN_3	WB; fluorescent staining	Sigma	variable
p-Coumaric acid	Western blot	Sigma	0.68mM
Penicilline	Cell culture		100U/ml
Pfu polymerase	DNA modification	Promega	variable
Pfu polymerase Turbo	DNA modification	Stratagene	variable
Polybrene	Retroviral infection	Sigma	8µg/ml
ProteinA sepharose	IP	Sigma	variable
Radioactive NaJ	Iodination	Amersham	variable
Restriction enzymes	Cloning	MBI Fermentas New England Biolabs	variable
RNase	DNA preparation	Roth	variable
SDS-7B	SDS-PAGE marker	Sigma	variable
Skim milk powder	WB	Fluka	3-5%
Sodium butyrate	Retroviral infection	Sigma	10mM

	Used in	Company	concentration
Streptavidin-HRP	Biotinylation	Pierce	1:10000
Streptomycin	Cell culture		100µg/ml
Taq polymerase	DNA modification	Promega	variable
Tween-20	variable	Merck	variable

Table 10: Other reagents

2.3.6 Antibodies

	origin	used in	dilution	obtained from
Histone H3	rabbit	**WB** TBS 0.1%Tween 5% milk o.n.	1:1000	Cell signaling (Beverly, MA)
Lamin antisera	human anti-human	**WB** 10% milk o.n.	1:200 in TBS-T 1h RT	Prof. Scheer Biocenter, Würzburg
P-p38	rabbit	**WB** TBS/1%BSA 38kD	1:2000 in TBS/1%BSA 2h RT	Promega
P-SAPK/JNK	rabbit	**WB** TBS 0.1%Tween 5% milk o.n.	1:1000	Cell signaling (Beverly, MA)
αCD28 (clone 37.51)	αmouse	**T cell stimulation**	5mg/ml	BD Pharmingen (San Diego, CA).
αCD3ε (clone 145-2C11)	αmouse	**T cell stimulation**	2µg/ml	BD Pharmingen (San Diego, CA).
αPSmad2(SSXS)	rabbit	**WB**	1:1000 in TBS/0.5% tween	Peter ten Dijke (The Netherlands Cancer Institute, Amsterdam)
αPsmad3	rabbit	**WB**	1:1000 in TBS/0.5% tween	Peter ten Dijke (The Netherlands Cancer Institute, Amsterdam)

	origin	**used in**	**dilution**	**obtained from**
αrabbit IgG (peroxidase konjugated)	Goat αrabbit	**WB** (2° ab)	1:20.000 in TBS/T	Dianova (Hamburg, Germany)
αSmad2(linker)	rabbit	**WB**	1:1000 in TBS/0.5% tween	Peter ten Dijke (The Netherlands Cancer Institute, Amsterdam)
αSmad3	rabbit	**WB**	1:1000 in TBS/0.5% tween	Peter ten Dijke
αSmad4	rabbit	**WB**	1:1000 in TBS/0.5% tween	Peter ten Dijke
αTβRII	rabbit	**IP**	1:50	[Moustakas A #219]
αHA	mouse	**WB,IP**	1:200	Hybridoma-produced

Table 11: Antibodies

2.4 Cell lines and expression vectors

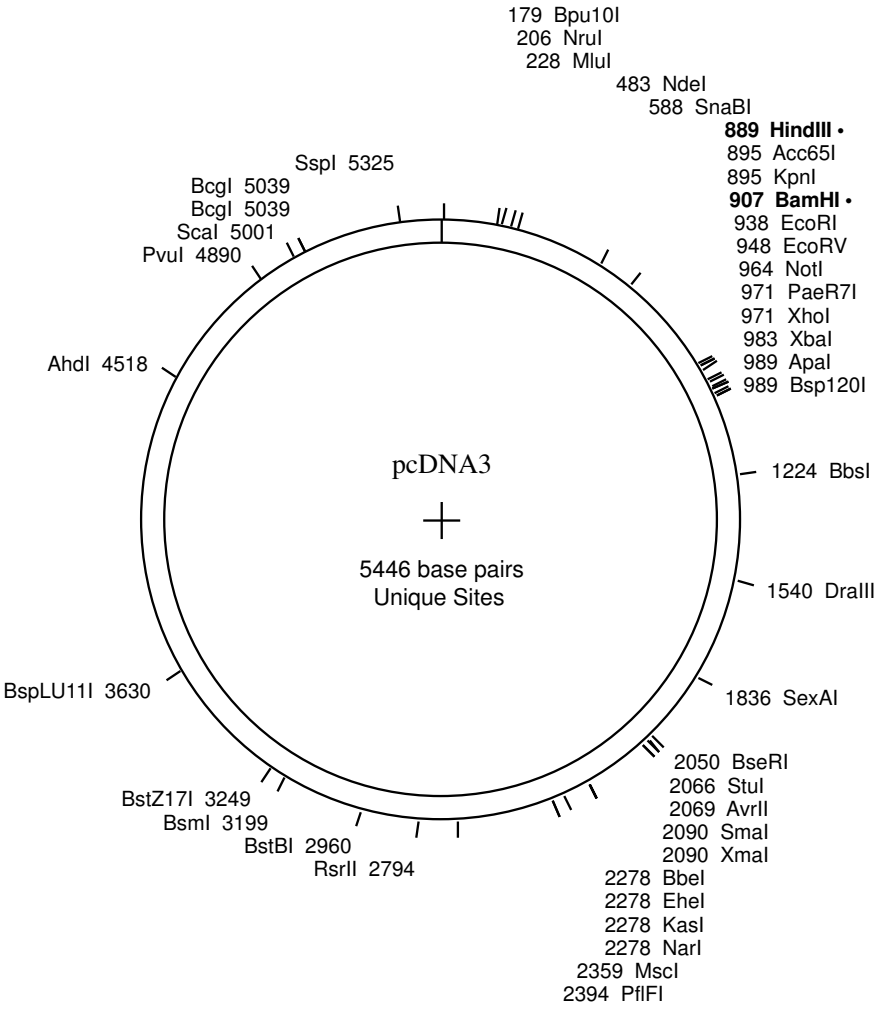
2.4.1 Expression vectors and plasmid maps

	insert	**# in Stab**	**resistance**
CMV-GFP	GFP	80	Amp
EGIRT ScFv	ScFv	107	Amp
EGIRT TβRII	TβRII	52	Amp
EGIRT TβRIIdcyt	TβRIIdeltcyt	90	Amp
EGIRT Tβ-TK	TβΔcyt-TK	250	Amp
FOV-7	HSV-TK	232	Amp
H20HA	TβRII-HA in pcDNAI	10	Amp/Tet
p3TPlux	SBE+luciferase	24	Amp/Tet
pcDNAIII		95	Amp
pczVSV-G wt	VSV-G	172	Amp
pet3d		148	Amp
pet3D TβΔcyt	TβΔcyt	206	Amp
pHIT60	gag-pol	81	Amp

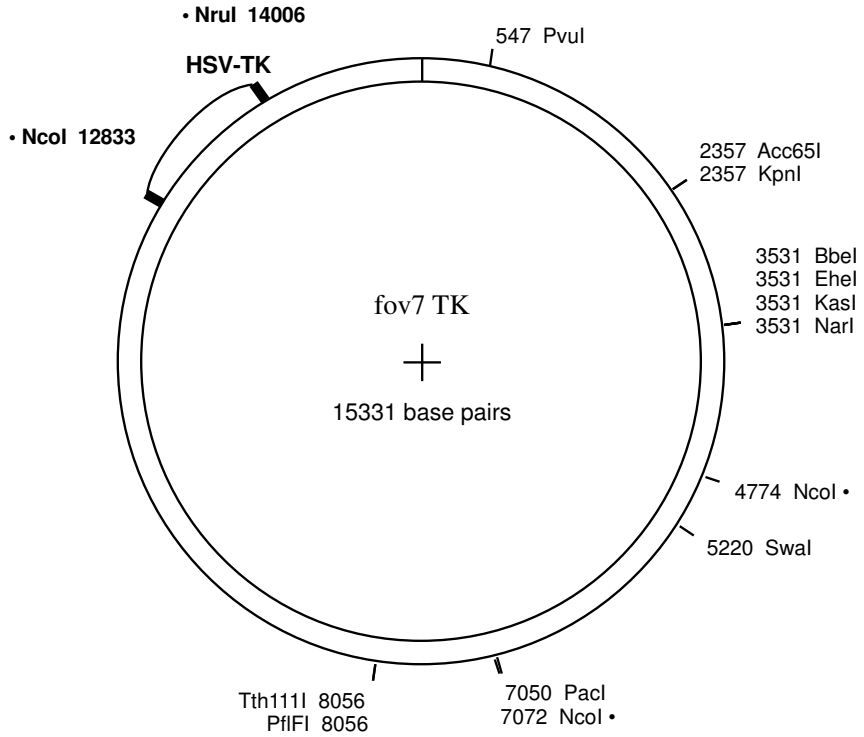
	insert	# in Stab	resistance
pLuc+	luciferase	149	Amp
pRLTK	Renilla	51	Amp
TβRI-HA	TβRI-HA	47	Amp/Tet
Tβ-TK in pcDNAIII	TβΔcyt-TK	231	Amp

Table 12: Expression vecors

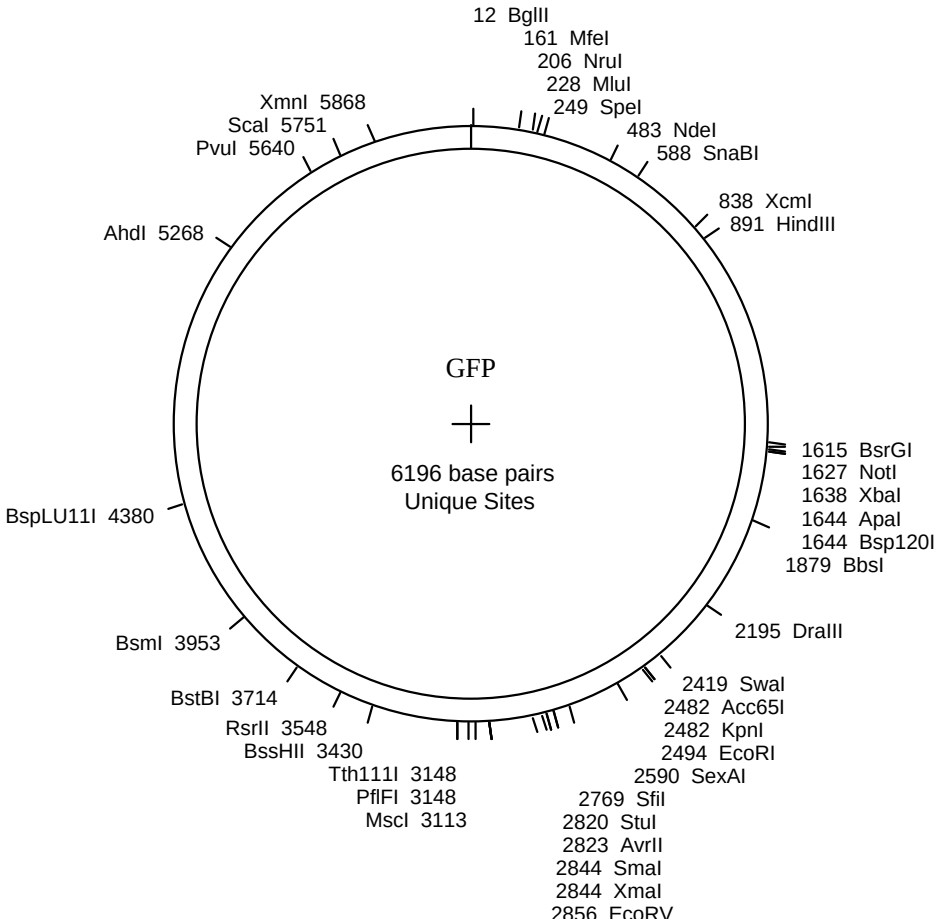
pcDNAIII:



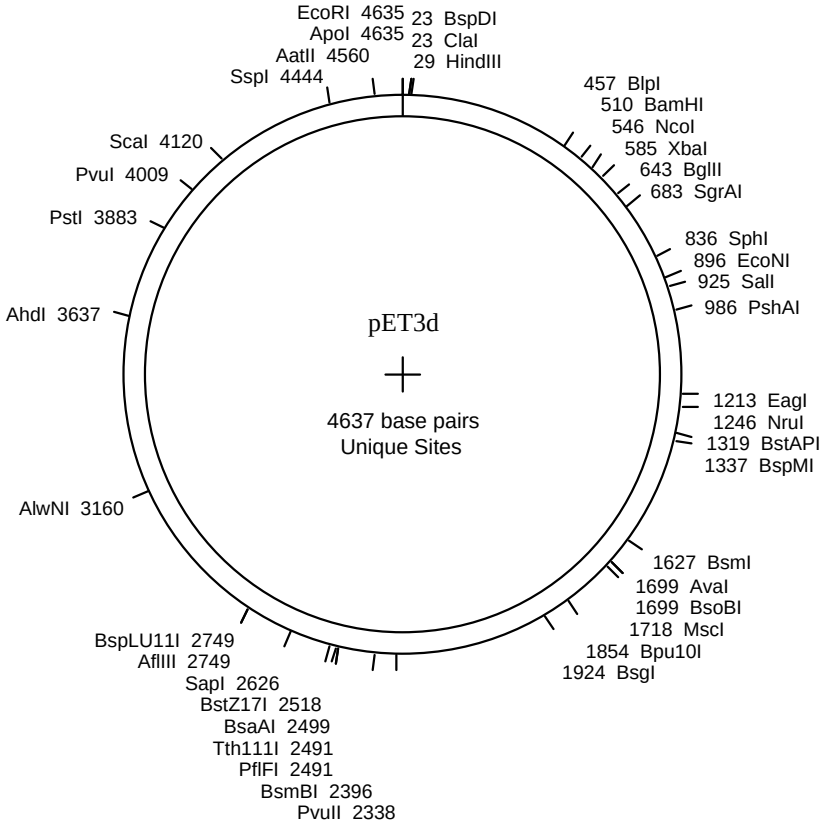
FOV-7 HSV-TK (obtained from Dr. Lindemann, Würzburg)**:**



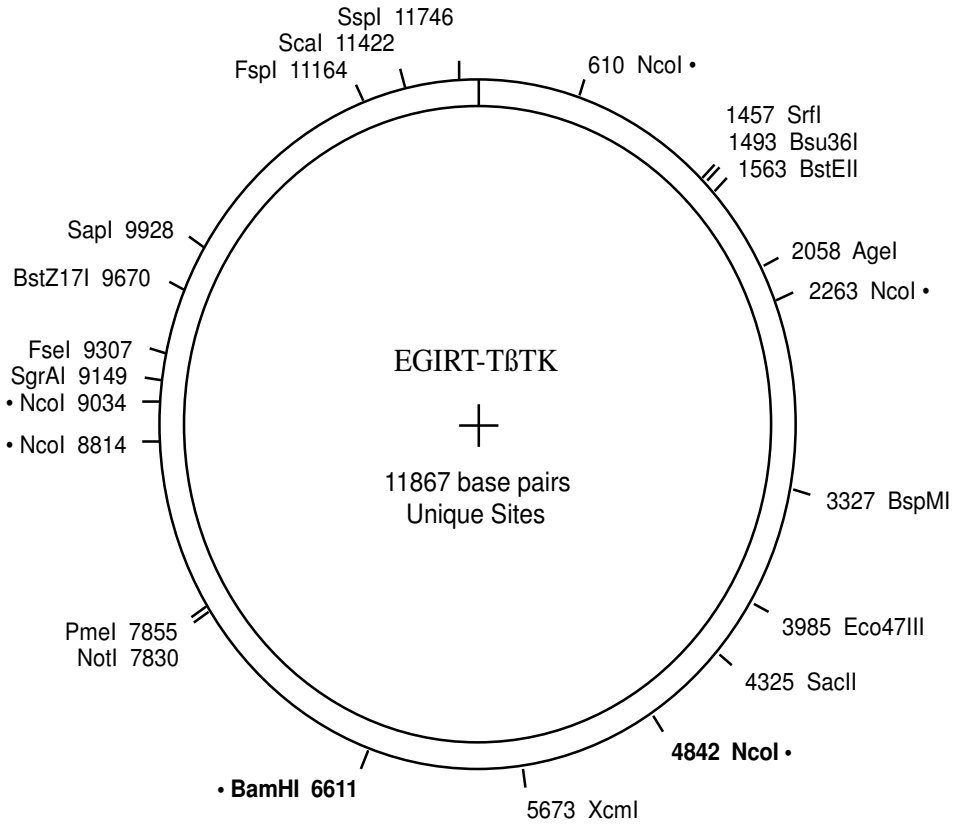
CMV-GFP:



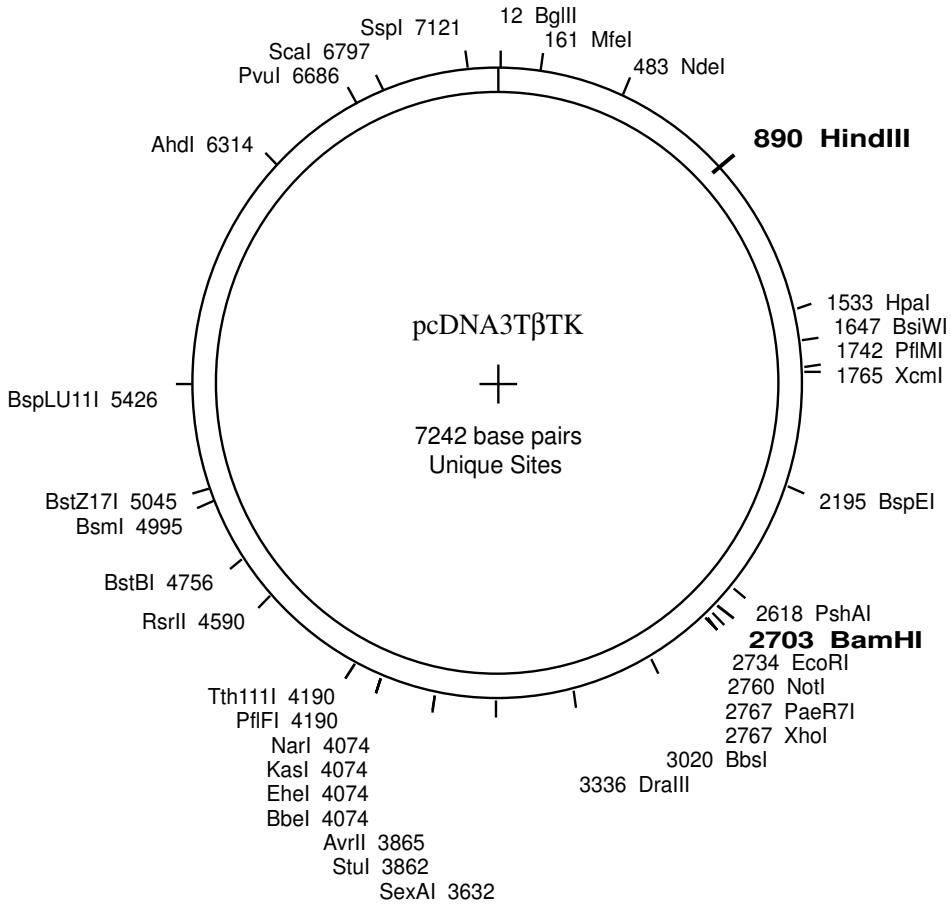
pet3d:



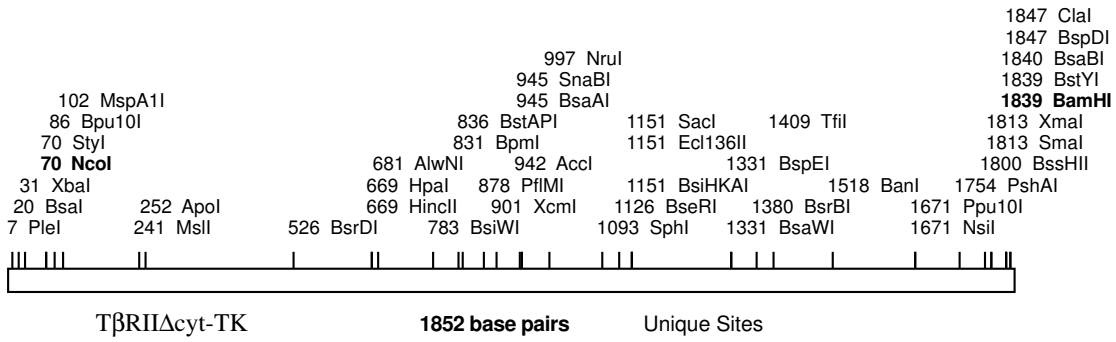
EGIRT-TβΔcyt-TK:



pet3d-TβΔcyt-TK:



TβΔcyt-TK fragment:



2.4.2 Oligonucleotides and sequences

Oligonucleotides for molecular cloning were synthesized by Interactiva and were HPLC grade. The lyophilized oligonucleotides were dissolved in dH_20 to obtain a final concentration of 100pM/µl.

P6: 5'-GGT GTT ATA TTC TTC TG-3'

T7: 5'-AAT ACG ACT CAC TAT AGG-3'

TTK1 5'-AAG CTG AGT **GGA GGT GGC GGG GGA GGA GGT GCT** TCG TAC CCC TGC CAT CAA CAC -3'

TTK2:5'-ATC GAT TAG GAT CCT CAG TTA GCC TCC CCC ATC TC -3'

TTK3:5'-GTA CGA AGC **ACC TCC CCC GCC ACC TCC ACT CAG** CTT CTG CTG CCG GTT AAC-3'

TTK4:5'-GGG AGA CCC AAG CTT CTA GAA ATA A-3'

Marked in **red**: complementary linker region

2.4.3 Cell lines

	media	**CO_2**	**splitting**	**origin**	**source**
293T	EMEM 10% FCS	5%	1:20 every 2nd day	Human fibroblasts	Dirk Lindemann, Würzburg Virology department
A 301	RPMI 10% FCS	5%	1:10 every 2nd day	Human T-lymphocytes	Dirk Lindemann
C2C12	DMEM 10% FCS	10%	1:5 every 2nd day	Murine mesenchymal precursor cells	ATCC
COS-7	DMEM 10% FCS	10%	1:5 every 2nd day	Monkey kidney	ATCC

	media	CO_2	splitting	origin	source
CTLL-2	RPMI 10% FCS 10% T-Stim™ 5%T-supplement	5%	1:5 every 2nd day	Murine cytotoxic T cells	ATCC
DR26	EMEM, 10%FCS NEAA	5%	1:10 every 2nd day	Mink lung epithelial cells; do not express TβRII [Laiho M #167]	Massague, New York
EL-4	RPMI 10% FCS	5%	1:10 every 2nd day	Murine T cell lymphoma	Lindemann
Jurkat	RPMI 10% FCS	5%	1:10 every 2nd day	Human T cell lymphoma	Lindemann
MD45	DMEM 4g sucrose 2mM NaPyr 10% FCS	5%	1:10 every 2nd day	Murine Hybridoma cells	Weizmann Institute, Rehovot, Israel
MvLu	EMEM, 10%FCS NEAA	5%	1:10 every 2nd day	Mink lung epithelial cells	ATCC
P815	RPMI 10% FCS	5%	1:10 every 2nd day	murine mastocytoma (DBA/2)	DSMZ

Table 13: Cell lines

MD45

MD45 cells are derived from fusing murine lymphoma cells with cytotoxic T cells from Balb/c mice. The CTL were specific for EL-4 mouse tumor cells. The cells are described to need no T cell growth factors and to be cytotoxic even after months of culture. The cells are roughly double the size of parental lymphoma cells, and thrice the size of parental CTL. They do not display NK-activity as determined by YAK-cells and do not show antibody-dependent cytotoxicity. MD45 are reported to be transfectable by electroporation using 20µg of DNA

CTLL-2

CTLL-2 cells need T-Stim™ as supplement, as these cytotoxic T cells do not produce IL-2 and other cytokines by themselves. T-Stim™ is media supernatant from ConA stimulated rat splenocytes and contains predominantely IL-2, but also other cytokines. They should be frozen in culture media containing an additional 10% FCS and 5% DMSO. After thawing, the cells need about a week to start growing.

2.5 Solutions & Buffers

AA/Bis-AA	30% Acrylamide 1% N,N'-methylenebisacrylamide
Agarose gel electrophoresis	
TAE 1x	40mM Tris-acetate 1mM EDTA
Bacterial media	
LB-media (pH 7.4)	10g/l Bacto-Trypton 5g/l Yeast-extract 10g/l NaCl
SOB media	20g/l bacto tryptone 5g/l yeast extract 0.5g/l NaCl 0.83mM KCl
SOC media	100ml SOB + 1ml 40% glucose 1ml 1M $MgCl_2$ 1ml 1M $MgSO_4$
Tfb1	30mM KCl 100mM RbCl 10mM $CaCl_2$-$2H_2O$ 50nM $MnCl_2$-4 H_2O +75ml glycerine (86%) ad 500ml H_2O
Tfb2	10mM MOPS 75mM CaCl2-$2H_2O$ 10mM RbCl (pH6.5)

	+15ml gycerine ad 100ml H_2O
Biotinylation	
TBS	0.05M Tris/HCl pH 7,4 0.15M NaCl
TBS-T	0.05M Tris/HCl pH 7,4 0.15M NaCl 0.1% Tween-20
Cell lysates	
TNE lysis buffer (Smads)	20mM Tris/HCl pH 7.4 150mM NaCl 1% triton-X-100 1mM EDTA 50mM sodiumfluoride 1mM sodiumorthovanadate 10mM $Na_4P_2O_7$ protease and phosphatase inhibitors
Triton-Lysis buffer (receptors)	1mM EDTA 1% triton-X-100 PBS
Cellular fractionation	
Buffer A	10mM HEPES (pH7.9) 1.5mM $MgCl_2$ 10mM KCl
Buffer B	20mM HEPES (pH7.9) 25% glycerol 0.42M NaCl 1.5mM $MgCl_2$ 0.2mM EDTA

DNA preparation	
Solution1 (pH 8.0)	25mM Tris/HCl
	50mM glucose
	10mM EDTA
	5mg/ml lysozyme
Solution2	0.2N NaOH
	1% SDS
Solution3 (pH4.8)	3M KAc
DNA sample buffer (6x)	0.25% bromophenole blue
	0.25% xylene cyanole
	30% gycerine
Erythrocyte lysis buffer	0.16M NH_4Cl
	0.017M Tris/HCl pH 7,65
Fluorescent staining	
PIN-solution	1x PBS
	0.1% BSA
	0.005% NaN3
Iodination and Cross-link	
Elution buffer	4mM HCl
	75mM NaCl
	0.1% BSA
KRH (pH 7.5)	50mM HEPES
	128mM NaCl
	1.3mM $CaCl_2$
	5mM $MgSO_4$
	5mM KCl
Luminol detection	
Sol A	5.4mM H2O2
	0.1M Tris/HCl pH 8.5
Sol B	2.5mM Luminol
Luminol stock	2.5mM Luminol
Coumaric acid stock	90mM coumaric acid

PBS	120mM NaCl 2mM KCl 3mM KH_2PO_4 7mM Na_2HPO_4
Retroviral infection **2x HBS**	 50mM HEPES (pH 7.05) 10mM KCl 12mM α-D-Glucose 280mM NaCl 1.5mM Na_2HPO_4 adjust to pH 7.05 with 1N NaOH
SDS Gel **running buffer** **4x lower Tris(pH 8.8)** **4x upper Tris**	 25 mM Tris/HCl pH 8,6 1M glycine 0,15% SDS 1.5M Tris/HCl 0.4% SDS 0.5M Tris/HCl 0.4% SDS
SDS-PAGE sample buffer (2x), **pH 8.6**	62.5mM Tris/HCl 2% SDS 20% gylcerine (stock 87%) 1% bromophenole blue 5% 2-ME

WB	
Transfer buffer	25mM Tris/HCl pH 8,6
	192 mM glycine
	20% methanol
Stripping buffer	5mM phosphate buffer
	2% SDS
	0.014% 2-ME
Washing buffer	10mM Tris pH8.0
	150mM NaCl
	0.5% Tween

Table 14: Solutions and buffers

SDS-gel concentrations

	recommendend for:	**H_2O**	**acrylamid/bis-acrylamid**	**buffer**
7.5%	200-60 kD	6ml	3ml	lower Tris 3ml
10%	120-50 kD	5ml	4ml	lower Tris 3ml
12.5%	100-10 kD	4ml	5ml	lower Tris 3ml
stacking gel		5ml	1ml	upper Tris 1ml

To the stacking gel, 10µl of each APS/TEMED is added.

Table 15: SDS-gel concentrations

3 Methods

3.1 Microbiology

3.1.1 Bacterial cell culture

Bacterial cultures are either picked from agar plates, taken from frozen gycerol stocks or taken from already existing liquid cultures. The bacteria are then cultured overnight at 30°C (C600) or 37°C (DH5α). As media, LB media is used, including the appropriate antibiotics.

3.1.2 Heat shock transformation

First, competent bacteria have to be produced. For this, the bacteria are cultured in 100ml LB media at 37°C until the culture reaches an OD of 0.2-0.3. After briefly cooling the bacteria on ice they are centrifuged (4°C, 2500rpm, 10min). The pellet is then resuspended in Tfb1 and again centrifuged. The cells are then resuspendend in Tfb2 and incubated for 15min on ice. Then, aliquots are frozen in liquid nitrogen and stored at –80°C.

To transform these bacteria with the desired plasmid, the bacteria are thawed on ice. 1-10ng of the plasmid DNA (or the ligation) are added to 100µl of the thawed bacteria and kept on ice for 30min. This is followed by the heat shock, ar 42°C for 90sec. Afterwards, the bacteria are incubated in 1ml of SOC media for 1h at 37°C and may then be selected on agar plates containing antibiotics.

3.2 Molecular biology

3.2.1 DNA preparation (alkaline lysis)

Mini-Prep

The bacteria are centrifuged at 6000rpm in an eppendorf centrifuge. To 100µl of solution I 5mg/ml lysozyme is added, + 3µl RNAse. The cells are then incubated for 5min on ice. 100µl of solution II is added, followed by further incubation for 5min on ice. Then, solution II is added (200µl), followed by incubation for 10min on ice. Then, the probe is centrifuged for 15min, 13000rpm. The supernatant is transferred to a new tube, and 180µl of isopropanole are added for 10min at RT. After centrifugation (10min, 14000rpm) the pellet is washed with 80µl of 70% EtOH, centrifuged again, dried in the SpeedVac and resuspendend in 20µl of H_2O (sequencing) or TE.

Midi-Prep

The bacteria are centrifuged for 10min at 6000rpm in the JA-20 centrifuge rotor. To 5ml of solution I, 5mg/ml lysozyme is added, + 50µl RNAse. The cells are then incubated for 5min on ice. 5ml of solution II is added, followed by further incubation for 5min on ice. Then, solution III is added (10ml), followed by incubation for 10min on ice. Next, 9ml LiCl (5M) are added (10min ice). Then, the probe is centrifuged for 15min, 14000rpm. The supernatant is filtered, and 0.6 volumes of isopropanol are added for 10min at RT. The probe is centrifuged in a Falcon centrifuge at 4000rpm for 30min. After centrifugation , the pellet is washed with 500µl of 70% EtOH, centrifuged again for 2min, dried for 1h at RT and

resuspendend in 1ml of H_2O. The solution is transferred to an eppendorf tube. 50µl of RNase is added, and incubated for 1h at 37°C. For phenole extraction, 1ml of phenole is added to the DNA, mixed and centrifuged for 5min at 14000rpm. The upper phase (water) is transferred to a new tube. Then, 1ml of 24:1 chloroforme/isoamylalcohol is added, and the extraction step is repeated. Next, the DNA is isopropanole precipitated in order to desalt and recover the nucleic acid. Per cap, 100µl of 3M NaAc and 300µl isopropanol is added and incubated for 10min at RT. After 10min of centrifugation (14000rpm), the pellet is subjected to a further EtOH-wash, dried in the SpeedVac, and resuspended in 100µl of H_2O (sequencing) or TE. The concentration of the nucleic acids may be analyzed by photo-spectrometry. The absorption wavelength is 260nm, and the spectra are recorded from 240-320nm. One absorption value (A_{260}) equals 33µg/ml of oligonucleotides, 40µg/ml for RNA or ssDNA, and 50µg/ml with dsDNA.

3.2.2 DNA modification

Digestion with restriction enzymes

Restriction endonucleases are able to recognize specific palindromic sequences on the DNA and cut these sequences on dsDNA. All restriction enzymes as mentioned in the "results" section were used according to the manufacturers instructions.

Ligation

For ligating DNA fragments, T4 DNA ligase (Promega) was used. This enzyme catalyses the formation of phospho-diester bonds between the 3'hydroxy-and 5'-phosphate groups of DNA fragments. The ligation protocol as provided by the distributor was followed throughout the procedure. The ligation was either incubated for 2-3h at RT, or at 16°C overnight.

PCR

The PCR reaction amplifies defined DNA fragments. To perform a PCR, the ds DNA strand has to be denatured, and serves then as template for the synthesis of identical fragments by the DNA polymerase. As primers, synthetic oligonucleotides are used. The denaturing step is followed by the annealing phase, in which the polymerase produces the fragments from the added dNTPs. In the last phase, the extension step, the DNA fragments are filled up. Then, the next cycle is being started with –again- the denaturation of the dsDNA. The annealing temperature is dependent on the GC content of the DNA fragments: The higher the GC content, the higher the annealing temperature has to be chosen.

For an analytical PCR, bacteria are used directly in the PCR reaction (20µl), and the Taq polymerase (0.5U/reaction) without proofreading capacity was chosen. As this serves solely

to check on the succes of cloning, this is sufficient. For preparative PCR reactions, the product is further needed for cloning or sequencing. Therefore, the reactions have to be greater in volume, and a polymerase with high proof reading capacity (e.g. Pfu, 1U/reaction) has to be used, in order to minimize the mutation rate.

The reaction for a preparative PCR contains:
- 10pmol dNTP
- 1/10 volume 10x reaction buffer
- 100pmol of primer A and B
- 1-10ng template DNA
- DNA polymerase

Recombinant PCR

This type of PCR is used in order to actively mutagenize DNA. The primer may contain additional epitopes, restriction enzyme recognition sites or mutations. By this technique, overlapping DNA fragments are amplified. These overlapping fragments then are used in a further PCR, where they anneal to each other and are amplified as one fragment. This way, they function as templates, and by using the outer primers again, the DNA fragments are further amplified and exist as ds DNA.

3.2.3 Agarose gel electrophoresis

Analytical agarose gels allow separation and identification of nucleic acids based on charge migration. In an electric field, nucleic acid molecules migrate towards the anode due to negatively charged phosphates along the backbone. The migration is determined by their size and conformation, allowing nucleic acids of different sizes to be separated. However, the relation between the fragment size and rate of migration is non-linear, since larger fragments have greater frictional drag and are less efficient at migrating through the polymer. Agarose gel electrophoresis is efficient for analyzing DNA fragments between 0,1 and 25kB. The concentration of the agarose depends on the size of DNA fragments to be analyzed:

Agarose concentration (%w/v)	**DNA fragment range (kb)**
0.3	5-60
0.5	1-30
0.7	0.8-12
1.0	0.5-10

Agarose concentration (%w/v)	DNA fragment range (kb)
1.2	0.4-7
1.5	0.2-3
2.0	0.1-2

Table 16: Agarose gel concentrations with respect to DNA fragmet lengths

To perform the electrophoresis, the electrophoresis tank is filled with buffer. The agarose is boiled and cooled to 55-60°C. EtBr is added to allow the DNA to be visualized under UV light. The agarose is poured on the gel tray to a thickness of 3-5mm without air bubbles, and the comb is inserted. The gel has to set now for 30min.Then, the gel is inserted into the electrophoresis chamber, and the comb is removed. The buffer has to cover the gel with a depth of approximately 1mm above the gel. If too much buffer is used, the current will flow through the buffer instead of the gel

3.2.4 Elution of DNA fragments

For the isolation of DNA fragments from agarose gels, the geneclean-kit was used. The desired DNA bands are excised from the agarose gels, and the gel compartments containing the desired DNA are weighed. Then, thrice the volume NaJ is added. The agarose is melted at 55°C. After the agarose has melted, 10µl of glassmilk is added, and incubated for 15min at RT, mixing every 1-2 minutes to ensure that the glassmilk stays suspended. The glassmilk then is briefly spun down, and washed 3x with NEW Wash. After the 3rd wash, the liquid is totally removed. Then, the DNA is eluated twice with each 10µl of ultrapure H_2O at 55°C for 5min. The glassmilk is centrifuged for 5min, and the supernatant is transferred to a new tube.

3.3 Cell biology

3.3.1 Cell culture

The cells were cultured according to their needs for media and special additives as described in “cell lines”. Most cells were propagated in 75cm ^{2} tissue culture flasks and splitted 3 times per week. The media always contained Pen/Strep. Adherent cells were splitted by using trypsine, suspension cells were centrifuged and splitted in new media. The cells were counted by using typan blue exclusion: 0.5% trypan blue solution is unable to perfuse live cells, but

apoptotic or necrotic cells are stained blue. The cells were then counted in a Neubauer counting chamber counting two 4x4 quadrants.

3.3.2 Transfection Methods

By transfecting cells, plasmid DNA is transferred into the living cells, and the genes are transcribed and translated. Different methods exist based on creating pores in the cell membrane or the ability of cells to integrate lipid biliyers into their cell mebrane.

Lipofectamine

In a 6-well-plate, $1x10^5$ cells are plated on day 1, to be 50-80% confluent on day 2. For solution A, 2µg of DNA are diluted in 100µl of media free of PenStrep, containing 0,2% FCS (SF-media). For solution B, 2-25µl lipofectamine are diluted in SF-media. Both solutions are mixed and incubated for 15-25min, while the complexes assemble. Meanwhile, the cells are washed with SF-media. After the indicated incubation time, 800µl of SF-media are added to solution A/B and pipetted on the cells. The cells are thus incubated for at least 5h or overnight. Following this incubation period, growth media containing 20% FCS is added. On day3, SF-media together with ligand is added, and cells can be analyzed further by luciferase on day 4.

DEAE-Dextran

The COS-cells ought to be 50-60% confluent. On the day of transfection, the cells are washed 3x with transfection buffer (view table "Solutions and Buffers"). 7µg of DNA are diluted in transfection buffer to the amount of 1140µl. To this solution, 60µl of 10mg/ml DEAE-dextran are added. The cells are incubated with the DEAE-dextran/DNA solution for 30min with repeated gentle shaking. After this incubation, 14ml of chloroquine media are added, consisting of DMEM + 10% HS and 80µM chloroquine. The cells are incubated in this media for 3h, followed by DMSO-shock: The media is aspirated, and 3ml of growth media + 10% DMSO are added for 2 ½ min. Then, the DMSO-media is aspirated, and 10ml of normal growth media is added. After 48h, the cells can be submitted to various assays.

Electroporation

As cuvettes, 4mm gap cuvettes from Pequlab were used. The CTLL-2 cells are harvested during logarithmic growth, and washed 2x in HBS and resuspended in HBS at a concentration of $2x10^7$ cells/ml. 400µl of this cell suspension is transferred to a cuvette, and 40µg of DNA is added. The parameters are set at Low Voltage, 200V, 950µF. After electroporation, the cells are seeded in 2 wells of a 6-well-plate in each 3ml of prewarmed media.

3.3.3 Production of replication deficient MuLV retroviruses by transient transfection of 293T cells

Transfection of 293T

On day 1, $2x10^6$ 293T cells are plated in 5ml EMEM into 6cm cell culture dishes. On day 2, first the EMEM media has to be equilibrated in the incubator for at least 30min. Then, the media from the dishes is aspirated, and 4ml of fresh EMEM is added. Altogether, 15µg DNA is used per dish, each 5µg of envelope-DNA, gag-pol and retroviral DNA. The combined DNA is brought to 438µl in sterile water. 62µl of 2M CaCl2 is added to the water, and 500µl of 2xHBS is added by bubbling. Immediately the transfection solution is added dropwise to the plates, and the plates are gently shaken to distribute the solution evenly. The plates are then returned to the incubator for 7-10h. After the indicated incubation time, the media is aspirated and replaced with 5ml of fresh media. The next day, the media is replaced by EMEM containing 10mM sodiumbutyrate, and the cells are incubated for 8-12h, followed by a washing step and the addition of 3ml of fresh media.

Infection of cells

Adherent cells

The media of the transfected 293T is taken off with a syringe and filtered through a 0,45µm syringe filter to dispose of cell debris. To the virus-containing supernatant, a final concentration of 8µg/ml polybrene is added. From the adherent target cells that had been plated into 6well-plates the day before, the media is aspirated, and the virus-containing supernatant is added. After 4-8h of incubation, the media is exchanged for fresh media.

Suspension cells

The target cells are brought to $1x10^5$/ml, centrifuged, and the virus-containing supernatant is added. After the indicated incubation time, the cells are spun down and supplied with fresh media. The infection is repeated samewise the next day. On day 6 or 7, the expression of GFP for determining the infection efficiency is measured in a flow cytometer.

3.4 Protein chemistry

3.4.1 Cellular lysates

For each lysate, $5x10^5$ (purified CD8+ T cells) or $1x10^6$ mixed splenocytes were harvested on day 3 of culture, washed with fresh media, resuspended in fresh media and returned to the incubator to be starved for TGF-β1 for 3 hours. Then, cells were washed again, resuspended

in 500µl of fresh media and pulsed with 240pM TGF-β1 for 15 minutes. The reaction was stopped on ice, cells were centrifuged and the supernatant discarded. Cell pellets were resuspended in 30µl of lysis buffer consisting of TNE-Buffer, PMSF, protease inhibitor mix (Complete™, Roche, Mannheim, Germany), 50mM sodiumfluoride, 1mM sodiumortho-vanadate and 10mM $Na_4P_2O_7$. After 10 minutes on ice lysates were centrifuged at 14.000rpm in an eppendorf centrifuge at 4°C for 10 minutes. The supernatant was then mixed 1:1 with loading buffer containing 2% SDS and 5% β-mercaptoethanol, boiled for 10min, centrifuged again, and subjected to SDS-PAGE.

3.4.2 Immunoprecipitation

Of the cleared cellular lysates, aliquots of 500µl are placed into 1.5ml eppendorf tubes. Then, the desired antibody is added to a concentration of 1-4µg/ml. The tube is incubated with agitation in the cold room at 4°C. Then, 30µl of protein A sepharose beads (to which the antibodies bind) in a 1:1 solution in PBS is added, and the tube is again incubated on the roller in the cold room over night. The next day, the beads are washed twice with lysis buffer, and once with ice cold PBS. Then, 50µl of SDS-PAGE sample buffer is added, vortexed, and boiled for 10min. Then, the samples are centrifuged for 5min at 14.000 rpm and transferred to a new tube. Now they can be loaded on the SDS-gel.

3.4.3 SDS-PAGE

SDS-PAGE involves the separation of proteins based on their size. By heating the sample under denaturing and reducing conditions, proteins become unfolded and coated with SDS detergent molecules. By this, they acquire a high net negative charge that is proportional to the length of the polypeptide chain. When loaded to the gel matrix, and placed into a electric field, the negatively charged proteins migrate towards the positively charged electrode and are separated by a molecular sieving effect. The concentration of acrylamide used for the gel depends on the size of the proteins to be analyzed. Low acrylamide concentrations are used to separate high molecular weight proteins, and high concentrations are used to separate low molecular weight proteins (see section "Solutions and Buffers). Improved resolution of protein bands is achieved by the use of a discontinuous gel system having stacking and separating gel layers.

3.4.4 Western Blot

Blotting

The blotting procedure was wet type transfer in WB transfer buffer on a nitrocellulose membrane.

Incubation and development

The membrane is blocked according to the antibody to follow with skim milk or BSA in TBS-T for at least 30min at RT. After briefly rinsing the membrane with TBS-T, the 1° antibody is incubated o.n. or 1h at RT, followed by 3x washing for 5min in TBS-T. The 2° antibody is incubated for 1h RT, again followed by 3 washing steps. ECL solutions A and B are combined and added to the mebrane for 60s. The excess fluid is drained, and the membrane exposed to the film.

Stripping

For the removal of the antibody from the membrane 10ml of stripping buffer + 1.35µl 2-mercaptoethanol are added and and the membrane is incubated, sealed in a plastic bag, for 30min at 56°C. After incubation, the stripping buffer has to be washed off thoroughly with PBS, and then the membrane can be blocked again as described.

3.4.5 Cellular fractionation

To all buffers, protease/phosphatase inhibitors have to be added as noted in section "Solutions & Buffers". $3x10^7$ cells are washed in a 15ml Falcon tube with ice cold PBS and transferred into 1,5ml safe-lock Eppendorf caps. The cells are centrifuged (1000g, 3300rpm) for 10min at 4°C in an Eppendorf centrifuge. The supernatant is discarded, and the cell pellet is resuspended in 50µl buffer A. The cells are then vortexed 20 times, each 2-5 sec., with a shearer, and the lysis is consequently followed by microscopy and trypan blue staining. After >90% of the cells are lysed, the cells are centrifuged (1000g, 3300rpm) for 10min at 4°C. The supernatant is declared as "cytoplasmic fraction", and another centrifugation step follows: 25000g (14.000 in JA20 with Eppi-inserts), 20min at 4°C. The supernatant is added to the cytoplasmic fraction. The remaining pellet is resuspended in 25µl of buffer B, vortexed 20 times with a shearer as before, and rotated for 30min at 4°C. Then the lysate is centrifuged at 25000g, 20min at 4°C, and the supernatant is declared as "nuclear fraction". The purity of the fractionation may be verified using anti-Lamin-antibody as described in the "antibodies" section.

3.4.6 Biotinylation of surface proteins

Preparation of the lysates

$2x10^7$ cells are washed in 15ml Falcon tubes 2x with ice cold PBS (pH 8,0) and centrifuged at 1200rpm for 5min. In the meantime, Biotin can be dissolved in PBS: 0,5mg/ml; 500µl per falcon tube suffice. The PBS-wash is discarded thoroughly, and the cells are resuspended carefully in the biotin-solution. After incubation for 30min at RT, the cells are washed again 2x with cold PBS. Finally, the cells are lysed in 100µl of lysis buffer (1% triton) and rotated 45min at 4°C. Cleared lysates are prepared by spinning down the probes at 13000rpm for 10min (4°C).

Immunoprecipitation

To the supernatant antibodies are added (αTGFβRII: 1:100), and rotated for 1h at 4°C. Then, protein A sepharose is added (20µl), and the lysate is further rotated at 4°C overnight. The next day, the sepharose is spun down briefly, washed 2x with lysis buffer and 2x with PBS. Then, 30µl of SDS buffer is added, the sepharose is boiled for 10min and spun down for 5min at 14000rpm.

Western blot and development

The lysates are run on an 12,5% SDS-PAGE and blotted. The membrane is blocked for 30min in TBS + 5% BSA, and incubated with HRP-Streptavidine in TBS-T (1:10000) for 20min. Then, the membrane is washed 3x 5min with TBS-T, and developed with ECL.

3.4.7 Iodination of TGF-β

Information

The radioactive labelling is based upon the Chloramine T method. Chloramine T oxidizes J- to J2, which then can bind to tyrosine residues on the protein. Acetyltyrosine is thought to quench the residual NaJ, and residual chloramineT is reacting with KJ.

Preparation

The desired amount of TGF-β (carrier free!!) is dissolved in 10µl of 1M $NaPO_4$ (pH 7.4). 2µl (0.2mCi) 125-J are added. The mixture should not be pipetted due to the volatility of the Iodine. 5mg/ml ChloramineT is further diluted 1:100 and added stepwise to the mixture: After 2 min, 90s and 60s 2µl of ChloramineT is added, then subsequently 10µl 100mM acetyltyrosine, 100µl 100mM KJ and 100µl of a saturated solution of urea in acetic acid (1,2g/ml) is added. 2µl of this mixture is transferred to cap0. The rest is transferred to the Sephadex-column, and after 2min of incubation, the column is eluted with 670µl of elution buffer (view section "Solut ions and Buffers), collecting the eluate in cap2. This is repeated up

to cap4. For thin-layer chromatography, 1µl of the solution from each cap is pipettet on a chromatography paper strip, and submitted to chromatography in 10% TCA. The upper and lower half are counted in a γ-counter, and the amount of radioactively marked protein is calculated.

3.4.8 Binding and cross-link (suspension cells)

Preparation

The cells are washed in a 15ml Falcon tube for 2 times with prewarmed KRH + 0.5% fatty acid free BSA to clear all endogeneous TGF-β. The cells are incubated in KRH/BSA for 30min at 37°C, and placed on ice afterwards.

Binding

For the preparation of the binding solution, 500pM ^{125}J-TGF-β are added to KRH/BSA. The cells are centrifuged and resuspended in 1ml of the binding solution. The cells have to be incubated with further rolling at 4°C for 4h, followed by 4x washing with cold KRH.

Cross-link

The crosslinker (DSS) is dissolved in DMSO (10mg/ml), and further diluted right before the end of the binding time 1:100 in KRH. After the washing step, the cells are spun down again and resuspended in 1 ml of the crosslinker solution. The cells have to be incubated in the crosslinker for 15min.

Lysis and IP

The cells are centrifuged and 1ml of lysis buffer (+ PMSF/PI) is added, followed by rolling for 40min at 4°C. Then, the lysates are transferred into Eppendorf caps. The lysates are centrifuged, and to the supernatant the antibody is added. Further steps are as decribed for "Biotinylation"

3.4.9 Luciferase Assay (Dual-Luciferase® Reporter, Promega)

Theory

The term "dual" refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system, an "experimental" reporter and a "control" reporter plasmid that serves as a baseline response, thus monitoring transfection efficiency. As "experimental" activity, the luciferase of firefly (*Photinus pyralis*, Beetle luciferin → Oxyluciferin) is used, and activity of *Renilla reniformis* (Coelenterazine → Coelenteramide) serves as control. First the firefly reporter is measured by adding LAR (luciferase assay reagent), followed by measurement of Renilla activity by adding Stop&Glo®. As common

contents of lysis buffers like triton intensify autoluminescence of Coelenterazine, the use of a different passive lysis buffer (PLB) is required.

Performance of the assay

First the 5x concentrate of PLB is diluted in water. The cells are washed in PBS, and a suitable amount of PLB is added to the cells, the cells are resuspended and left on ice for 10min. Then, the lysates are briefly centrifuged. In translucent luciferase tubes, each 100µl of LAR is predispensed. 20µl of the lysate are transferred to the tube and mixed briefly. The firefly is measured. Now to the same tube 100µl of Stop&Glo® are pipetted, followed by recording renilla activity.

Luminometer program:

2s premeasurement
10s measurement } firefly

2s premeasurement
10s measurement } renilla

3.5 Immunology

3.5.1 T cell preparation

As a source for splenic T cells, 4-6 weeks old female C57BL/6 (H-2b) (Charles River, Sulzfeld, Germany) were used.

Preparing a single-cell suspension

After sacrificing the mice, the spleens are transferred to ice-cold RPMI media as soon as possible. In 3cm petri-dishes, media is prepared, and the spleens are transferred to the petri-dish. With the pistil of a syringe, the spleen structure is carefully destroyed, and the cells are filtered through a 70µm cell strainer into a 50ml falcon tube. The cells are centrifuged at 1200rpm for 10min (due to the small size). Lysis of the RBC is performed by hypotone Tris/NH_4Cl-buffer (view "Solutions and Buffers"): The pellet is resuspended, and per 100µl cell solution, 1ml of hypotone erythrocyte lysis buffer is added. After exact 2min of incubation, the Falcon tube is filled with media, and the cells are centrifuged. The pellet can be resuspended to a spleen cell single cell suspension. (Contains approximately 15% CD4+ T cells, and 5% CD8+ T cells)

Enriching the splenocytes for T cells via nylon-wool columns

For this step, the nylonwool columns are first wetted by filling them with a syringe through the rubber cap. The residual media is then forced out of the nylon wool column by filling the syringe with air and pushing the media out of the column. Then, the cell pellet is resuspendend in 2ml of fresh media and pipetted onto the nylon wool. It has to be considered that not more than the cells equal to three spleens are loaded onto the columns. Then the columns are tightly closed and placed horizontally in the incubator for 45min. After this time period, the cells are flushed out of the nylon wool column with fresh media, and centrifuged again. The suspension now contains approximately 35% T cells, and 15% CD8+ T cells.

3.5.2 Preparation of CD8+ T cells

To obtain purified CD8+ T cell cultures, Dynal CellLection Mouse CD8 Dynabeads (Dynal, Oslo, Norway) were used according to the manufacturers instructions, resulting in generally >90% CD3+CD8+ T cells.

Pre-treatment of cells

In advance, the splenocytes are prepared as described. As it is of great importance for purification of cells with Dynabeads to indeed start with a single-cell suspension, it is recommended to perform a DNase digestion step on the splenocyte suspension. Otherwise, DNA derived from sheared cells will clump the cells. Therefore, to splenocytes at a cell density of $2x10^7$cells/ml in a 50ml Falcon tube 120U/ml DNase is added, followed by 15min of gentle agitation at RT. Then, the tube is filled with media and cetrifuged (1200rpm, 10min). The cells are resuspendend in cold media at $2x10^7$cells/ml.

Pre-treatment of Dynabeads

For each $2x10^7$cells to be purified, 25µl of Dynabead-suspension are needed. The desired amount is to be transferred into a 15ml Falcon tube and placed in the magnet. The supernatant containing NaN_3 is discarded, and the beads are washed with 500µl buffer (RPMI 1%FCS) per 25µl of beads. The buffer is discarded, and the Dynabeads are incubated with the splenocytes on a roller for 20min at 4°C.

Selection of cells

After the splenocytes have been incubated for 20min, the tube is placed in the magnet for at least 1min, and the residual cells are discarded. Per ml cells used, the beads are resuspended in 500µl of buffer, and the beads are transferred to a new tube. The original tube is rinsed with another 500µl buffer per ml cells used, and both aliquots of buffer are combined. The beads are placed in the magnet again, and washed twice.

Releasing the cells

Following the final washing step, the beads are resuspendend in 200µl/ml cells originally used, and 4µl of releasing buffer is added. The beads are incubated for 15min at RT with gentle shaking, and afterwards flushed rapidly through a pipet tip. The beads are placed in the magnet once again for 1min, and the released cells can be pipetted off. Repeat this step 1-2 times.

As the CD8 antibodies are coupled to the Dynabeads via a DNA linker, the cells are released by DNase digestion. Alas, the antibody still clings to the cells this way after the purification, and thus the cells can not be stained with CD8 antibodies for some time afterwards.

3.5.3 T cell culture

Viability of the cells measured with trypan blue was > 95%. Following preparation, the cells were cultivated in RPMI plus 10% heat-inactivated FCS supplemented with 2mM L-glutamine, 100U/ml penicilline and 100µg/ml streptomycin and 5µM β-mercaptoethanol (cRPMI). Additionally, non-essential aminoacids, 2mM L-glutamine, 1mM sodium pyruvate, 10mM hepes and 4g/l glucose were added. Total splenocytes were cultured at $1x10^6$/ml, CTL at $4x10^5$/ml. For stimulation, IL-2 (500ng/ml), ConA (2µg/ml) + IL-2 (250 ng/ml) or anti-CD3 (1µg/ml) + IL-2 (250ng/ml) were used. Concentration of TGF-β1 was 240pM. Much lower doses however are effective, as shown in Figure 16.

Stimulation with IL-2 alone induces the growth of T cells, but without triggering the TCR. αCD3 stimulation triggers specifically and polyclonally the TCR of all T cells. Addition of ConA as a lectin crosslinks unspecifically the sugar chains of extracellular proteins such as the TCR, but also numerous other (unknown) pathways are activated. The stimulation with ConA or αCD3 without addition of IL-2 is poor. Strongest proliferation is observed upon stimulation with αCD3 (2µg/ml) + IL-2 (250ng/ml).

PD98059 dissolved in DMSO was used at 50µM final concentration and was added to the cells on day 0 1h before addition of other stimuli. Controls with equal DMSO concentrations were unaffected.

3.5.4 Proliferation assay

For determining the proliferation of cells, the CellTiter 96® AQ_{eous} One Solution Cell Proliferation Assay (Promega) is used.

Background information

This assay is a colorimetric method for determining the number of viable cells in proliferation assays. It contains a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS[(a)]) and an electron coupling reagent (phenazine ethosulfate; PES). PES has an enhanced chemical stability, which allowes it to be combined with MTS to form a stable solution. MTS is bioreduced by cells into a coloured formazan product that is soluble in tissue culture media, presumably by NADPH in metabolically active cells. The quantitiy of the formazan product is directly proportional to the number of living cells in culture

Performance of the assay

Cells are plated at recommended densities into 96well plates in triplicates or quadruplicates, 100µl per well, including cytokines, growth factors or chemicals. After 72h, 20µl of the assay reagent is added, incubated at 37°C for 2-3h and the OD is recorded using a standard ELISA reader at 490nm.

3.5.5 Cytotoxicity assay

General information

To measure cytolytic responses of cytotoxic T cells, the CytoTox 96 Non-Radioactive cytotoxicity Assay (Promega) was used.This assays quantitatively measures the release of lactate dehydrogenase from lysed cells.

Primary murine splenocytes are stimulated and cultured as described in “T cell culture”, but for the cytotoxicity assay a 5d incubation time has to be followed. The activated blasts are very sensitive to shear forces, thus it is recommended to cut the pipet tips, thus decreasing shear forces during pipetting the cells. In every cytotoxicity test, specific and unspecific target cells have to be defined, specific target cells to be of a different H2-background, unspecific target cells being of the same H2-background. For the C57BL/6 mice, themselves bearing the H-2b, P815 as specific target cells bearing H-2d are used, and EL-4 bearing H-2b molecules are classified as unspecific target cells.

In order to enable the effector cells to easily access the target cells, 96well U-bottom plates are used. To lower background absorption values, the overall FCS concentration in the assay is reduced to 5%.

Assay-setup:

Controls:

In order to enable the calculation of the specific lysis, the following controls are indispensable: Effector-cell spontaneous release including splenocytes only, and target cell spontaneous release, plating only target-cells. For determination of the target-cell maximum release, target cells alone are plated, and lysed with lysis buffer. For this, an additional volume correction control has to be included, as the lysis buffer changes the absorption of the target-cell maximal release wells. Thus, media alone is treated with lysis buffer, and this is used as media correction control for the maximal values. For all other wells, a normal media control with media only is set up.

Experimental wells:

To plate the target cells, the P815 cells and EL-4 cells are brought to a concentration of $4x10^5$, as the effective concentration is $2x10^5$, and they will be diluted with the effector cells. In each assay well 100µl of either P815 or EL-4 is pipetted. The target cells are set up this way, that in the final wells the ratio of effector-cells to target-cells decreases from 80:1 down to 2.5:1. The assay plates are spun down at 1000rpm with a plate centrifuge, and returned to the incubator for 4-5 hours.

30min before the end of the incubation time, to the maximal release wells lysis buffer is added. After the incubation time, the plates are spun down, and 50µl aliquots of each well are transferred to a flat-bottom plate. The substrate mix is dissolved in assay buffer, and 50µl of the substrate is pipetted to the aliquots. After an incubation time of 30min at RT, Stop-Solution is added, and the plates can be recorded in the ELISA-Reader (490nm). Unspecific lysis is subtracted from specific lysis, and the % specific lysis is calculated as follows:

$$\%\ \text{cytotoxicity} = \frac{\text{experimental - effector spontaneous - target spontaneous}}{\text{target maximum – target spontaneous}}$$

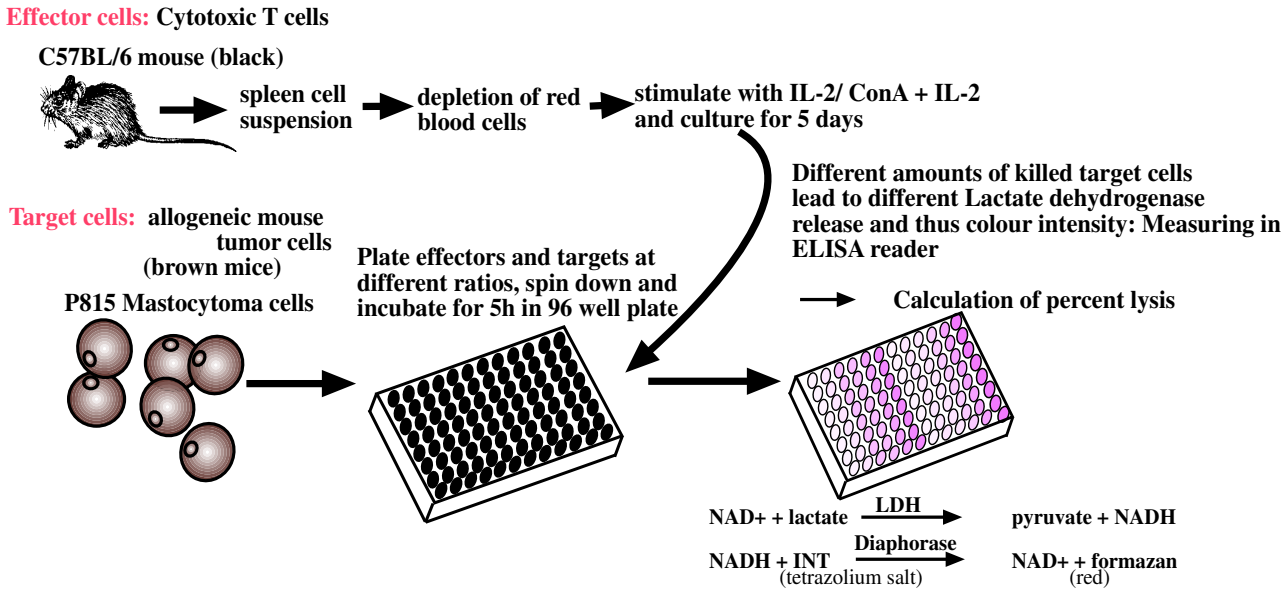


Figure 46: Scheme of the setup of a cytotoxicity assay.

3.5.6 Flow Cytometry

On the function of fluorescence activated cell sorters

The cells in the sample are stained with specific fluorescent dyes to detect surface molecules and are then introduced into the vibrating flow chamber of the FACS, as illustrated in Figure 47. The cell stream passing out of the chamber is encased in a sheath of buffer fluid. The stream is illuminated by laser light and each cell is measured for size (forward scatter) and granularity (90° light scatter=side scatter), as well as for red and green fluorescence, to detect different surface markers. In a cell sorter, the vibration in the cell stream causes it to break into droplets which are charged and may then be steered by deflection plates under computer control to collect different cell populations.

To determine the surface expression of CD3, CD4 and CD8, triplicates of stimulated cells were plated in 24well plates for 3 days.

Antibody staining

In FACS-tubes, $5x10^5$ cells are washed with ice-cold PIN solution. The cells are centrifuged at 1200rpm, 5min. The PIN solution is decanted, and the cells are resuspended. To the remaining 100µl the antibodies are added:

αCD3-FITC	1:100	Pharmingen (San Diego, CA)
αCD4-Cy	1:50	Pharmingen
αCD8-PE	1:100	Pharmingen
αCD3-Cy	1:50	Pharmingen

Table 17: Antibodies for fluorescence analysis

The cells are incubated with fluorescent antibodies on ice in the dark for 30min, then washed in ice-cold PBS, centrifuged again and resuspendend in 300µl of PIN

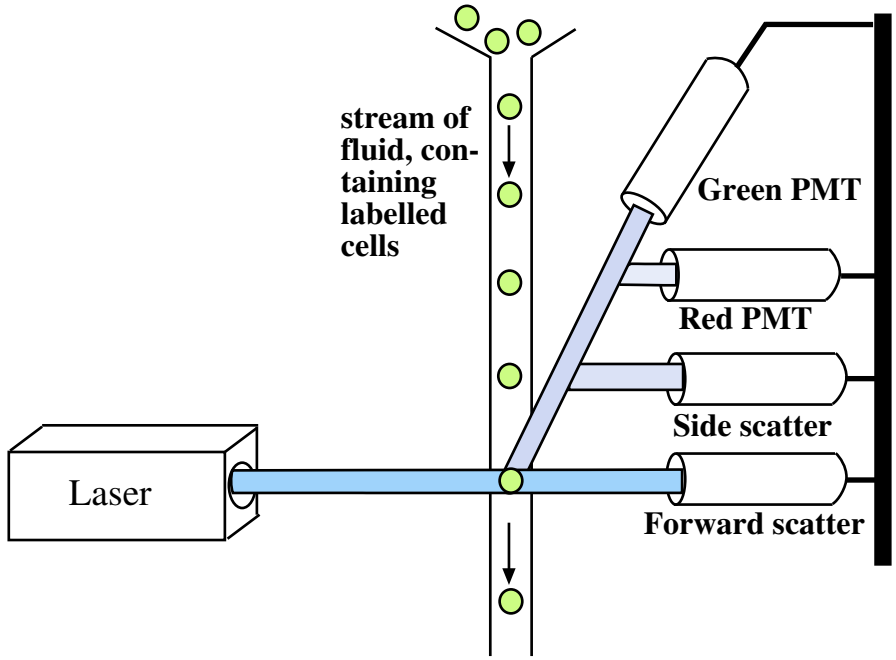


Figure 47: Flow cytometry. With this method, individual cells can be analysed. The cells are labelled with fluorescent antibody directed against a specific cell surface antigen. The labelled cells are forced through a nozzle in a single cell stream that passes a laser beam. Photomultiplier tubes (PMT) detect the scattered light (cell size and granularity), and the emissions from the different fluorescent dyes.

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VI Summary

Transforming-Growth-Factor-beta1 (TGF-β1) is a multifunctional cytokine that regulates cell growth and differentiation in many types of cells. TGF-β1 is especially known to exert a variety of regulatory functions in the immune system, such as T cell differentiation and T cell function. Signal transduction of TGF-β1 is mediated by phosphorylation of receptor-associated Smad proteins (R-Smads). R-Smads are phosphorylated by the activated type I receptor, which is itself phosphorylated by the high affinity type II receptor upon ligand binding. The phosphorylated R-Smads then associate with Co-Smads. Heterooligomers of R- and Co-Smads translocate into the nucleus where they regulate transcription of target genes in concert with other transcription factors such as CBP/p300 or AP-1. Recent findings suggest that the pleiotropic effects of TGF-β1 are conferred by crosstalks to other signal transduction pathways such as the MAP-kinases or the STAT-pathway.

Here we describe the effect of long-term exposure to TGF-β1 on the effector function of differentially stimulated primary murine splenocytes and purified primary murine CD8+ cytotoxic T cells. Long-term exposure to TGF-β1 results in non-responsiveness to TGF-β1-induced Smad2 phosphorylation. This is seen either by no phosphorylation or sustained phosphorylation of Smad2. Furthermore, we observed a strong correlation between sustained Smad2 phosphorylation and resistance to TGF-β1 mediated growth inhibition. In contrast, splenocyte cultures strongly growth inhibited by TGF-β1 showed no Smad2 phosphorylation. Lytic activity of these cultures, however, was found to be suppressed regardless of proliferation properties and Smad2 phosphorylation pattern. We also describe that a functional MEK-1 pathway is a prerequisite for rendering murine splenocytes unresponsive to TGF-β1 mediated growth inhibition, and that inhibition of the MEK-1 cascade alters the Smad2 phosphorylation pattern. In addition, we show that resistance to TGF-β1 mediated growth inhibition correlates with the activation of the JNK pathway. However, the resistant phenotype was found unable to be reverted upon administration of exogeneous IFNγ and/or αCD28 antibody. In human or mouse T cell lines, however, the described correlation between the type of stimulation and TGF-β growth resistance or growth sensitivity is not present. Thus, this correlation is specific for primary T cells. We also cloned a chimeric dominant-negative TGF-β receptor which is coupled to a suicide gene, in order to render T cells resistant to TGF-β mediated effects.

These findings shed light on how TGF-β1 mediates its immunosuppressive role, and may help to gain knowledge of averting these TGF-β1 effects in the course of tumor therapy.

VII Zusammenfassung

Transforming-Growth-Factor-beta1 (TGF-β1) ist ein multifunktionelles Zytokin, welches insbesondere Zellwachstum und Zelldifferenzierung koordiniert. TGF-β ist vor allem dafür bekannt, Zellen des Immunsystems zu beeinflussen. TGF-β steuert zum Beispiel die Differenzierung von T-Zellen und und deren Effektorfunktionen. Die Signaltransduktion von TGF-β wird vermittelt durch die Phosphorylierung von Rezeptor-assoziierten Smad-Proteinen (R-Smads). R-Smads werden vom Typ I Rezeptor aktiviert, der seinerseits vom hochaffinen Typ II Rezeptor phosphoryliert wird, sobald der Ligand bindet. Die phosphorylierten R-Smads assoziieren darauf mit Co-Smads. Heterooligomere von R-Smads und Co-Smads wandern dann in den Zellkern, wo sie im Zusammenspiel mit Transkriptionsfaktoren wie CBP/p300 oder AP-1 die Transkription TGF-β-spezifischer Zielgene koordinieren. Neue Erkenntnisse lassen vermuten, daß die pleiotropen Effekte von TGF-β durch das Interagieren mit anderen Signalkaskaden entstehen, zum Beispiel mit dem MAP-Kinase-Weg oder der STAT-Kaskade.

Wir beschreiben hier den Effekt von TGF-β auf die Effektorfunktionen unterschiedlich stimulierter primärer Maus-Milzzellen und aufgereinigten zytotoxischen CD8+ Maus-T-Zellen. Langzeitbehandlung mit TGF-β resultierte in der Unfähigkeit der Zellen, Smad2 ligandeninduziert zu phosphorylieren. Entweder wurde überhaupt keine Phosphorylierung beobachtet, oder eine anhaltende Phosphorylierung von Smad2 unabhängig vom Vorhandensein des Liganden. Des weiteren stellten wir einen Zusammenhang zwischen anhaltender Smad2-Phosphorylierung und der Resistenz gegenüber TGF-β induzierter Wachstumshemmung fest. Im Gegensatz dazu zeigen Zellen, die sensitiv sind gegenüber TGF-β vermittelter Wachstumshemmung, keine Smad2-Phosphorylierung mehr. Bezüglich ihrer zytotoxische Aktivtät waren allerdings beide Phänotypen nicht mehr lytisch wirksam, unabhängig von der jeweiligen Smad2-Phosphorylierung. In dieser Arbeit zeigen wir auch die Notwendigkeit eines funktionalen MEK-1-Signalweges auf, der unabdingbar ist, damit T-Zellen keine Wachstumsinhibierung durch TGF-β mehr erfahren. Das Blockieren dieses Signalweges führt darüberhinaus bei diesen Zellen ebenfalls zu einem veränderten Smad2-Phosphorylierungsmuster. Bezüglich des JNK-Signalweges konnten wir feststellen, daß ein funktional aktiver JNK-Signalweg mit der Resistenz gegenüber TGF-β vermittelter Wachstumsinhibierung einhergeht. Allerdings führt die Zugabe von IFNγ und/oder αCD28-Antikörper nicht zu einer veränderten Sensitivität gegenüber TGF-β. Im Gegensatz zu

primären Zellen können die beschriebenen Zusammenhänge in Zellkulturen vom humanen und murinen T Zellen nicht beobachtet werden, und sind somit spezifisch für primare T-Zellen. Wir beschreiben auch die Klonierung eines chimären dominant-negativen Typ II Rezeptors, der an eine Kinase gekoppelt ist, die bei Aktivierung Zelltod auslöst. Damit soll es in Zukunft möglich sein, T-Zellen gegenüber TGF-β Resistenz zu verleihen.

Die hier geschilderten Ergebnisse vertiefen die Kenntnisse über molekulare Mechanismen der Wirkung von TGF-β auf T-Zellen und können vielleicht dazu beitragen, negative Effekte von TGF-β, zum Beispiel in der Tumortherapie, gezielt abzuwenden.

VIII Schriftenverzeichnis

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IX Lebenslauf

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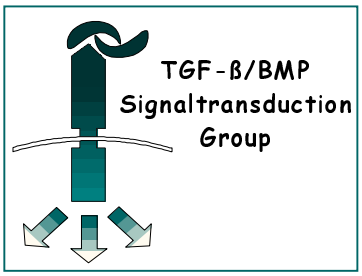
Oberkochen, 12.07.02

Kristina Feldmann

X Danksagungen

Hiermit möchte ich mich bei allen Personen bedanken, die an der Entstehung dieser Arbeit beteiligt waren. Mein erster Dank gilt Herrn Professor Sebald für die Möglichkeit, an seinem Lehrstuhl zu arbeiten, und insbesondere für sein stetes Interesse an meiner Arbeit.



Frau Dr. Petra Knaus danke ich sehr herzlich für die Vergabe des interessanten Themas, die durchgängig gute Betreuung und die familiäre Atmosphäre im Labor unserer „Signaltransduction Group“.

Mein Dank gilt auch meiner Kollegin Marion Lutz, deren Konstrukte ich zum Teil verwendete, die mich ich die Arbeit mit Retroviren einwies und bereitwillig meine Veröffentlichung und meine Dissertation Korrektur las. Den technischen Assistenten im Labor danke ich für ihre hilfreiche Unterstützung meiner Arbeit , vor allem Yvonne Kehl und Simone Schmitt. Wolfgang Hädelt danke ich für die Sequenzierung meiner Konstrukte. Ein „Dankeschön“ geht auch an meine F -Praktikanten, hier vor allem Jan Schülke, der fleißig an vielen Western Blots arbeitete.

Bedanken möchte ich mich auch bei meinen anderen Kollegen im Labor für hilfreiche Diskussionen und praktische Unterstützung: Marai Sammar, Sylke Haßel, Martin Roth und Diana Rotzer.

Weiterhin bedanken möchte ich mich bei allen anderen Mitgliedern der benachbarten Arbeitsgruppen für Tipps und aufmunternde Worte, im speziellen bei Joachim Nickel, Markus Klein, Werner Schmitz, Thomas Hom und der Arbeitsgruppe Hoppe. Mein besonderer Dank gilt auch den Sekretärinnen, Frau Meyer, Frau Borst und Frau Lakeit, die immer mit Rat und Tat zur Seite standen.

Ganz besonders bedanken möchte ich mich auch bei meinem Ehemann Heiko und bei meinen Eltern, die mir dieses Studium ermöglichten.

Mein Dank gilt auch der Studienstiftung des deutschen Volkes, die mich für 3 Jahre finanziell und ideell optimal förderte.

XI Ehrenwörtliche Erklärung

Hiermit erkläre ich ehrenwörtlich, daß die vorliegende Arbeit von mir selbständig und nur unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde. Weiterhin habe ich noch keinen Promotionsversuch unternommen oder diese Dissertation in gleicher oder ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt.

Würzburg, den

Kristina Feldmann