The EMBO Journal Vol.18 No.5 pp.1280–1291, 1999

Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-β

Xiao Yang[1,2], John J.Letterio[3], Robert J.Lechleider[3], Lin Chen[1], Russ Hayman[4], Hua Gu[4], Anita B.Roberts[3] and Chuxia Deng[1,5]

[1]Genetics of Development and Disease Branch, 10/9N105, NIDDK, [3]Laboratory of Cell Regulation and Carcinogenesis, NCI, [4]Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, MD 20892, USA and [2]Institute of Biotechnology, 20 Dongdajie Street, Beijing 100071, China

[5]Corresponding author
e-mail: chuxiad@bdg10.niddk.nih.gov

X.Yang and J.J.Letterio contributed equally to this work

SMAD3 is one of the intracellular mediators that transduces signals from transforming growth factor-β (TGF-β) and activin receptors. We show that SMAD3 mutant mice generated by gene targeting die between 1 and 8 months due to a primary defect in immune function. Symptomatic mice exhibit thymic involution, enlarged lymph nodes, and formation of bacterial abscesses adjacent to mucosal surfaces. Mutant T cells exhibit an activated phenotype *in vivo*, and are not inhibited by TGF-β1 *in vitro*. Mutant neutrophils are also impaired in their chemotactic response toward TGF-β. Chronic intestinal inflammation is infrequently associated with colonic adenocarcinoma in mice older than 6 months of age. These data suggest that SMAD3 has an important role in TGF-β-mediated regulation of T cell activation and mucosal immunity, and that the loss of these functions is responsible for chronic infection and the lethality of *Smad3*-null mice.

Keywords: bacterial infections/gene targeting/ inflammation/SMAD3/TGF-β signaling

Introduction

The transforming growth factor-β (TGF-β) superfamily constitutes a large family of secreted signaling molecules which are known to have important roles in regulating a wide variety of cellular processes including proliferation, differentiation, adhesion and migration (Roberts and Sporn, 1990; Kingsley, 1994). All known receptors of this superfamily signal through a heteromeric complex of type I and type II transmembrane receptor serine/threonine kinases which act in series (Derynck and Feng, 1997; Massague, 1998). Despite extensive knowledge about the receptor activation mechanisms, which involve recruitment and activation of the type I receptor kinase by the ligand-activated type II receptor kinase, the downstream signaling pathways from the activated type I receptor are not yet clearly defined. Recently, a set of novel mammalian proteins, termed SMADs, has been identified based on their high homology to the *Drosophila* Mad and the *Caenorhabditis elegans* Sma proteins, which were previously identified in genetic screens and shown to act downstream of the TGF-β family ligands dpp and daf7, respectively (Newfeld *et al*., 1996; Savage *et al*., 1996; Wiersdorff *et al*., 1996). SMAD proteins have now been demonstrated to mediate a short-circuit signaling pathway from the receptor serine/threonine kinases to the nucleus (Heldin *et al*., 1997).

To date, nine different *Smad* genes have been described, which fall into three distinct functional sets: signal-transducing, receptor-activated SMADs, which includes SMADs 1, 2, 3, 5, 8 and 9; a single common mediator SMAD, SMAD4/DPC4, and inhibitory SMADs, SMADs 6 and 7 (Heldin *et al*., 1997; Massague, 1998). The present model for downstream signaling which is emerging from these studies is that (i) receptor-activated SMADs bind to the type I receptor kinase and are phosphorylated on two C-terminal serine residues in their MH2 domain (Abdollah *et al*., 1997; Souchelnytskyi *et al*., 1997); (ii) the phosphorylated, pathway-specific SMADs (SMAD-P) then form a heteromeric complex in the cytoplasm with the common mediator, SMAD4 (Lagna *et al*., 1996); and (iii) the SMAD-P/SMAD4 complex is then translocated to the nucleus where it participates in a transcriptional complex and mediates activation of the target genes (Chen *et al*., 1997; Liu,F. *et al*., 1997). Inhibitory SMADs, induced by TGF-β family ligands, function in a negative feedback loop to terminate or reduce the strength of the signal (Hayashi *et al*., 1997; Imai *et al*., 1997; Nakao *et al*., 1997b).

Analysis of *Smad* genes by targeted mutagenesis is beginning to provide insights into SMAD functions during vertebrate development and tumorigenesis. SMAD4 null mice die around embryonic day 6.5 (E6.5) without any sign of gastrulation (Sirard *et al*., 1998; Yang *et al*., 1998). They show little or no elongation in the extraembryonic portion of late egg cylinder stage embryos, and have decreased proliferation *in vivo* and *in vitro* (Sirard *et al*., 1998; Yang *et al*., 1998). When rescued by aggregation, *Smad4* mutants show severe anterior truncation, indicating that SMAD4 is also involved in anterior patterning during gastrulation (Sirard *et al*., 1998). The severe phenotype of these mice is not unexpected, given the predicted central role of SMAD4 in partnering with each of the receptor-activated SMAD proteins, and inferentially in mediating all SMAD signaling from the entire TGF-β superfamily of ligands.

In contrast to SMAD4, targeted deletions of the receptor-activated SMADs might be expected to affect a more restricted set of target genes. For example, of the set of six receptor-activated SMAD proteins, only SMAD2 and 3 have been shown to bind to TGF-β and activin receptors and to induce dorsal mesoderm in *Xenopus* embryos

(Eppert *et al*., 1996; Macias-Silva *et al*., 1996; Zhang *et al*., 1996; Liu,X. *et al*., 1997; Nakao *et al*., 1997a). Recent studies suggest that SMAD2 is also required for early embryogenesis, as demonstrated by the failure in the establishment of the anterior–posterior axis (Waldrip *et al*., 1998) and the induction of mesoderm during gastrulation (Nomura and Li, 1998; Weinstein *et al*., 1998). To address whether SMAD3 might also be required for early embryogenesis, and to understand the specificity of the downstream targets of these two highly homologous SMAD proteins, we disrupted exon 8 of the *Smad3* gene in mice. This gene, also known as JV15-2 (Riggins *et al*., 1996), encodes a 424 amino acid protein, which is ~95% homologous to SMAD2 based on the amino acid sequence (Zhang *et al*., 1996). In contrast to the early effects of SMAD2 deletion, disruption of the *Smad3* gene by homologous recombination in embryonic stem (ES) cells generated viable mutant mice which develop a progressive illness, with onset around the time of weaning. Symptomatic mutant mice exhibit leukocytosis, with massive inflammation and pyogenous abscess formation adjacent to mucosal surfaces. Abscesses contain typically non-pathogenic microorganisms known to cause infection only in the setting of immune deficiency. Although proliferation of mitogen-stimulated $B220^+$ B cells remains sensitive to the growth inhibitory effects of TGF-β, T cell receptor-induced activation of mutant thymocytes and peripheral T cells is completely resistant to inhibition by TGF-β. Furthermore, the normal chemotactic response of mature neutrophils to TGF-β is significantly impaired in mutant mice. These data reveal an important role of SMAD3 in mediating TGF-β signals in T lymphocytes and in neutrophils, and demonstrate that SMAD3 deficiency results in immune dysregulation and susceptibility to opportunistic infection, ultimately leading to the lethality of SMAD3-null mice. The phenotype presented here is distinct from that recently reported for a mouse mutant created by targeted disruption of exon 2 of the *Smad3* gene (Zhu *et al*., 1998). The $Smad3^{ex2/ex2}$ mice developed invasive colonic adenocarcinomas, but were not evaluated for status of immune function. Potential reasons for these distinct phenotypes will be discussed.

Results

Targeted disruption of the Smad3 gene

The targeting construct *pSmad3neo* (Figure 1A) was used to delete exon 8 of the *Smad3* gene by homologous recombination. A stop codon was introduced immediately following exon 7, which truncated 89 amino acids from the C-terminal end. This deleted portion of the SMAD3 C-terminal end contains an SSVS consensus phosphorylation site and an L3 loop, which is essential for interacting with TGF-β receptors (Lo *et al*., 1998). The targeted truncation should, therefore, create a candidate null allele of the Smad3 locus. Of 130 G418 and FIAU double resistant ES clones analyzed by Southern analysis for homologous recombination, nine contained a correct targeting event (Figure 1B). Two targeted ES clones were injected into C57BL/6J blastocysts to obtain germline transmission. Southern blot analysis of offspring from mice with a high degree of chimerism indicated that ~50% of them were heterozygous for the targeted mutation ($Smad3^{ex8/+}$).

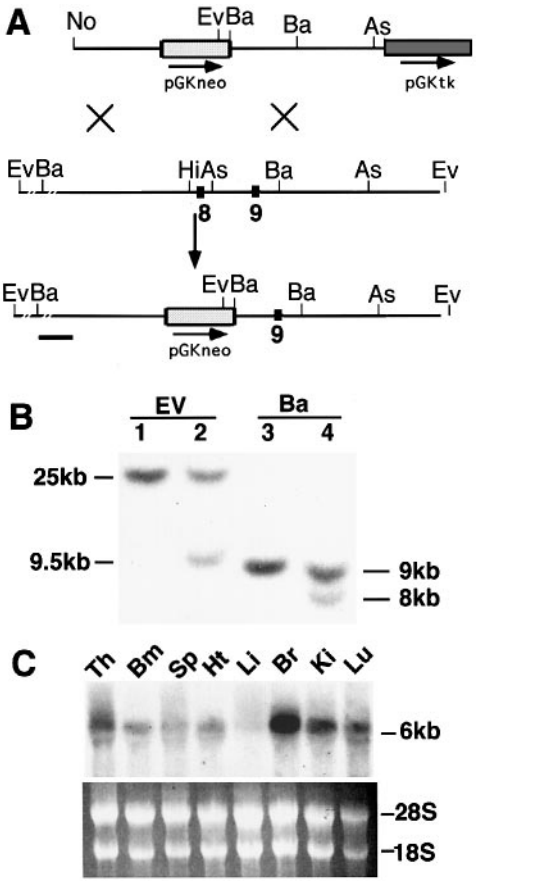

Fig. 1. Targeted disruption of the *Smad3* gene. (**A**) This targeting construct contains an 8 kb genomic sequence with a pLoxpneo cassette (Yang *et al*., 1998) inserted through *Hin*dIII and *Asp*718 sites which eliminates a 1 kb genomic fragment containing exon 8 of the *Smad3* gene. Transcriptional directions of *neo* and *tk* are indicated by arrows. Prior to electroporation, the targeting vector was linearized at the unique *Not*I site indicated. Homologous recombination within the *Smad3* locus would introduce the *neo* gene and delete the endogenous exon 8. (**B**) Southern blot analysis of DNA isolated from untargeted ES cells (lanes 1 and 3) and a targeted ES cell clone (lanes 2 and 4). As expected, the *Eco*RV (lanes 1 and 2) restriction fragment size change from >25 kb to 9.5 kb is seen in the targeted cells using the ^{32}P-labeled, 1 kb *Sal*I–*Eco*RI 5′ flanking probe. Correct targeting events were confirmed by *Bam*HI digestion and detected using the same probe (lanes 3 and 4). (**C**) Northern blot analysis of *Smad3* expression in adult tissues. Tissue types, size of transcripts and loading control were as indicated. Thirty micrograms of total RNA from each sample was used for the analysis. The filter was probed with a 0.7 kb *Asp*718–*Hpa*I fragment which is in the 3′ untranslated region of the *Smad3* gene. Th, thymus; Bm, bone marrow; Sp, spleen; Ht, heart; Li, liver; Br, brain; Ki, kidney; and Lu, lung.

The *Smad3* gene is expressed during embryonic development (not shown) and in the multiple adult organs and tissues examined (Figure 1C). To study the possible role of SMAD3 in murine development, mice heterozygous for the targeted disruption were intercrossed to produce homozygous offspring. These crosses gave rise to litters of normal size, with wild-type, heterozygous ($Smad3^{ex8/+}$) and homozygous ($Smad3^{ex8/ex8}$) offspring present at a frequency consistent with Mendelian inheritance (not shown).

To determine whether the targeted disruption of exon 8 of the *Smad3* gene generated a null mutation, we examined

expression and function of the mutant allele. Semi-quantitative RT–PCR analysis using primers located in exons 6 and 9 revealed a fragment of 284 bp in *Smad3*$^{ex8/ex8}$, and 431 bp in wild-type mice (Figure 2A). Sequencing of the mutant fragment indicated that the 284 bp mutant fragment was generated by direct splicing from exon 7 to exon 9. A stop codon four amino acids downstream of the fusion junction was created (Figure 2B). Translation of the aberrant transcripts would create a smaller protein of 339 amino acids. To determine whether this putative product was functional, we cloned the mutant cDNA into a Gal4 expression vector and performed *in vitro* analysis using a heterologous reporter assay. Gal4–SMAD fusion proteins have been shown to activate transcription from a promoter containing the GAL4 UAS in a ligand-dependent manner (Liu *et al*., 1996). We created Gal4–Smad3ΔC and analyzed its activity in transient transcriptional assays in NMuMg cells. Gal4–Smad3 activated transcription from a reporter containing five copies of the Gal4 UAS in a ligand- and dose-dependent manner, while Gal4–Smad3ΔC had no effect in these assays (Figure 2C). Additionally, when co-transfected with small amounts of Smad4 expression construct, Gal4–Smad3 was able to activate transcription in a ligand-independent manner, while Gal4–Smad3ΔC was inactive (Figure 2D). Other studies using the TGF-β responsive reporter 3TP-Lux failed to show any activation by the mutant construct (data not shown). However, we found that a 10-fold higher level of expression of Smad3ΔC suppressed TGF-β-induced 3TP-Lux reporter activity ~50%, while similar amounts of wild-type Smad3 constitutively activated basal (25-fold) and super-activated TGF-β-induced (4-fold above endogenous activation) reporter gene activity (data not shown). Taken together, these data show that the *Smad3* allele created has no ability to activate endogenous signaling pathways, but at high levels of expression may suppress signals from the TGF-β receptors. However, the result of semiquantitative PCR indicated that the Smad3ΔC is not expressed more highly than wild-type allele (Figure 2A); therefore, it is unlikely that the Smad3ΔC could created a dominant-negative effect in our mutant mice.

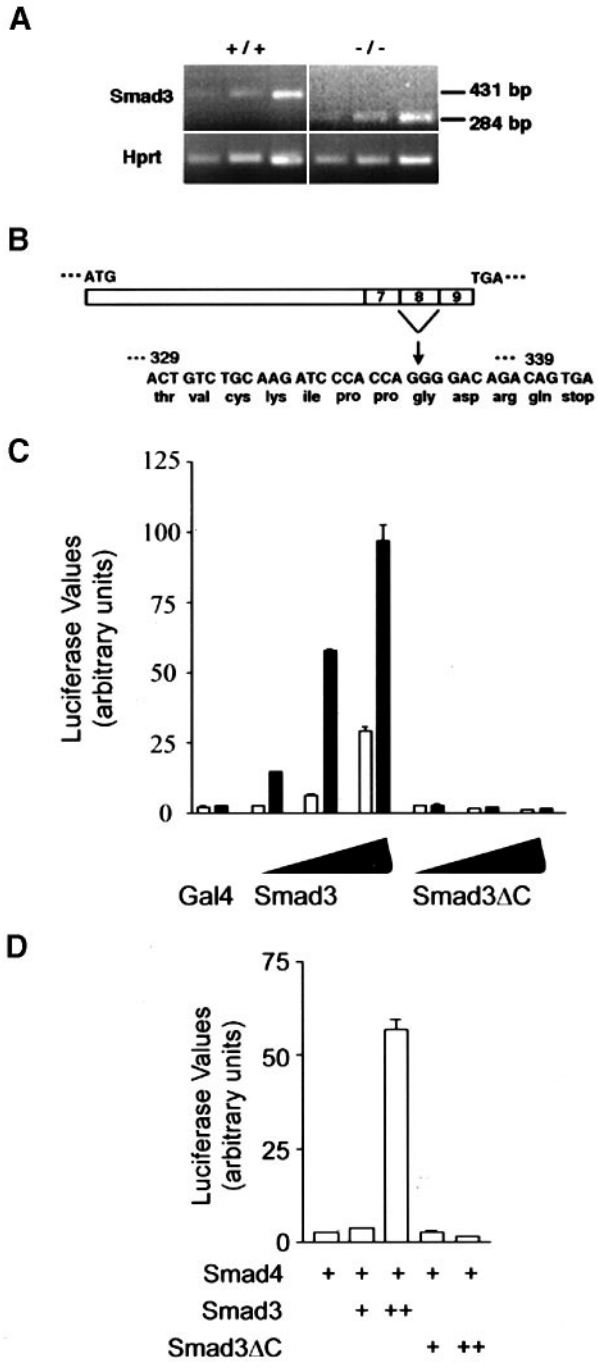

Fig. 2. Molecular analysis of the *Smad3*$^{ex8/ex8}$ allele. (**A**) Semi-quantitative RT–PCR of RNA from the wild-type (*Smad3*$^{+/+}$) or mutant (*Smad3*$^{ex8/ex8}$) mice using primers in exon 6 (5′-CTGGGCCT-ACTGTCCAA TGT-3′) and exon 9 (5′-CATCTGGGTGAGGACC-TTGT-3′). Equal amounts of reaction mixture was taken out for agarose gel electrophoresis after cycles 24, 27 and 30. The 431 bp band is the wild-type transcript and the 284 bp band is the mutant. Note that no wild-type transcript appears in RNA from the *Smad3*$^{ex8/ex8}$ mice, and the levels of expression are roughly equivalent. (**B**) Aberrant splicing found in *Smad3*$^{ex8/ex8}$ mice. The sequence of the mutant transcript and putative amino acids are indicated. (**C**) Activation of the Gal4 heterologous reporter. Wild-type or mutant Smad3 were transiently transfected in increasing amounts and ligand-dependent transcriptional activity determined. Wild-type Smad3 demonstrates both ligand-dependent (closed bars) and -independent (open bars) activation at the highest dose (1.0 μg) but the mutant is completely inactive. (**D**) Ligand-independent activation of Gal4–Smad3. When transiently transfected with small amounts of Smad4 (0.1 μg) Gal4–Smad3 activates transcription at the higher amount transfected (++, 1.0 μg) in the absence of ligand, but not at the lower dose (+, 0.1 μg). Gal4–Smad3ΔC has no effect at either dose.

Gross phenotype of homozygous Smad3$^{ex8/ex8}$ *mutants*

Smad3$^{ex8/ex8}$ mice are phenotypically indistinguishable from their littermate controls (wild-type and heterozygous littermates) at birth. However, ~70% of *Smad3*$^{ex8/ex8}$ mice are much smaller prior to weaning (Figure 3A), suggesting that they suffer growth retardation during the lactation period. During the third week postnatal, these mice usually develop a wasting syndrome, and typically die between 1 and 3 months of age. This syndrome is associated with multifocal formation of pyogenic abscesses, which are most often periorbital, periodontal, and within the wall of the stomach and the intestine (Figure 3B, C and D). Culture of abscess fluid detected the presence of the typically non-pathogenic Gram-negative *Providencia rettgeri*. Symptomatic mice typically have a kyphotic

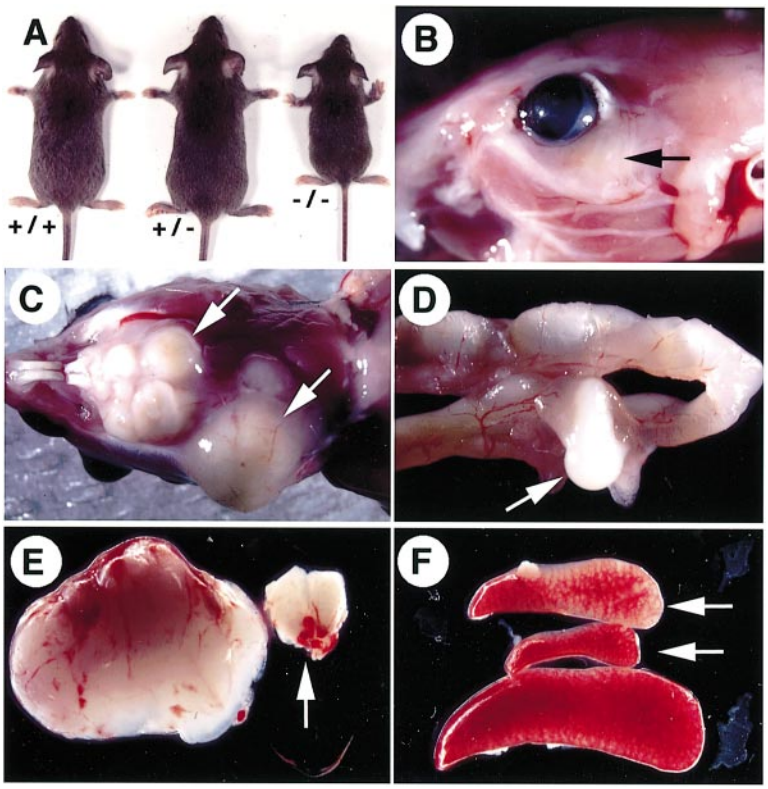

Fig. 3. Overall morphology of *Smad3*$^{ex8/ex8}$ mice. (**A**) About 70% of *Smad3*$^{ex8/ex8}$ mice exhibited reduced size compared with their littermate controls. Genotypes are as indicated. (**B**–**D**) Abscesses (arrows) developed near eyes (B), mandible (C), salivary gland (C) and intestine (D) of *Smad3*$^{ex8/ex8}$ mouse. (**E** and **F**) Thymus (E) and spleen (F) of control and mutant (arrows) mice.

posture, and enlarged lymph nodes, an involuted thymus and relatively small spleen (Figure 3G and H). The remaining 30% of the mutant mice died between 3 and 8 months, exhibiting a similar syndrome with a delayed onset. We have analyzed *Smad3*$^{ex8/ex8}$ mice from two strain backgrounds (129SVEV×C57BL/6 and 129SVEV×Black Swiss) and similar phenotypes were observed in both strains.

Pathological analysis of the Smad3$^{ex8/ex8}$ *mice*

Smad3$^{ex8/ex8}$ were examined further to find the cause of the lethality. Examination of >30 *Smad3*$^{ex8/ex8}$ mice between 1 and 8 months of age revealed inflammatory lesions in a number of organs, including the nasal mucosa, stomach, pancreas, colon and small intestine (Figure 4A–D). Immunostaining for CD3 revealed a significant increase in T cells infiltrating mutant intestines (Figure 4E and F). Brain, kidney, lung and heart are not commonly involved. Symptomatic mice exhibit a dramatic reduction in cellularity of the thymus (Figure 5A and B) and the spleen, where lymphoid depletion in B cell areas is often significant (not shown). In contrast, mediastinal, mandibular and mesenteric lymph nodes were enlarged in the majority of *Smad3*$^{ex8/ex8}$ mutants. These nodes displayed lymphoid hyperplasia, with extensive proliferation of T cells and an accumulation of plasma cells, effacing the normal node architecture (Figure 5E and F). T cells appear to invade normal regions of B cell development and germinal center formation (Figure 5G and H), reflected by a reduction in the number of B220 immunoreactive cells (Figure 5G and H). Extramedullary hematopoiesis within the liver and spleen is a common feature, particularly in mice which harbor abscesses (not shown), suggesting that this may represent a response to infection. None of these features were observed in age-matched control mice.

Hematology of Smad3$^{ex8/ex8}$ *mice*

Peripheral blood samples from six *Smad3*$^{ex8/ex8}$ and six control mice were analyzed for total numbers and differential distributions of leukocytes. There is a consistent elevation in absolute white blood cell counts in *Smad3*$^{ex8/ex8}$ mice relative to control littermates, with a significant increase within each subset (Table I). The increased number of circulating neutrophils and monocytes present in *Smad3*$^{ex8/ex8}$ mice is consistent with the inflammatory response observed, and with the existence of infection involving multiple organs. Red blood cell indices were also analyzed, and no significant differences were observed between the mutant and controls (not shown).

T and B cell development in Smad3$^{ex8/ex8}$ *mice*

Several wild-type, *Smad3*$^{ex8/+}$ and *Smad3*$^{ex8/ex8}$ littermates were sacrificed at 4–6 weeks of age for immunophenotyping of cells from thymus, spleen and lymph nodes. No discernible differences were observed in either the number of thymocytes expressing CD3, nor in the level of CD3 expression. Normal development of $CD4^+CD8^+$ thymocytes from $CD4^-CD8^-$ precursors is suggested by the presence of expected ratios of these progenitors with single positive thymocytes in asymptomatic mice. Analysis of spleen and lymph nodes failed to reveal any significant

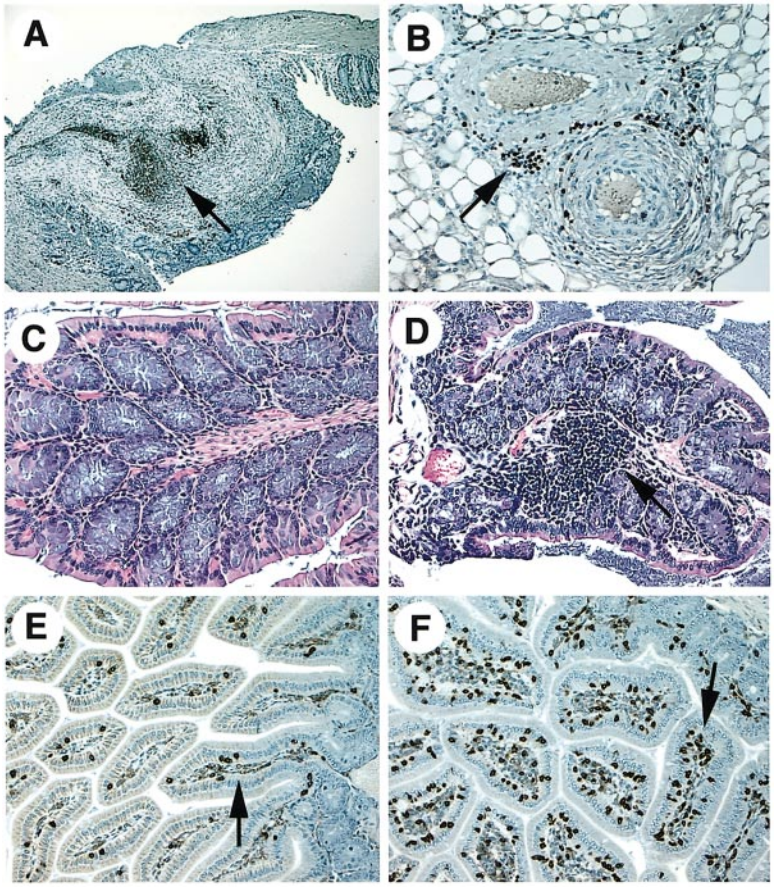

Fig. 4. Histopathological analysis of the *Smad3*$^{ex8/ex8}$ mice. (**A**) Formation of a chronic abscess within the submucosa of the colon, and (**B**) focal arteritis (inflammation adjacent to an arteriole) within fat tissue adjacent to submandibular gland of *Smad3*$^{ex8/ex8}$ mice. (**C** and **D**) Routine H&E staining of colonic villi from wild-type (C) and *Smad3*$^{ex8/ex8}$ mice (D) provides evidence of inflammation in the absence of SMAD3. (**E** and **F**) Immunohistochemical analysis of CD3^{+} T cells within the intestine of wild-type (E) and *Smad3*$^{ex8/ex8}$ mice (F) shows an accumulation of T cells within the intestinal infiltrates.

differences between the asymptomatic mutant mice and littermate controls, with normal numbers of CD3^{+}, CD4^{+} and CD8^{+} T lymphocytes, B220^{+} and IgM^{+} B lymphocytes, and NK1.1^{+} natural killer cells. However, lymph node T cells isolated from the enlarged submandibular and mesenteric lymph nodes of symptomatic *Smad3*$^{ex8/ex8}$ mice exhibit an activated phenotype, with an increased number of CD62L (CD62^{lo}) cells (Figure 6). This is also consistent with the observed increased proliferative indices (Figure 5E and F) and with an ongoing inflammatory response as revealed by tissue infiltration and abscess formation.

Proliferation of Smad3$^{ex8/ex8}$ *B cells is inhibited by TGF-β, but activated Smad3*$^{ex8/ex8}$ *T cells are resistant*

Although development of lymphocyte subsets appears to occur normally in *Smad3*$^{ex8/ex8}$ mice, susceptibility of these mice to infection and tissue inflammation suggests a defect in regulation of either the activation or function of mature leukocytes. We evaluated the TGF-β responsiveness of splenic B cells activated with lipopolysaccharide (LPS), and of thymocytes and splenic T cells activated with monoclonal antibodies to CD3 and the co-stimulatory receptor CD28. Surprisingly, the LPS-induced proliferative responses of wild-type and *Smad3*$^{ex8/ex8}$ B lymphocytes were equally inhibited by the addition of TGF-β1 (Figure 7A). However, TGF-β1 failed to inhibit the proliferation of *Smad3*$^{ex8/ex8}$ thymocytes activated by a combination of anti-CD3 and anti-CD28 antibodies, although similarly treated Smad3$^{+/+}$ thymocytes were strongly inhibited by TGF-β (Figure 7B). A purified population of T cells isolated from the spleen of *Smad3*$^{ex8/ex8}$ mice and activated by anti-CD3 alone were also completely insensitive to the inhibitory effects of TGF-β (Figure 7C).

Smad3$^{ex8/ex8}$ *neutrophils display impaired chemotaxis to TGF-β*

Periodontitis and abscess formation have been linked to defective neutrophil and monocyte motility in humans (Page *et al*., 1985). The infectious etiology of the abscesses in *Smad3*$^{ex8/ex8}$ mice was documented by bacterial culture (noted above) and by Brown and Hopps stain of sections through abscess tissue. The latter identified the presence of both Gram-positive cocci and Gram-negative rod-shaped organisms (Figure 8A). The presence of relatively few neutrophils within these abscesses, along with the severity of this phenotype in *Smad3*$^{ex8/ex8}$ mice, suggested that impaired neutrophil migration (chemotaxis) may be a contributing factor. We examined directed migration *in vitro* of wild-type and *Smad3*$^{ex8/ex8}$ neutrophils toward TGF-β using an under agarose chemotaxis assay, using neutrophils purified by density centrifugation (Figure 8B).

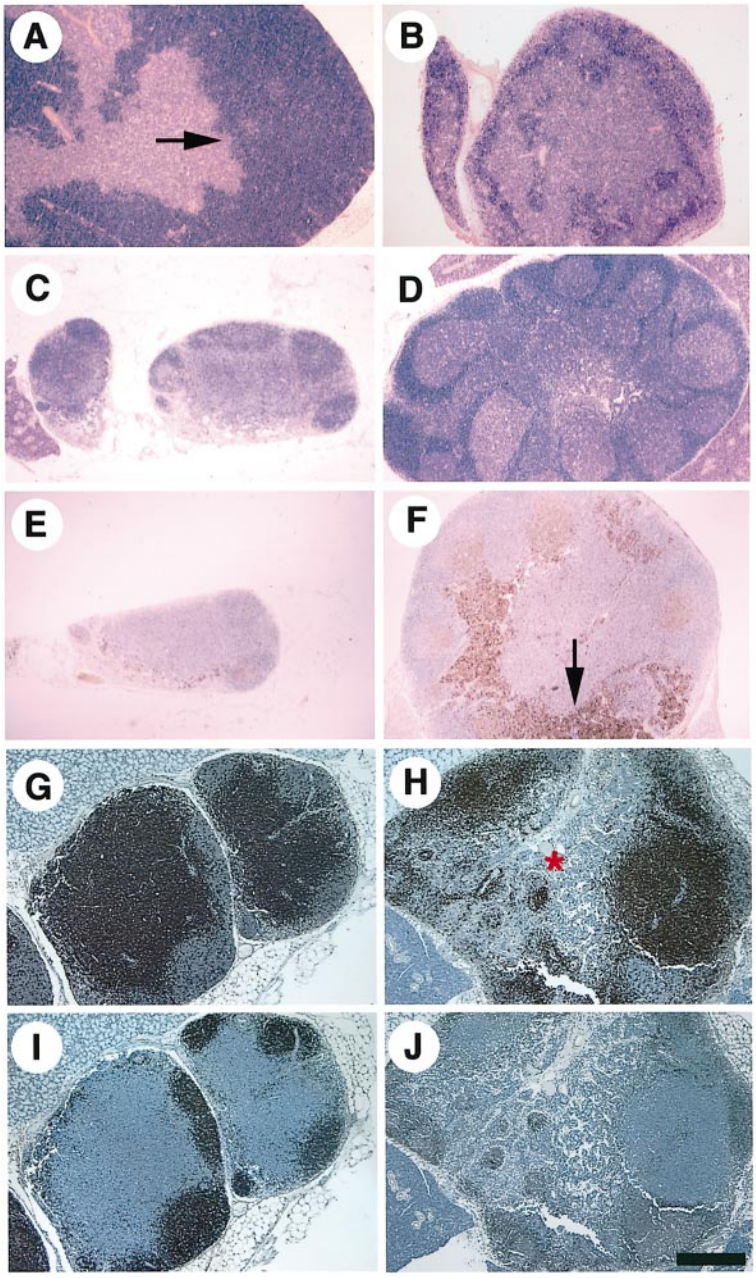

Fig. 5. Histological and immunohistochemical analyses of lymphoid organs in *Smad3*$^{ex8/ex8}$ mice. (**A** and **B**) The thymus of *Smad3*$^{ex8/ex8}$ mice (B) is often much smaller than that of normal littermates (A). Though the thymus shown in (B) exhibits mainly cortical atrophy, there is often preservation of cortical tissue despite the decrease in size. (**C** and **D**) Lymph node of mutant mice (D) is often enlarged compared with the wild-type controls (C). (**E** and **F**) Evidence of increased lymphocyte activation is provided by the increase in proliferating nuclear cell antigen (PNCA) immunoreactivity in mutant (F) relative to wild-type (E) lymph node. (**G** and **H**) The areas of proliferation are mainly composed of T lymphocytes, stained here with CD3 in wild-type (G) and mutant (H) submandibular lymph nodes. (**I** and **J**) The proliferation T lymphocytes invades normal B cell zones. Typical patterns of B220 immunoreactivity appear in the wild-type lymph node depicted in (I). In addition to decreased B220 immunoreactivity in lymph nodes from *Smad3*$^{ex8/ex8}$ mutants (J), a large portion of the medullary and cortical areas of the node have been replaced with plasma cells, and can be seen as a relative decrease of CD3 immunoreactivity in these areas of the node in (H). Bar = 640 m.

Although neutrophils isolated from wild-type mice exhibited a normal response to a concentration of 5 pg/ml of TGF-β, we observed no migration of neutrophils from *Smad3*$^{ex8/ex8}$ mice (Figure 8C and D). To determine whether such a defect could exist *in vivo*, we next examined the local response following subcutaneous injection of TGF-β in wild-type and mutant mice. An infiltrate of inflammatory cells could be detected within 12 h following a single injection of 200 ng of TGF-β in wild-type mice (Figure 8E), with substantially fewer cells observed at the site of injection in *Smad3*$^{ex8/ex8}$ mice (Figure 8F). The infiltrate was predominantly granulocytes in wild-type mice, while relatively few granulocytes were observed in sections from mutant mice (Figure 8G and H).

IgA production in plasma cells of Smad3$^{ex8/ex8}$ *mice*

The association of bacterial infections with selective IgA deficiency in humans, and the reported ability of TGF-β to induce immunoglobulin class switching and to enhance

IgA production (Coffman *et al*., 1989), suggested another potential defect which might contribute to the *Smad3*$^{ex8/ex8}$ phenotype. Moreover, a recent report has identified CAGA DNA motifs, shown to bind SMAD3 and SMAD4, in the promoter of the *IgA* gene (Dennier *et al*., 1998). We therefore examined the abundance of IgA$^+$ plasma cells within tissues and lymphoid organs of *Smad3*$^{ex8/ex8}$ mice and littermate controls as an indicator of secretory IgA. Relatively normal numbers of IgA$^+$ plasma cells were detected in the intestine, spleen and lymph nodes from asymptomatic mutant mice between 2 and 3 months of age. While an accumulation of these cells is occasionally evident in areas of intestinal inflammation, their numbers were often greatly reduced in the intestine of severely affected *Smad3*$^{ex8/ex8}$ mice (data not shown). Though it remains possible that TGF-β may induce class switching to IgA in a SMAD3-dependent pathway, it does not appear that this signal is required for production of IgA *in vivo*.

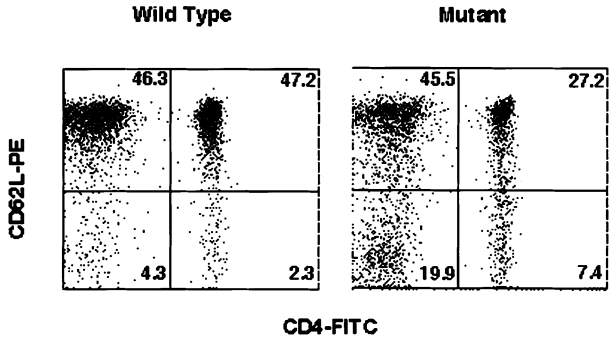

Fig. 6. T lymphocytes of *Smad3*$^{ex8/ex8}$ mice have an activated phenotype. Flow cytometric analysis of CD62L (L-selectin) expression on CD4$^+$ T cells is shown. Lymphocytes were isolated from submandibular lymph nodes of the wild-type (left panel) and *Smad3*$^{ex8/ex8}$ (right panel) mice, and co-labeled with anti-CD4-FITC and CD62L-PE conjugated antibodies. Decreased CD62L expression is characteristic of activated lymphocytes, and there is an ~4-fold increase in CD65L^{lo} CD4$^+$ T cells in lymph nodes of *Smad3*$^{ex8/ex8}$ mice.

Spontaneous tumorigenesis in Smad3$^{ex8/ex8}$ *mice*

We followed 30 chronically symptomatic *Smad3*$^{ex8/ex8}$ mice for >6 months prior to evaluating intestinal mucosa for the extent and progression of their inflammatory bowel disease. In one of the 30 mutant mice, we detected multiple invasive lesions within the colon and rectum (not shown). These adenocarcinomas were not observed in any of the mutant mice necropsied prior to 6 months of age, nor in older mice with minimal inflammation within the intestine. Histopathologic evaluation of the intestines of 13 *Smad3*$^{ex8/ex8}$ mice revealed chronic inflammation in ten, including the one mouse developing malignancy. It is possible that the evolution of this epithelial tumor in our *Smad3*$^{ex8/ex8}$ mice may result from the interaction of epigenetic events with disrupted SMAD3 expression, but further analysis will be required to clarify this issue. However, the rare appearance of this tumor in our mice appears to be consistent with a recent report which suggests that SMAD3 may not play an important role in the

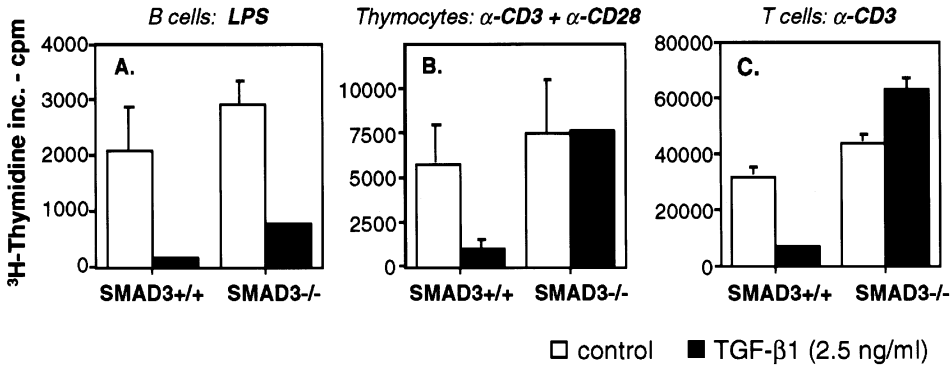

Fig. 7. Altered responsiveness of *Smad3*$^{ex8/ex8}$ lymphocytes to TGF-β. Inhibitory effects of TGF-β on B cells activated with LPS (**A**), thymocytes activated with immobilized anti-CD3 and anti-CD28 (**B**), T cells activated with anti-CD3 alone (**C**). Shown are the means and standard deviations of [^{3}H]thymidine incorporation (c.p.m.) from triplicate cultures. Genotypes are designated at the bottom of each plot. Although proliferation of *Smad3*$^{ex8/ex8}$ B lymphocytes can be maximally inhibited by TGF-β (A), *Smad3*$^{ex8/ex8}$ thymocyte activation by anti-CD3 plus anti-CD28 was unaltered in the presence of TGF-β (B); peripheral T cells isolated from *Smad3*$^{ex8/ex8}$ spleen were also completely refractory to TGF-β when activated on anti-CD3 (C).

Table I. Total numbers and differential distributions of blood leukocytes (10^3/mm^3)

Control mice							Mutant mice						
Mouse	Age	Sex	WBC	Lymphocyte	Neutrophil	Monocyte	Mouse	Age	Sex	WBC	Lymphocyte	Neutrophil	Monocyte
I789$^{+/-}$	51	M	11.7	8.78 (75.0)	2.57 (22.0)	0.23 (2.0)	I787$^{-/-}$	51	M	10.5	8.40 (80.0)	1.68 (16.0)	0.42 (4.0)
I742$^{+/+}$	55	F	11.8	10.27(87.0)	1.42 (12.0)	0.12 (1.0)	I786$^{-/-}$	51	M	12.6	9.95 (79.0)	2.27 (18.0)	0.38 (3.0)
I497$^{+/+}$	70	F	6.5	5.79 (89.1)	0.65 (10.0)	0.07 (0.6)	I730$^{-/-}$	50	M	19.0	14.83 (78.1)	3.42 (18.0)	0.57 (3.0)
I496$^{+/-}$	70	F	5.4	4.86 (90.0)	0.49 (9.1)	0.05 (0.9)	F499$^{-/-}$	70	F	15.7	9.42 (60.0)	5.97 (38.0)	0.31 (2.0)
F428$^{+/-}$	38	F	6.4	5.31 (83.0)	1.06 (16.6)	0.00 (0.0)	F433$^{-/-}$	38	F	8.7	5.31 (61.0)	2.87 (33.0)	0.44 (5.1)
F432$^{+/-}$	38	F	6.1	5.06 (83.0)	0.98 (16.1)	0.06 (0.9)	F429$^{-/-}$	38	F	10.6	7.84 (74.0)	2.33 (22.0)	0.42 (4.0)
mean			8.9	6.92 (84.5)	1.78 (14.3)	0.15 (0.9)				14.8	11.34 (72.0)	2.88 (24.2)	0.50 (3.5)
±SD			±4.0	±2.63 (5.5)	±1.12 (4.9)	±0.12 (0.7)				±5.9	±4.94 (9.2)	±0.77 (9.1)	±0.11 (1.1)
P value										<0.05	<0.05	<0.05	<0.01

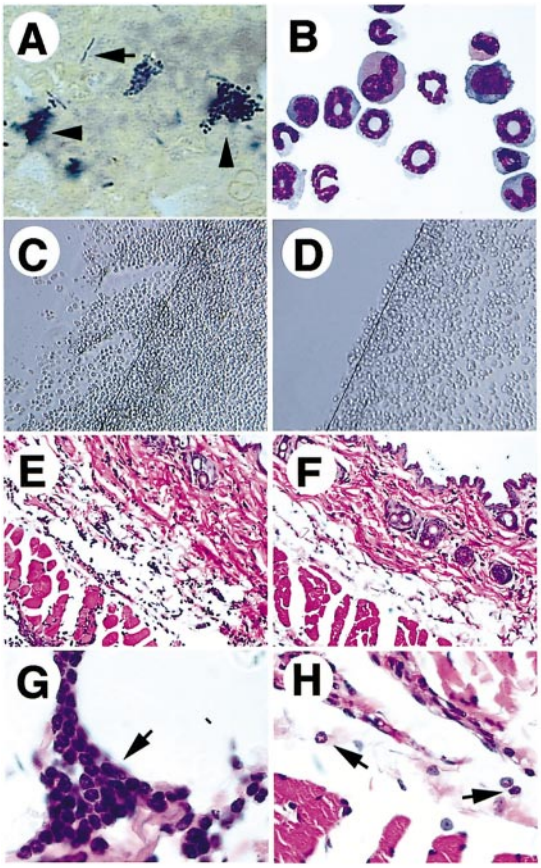

Fig. 8. Impaired neutrophil chemotaxis in *Smad3*$^{ex8/ex8}$ mice. (**A**) Brown and Hopps stain of section through chronic abscess tissue reveals Gram-positive cocciform (arrowheads) and Gram-negative rod-shaped organisms (arrow). (**B**) Cytospin of neutrophil preparation from *Smad3*$^{ex8/ex8}$ mice. (**C**) Normal migration of wild-type neutrophils under agarose toward 5 pg/ml of TGF-β (to the left of well edge, indicated by thin black line). (**D**) *Smad3*$^{ex8/ex8}$ neutrophils remain within well margins. (**E**) Arrow indicates the presence of inflammatory cells within the connective tissue and dermis of a wild-type mouse, 12 h following inoculation with 200 ng/ml TGF-β1, which consist predominantly of granulocytes (**G**). (**F** and **H**) Although inflammatory cells can be detected at the site of inoculation in *Smad3*$^{ex8/ex8}$ skin, the extent of the infiltrate and number of granulocytes per high power field is substantially less.

development of colorectal cancer in humans (Arai *et al*., 1998). The disparity between these observations and the tumor phenotype reported for *Smad3*$^{ex2/ex2}$ mice (Zhu *et al*., 1998) must be reconciled through further investigation in these model systems.

Discussion

We have disrupted the *Smad3* gene and found that SMAD3-deficient mice are normal during embryonic and early postnatal development. After weaning, these mice invariably develop an illness associated with progressive leukocytosis, periodontitis, gastritis, colitis and chronic infection with abscess formation adjacent to mucosal surfaces. Immunophenotyping of lymph node, spleen and thymus of young, asymptomatic mice demonstrates normal development of lymphocytes. Histological analysis of *Smad3*$^{ex8/ex8}$ lymph nodes of symptomatic mice revealed increased proliferation and activation of T cells, with T cell invasion of B cell zones and an accumulation of plasma cells. While isolated B cells activated in culture remained sensitive to the inhibitory effects of TGF-β when activated by LPS, thymocytes and peripheral T cells were totally resistant when stimulated by antibodies to the T cell receptor. In addition, the normal chemotactic response of neutrophils to TGF-β is impaired in mutant mice. These observations suggest that one function of SMAD3 is to mediate specific autocrine or paracrine effects of TGF-β which regulate the migration, activation and proliferation within specific leukocyte populations. A disruption of these events might lead to an immune deficiency state, as suggested by the presence of invasive bacterial infections in *Smad3*$^{ex8/ex8}$ mice, which is ultimately responsible for the lethality associated with homozygosity for our SMAD3 mutation.

However, these phenotypes are quite distinct, and in part contradictory to those exhibited by *Smad3*$^{ex2/ex2}$ mice (Zhu *et al*., 1998). According to the report by Zhu *et al*., 100% of *Smad3*$^{ex2/ex2}$ mice in an inbred 129SV background and 30% of mutant mice in mixed background (129SV×C57BL/6) developed metastatic colorectal cancer by the time they were 4–6 months old. No evaluation of the status of immune function was mentioned (Zhu *et al*., 1998). We have attempted to uncover a cause for the discrepancy in the tumor phenotype expressed in the two models. Our analysis indicates that differences in genetic background may not be the significant factor because the *Smad3*$^{ex8/ex8}$ mice in the two backgrounds (129SVEV×C57BL/6 and 129SVEV×Black Swiss) were virtually tumor free. Moreover, another mutant mouse in 129SVEV×C57BL/6, which lacks the first exon of the *Smad3* gene and makes no detectable protein, showed phenotypes similar to those described here (X.F.Wang, personal communication). We have carefully analyzed our mutant allele through rigorous tests of function (Figure 2C and D) and find that our *Smad3*^{ex8} null allele has no activity in assays using Smad3-responsive reporter constructs. It remains a formal possibility that protein translated from the message produced in the mutant cells can act in a dominant-negative fashion. Given that we were only able to suppress activation of the TGF-β-responsive reporter at high levels of Smad3ΔC expression, we doubt that this plays a significant role in the development of the observed phenotype. Zhu *et al*. (1998) tested their mutant allele by examining mesoderm in *Xenopus laevis*. Their data indicate that the first 77 amino acids of SMAD3 failed to induce mesoderm (Zhu *et al*., 1998). However, the effect of full-length SMAD3 on mesoderm induction was not examined, and there are no reports in the literature of mesoderm induction mediated by SMAD3. Moreover, they did not address the possibility that the remaining part of the mutant transcripts (i.e. from exons 4 to 9) may be translated. This possibility is suggested by the presence of two in-frame methionines located immediately after the fusion junction. This point is of critical significance, as it is known from previous studies in *Xenopus* that the C-terminal portion of SMAD2 is fully functional even in the absence of ligand (Baker and Harland, 1996).

Distinct developmental defects observed in SMAD2- and SMAD3-deficient mice

Our studies involving targeted disruption of the *Smad3* gene now permit comparison of the *in vivo* function of

SMAD3 and its close relative, SMAD2. These genes share a high degree of homology and are assumed to mediate signals from the identical set of ligands and receptors (Zhang *et al*., 1996; Nakao *et al*., 1997a; Lo *et al*., 1998). Although targeted deletion of SMAD2 resulted in failure of mesoderm formation (Nomura and Li, 1998; Waldrip *et al*., 1998; Weinstein *et al*., 1998), mutants null for the *Smad3* gene develop normally but succumb between 1 and 8 months from immune dysregulation and infection, indicating distinct roles of these genes in development. This hypothesis is also supported by our observation that the expression of Smad2 protein in $Smad3^{ex8/ex8}$ embryos and in tissues of adult mutant mice is identical to that detected in wild-type siblings (data not shown).

A growing number of *in vitro* studies now show not only selective activation of target genes by SMAD2 and SMAD3, but also striking differences in the mechanisms of transcriptional activation. The most studied example involves the activation by SMAD2 of *Mix2*, an immediate-early response gene, in *Xenopus* embryos, where SMAD2 has been shown to act as a bridge between the transcriptional activator SMAD4 and the forkhead transcription factor, FAST-1, which binds directly to the activin response element (Chen *et al*., 1996, 1997). In contrast, numerous studies suggest that SMAD3 can activate transcription by direct binding to DNA through its N-terminal MH1 domain (Yingling *et al*., 1997). Both SMAD3 and SMAD4, but not SMAD2, bind directly to a common DNA binding sequence, termed a CAGA box, in the plasminogen activator inhibitor-1 promoter (Dennier *et al*., 1998) and specifically recognize an identical 8 bp palindromic sequence which, when tandemly repeated, can confer TGF-β inducibility to a minimal promoter (Zawel *et al*., 1998). This may suggest a differential activation of downstream targets between SMAD2 and SMAD3, and this may underlie the observed phenotypic differences in $Smad2^{-/-}$ and $Smad3^{ex8/ex8}$ mice.

SMAD3 is required for mediating regulatory effects of TGF-β in T cells

T cells are essential for mounting cellular immune responses, for activating phagocytic cells and natural killer cells by secreted cytokines, and for regulating the generation of antigen-specific antibodies by B cells. Each of these T cell functions are impacted by both autocrine and paracrine TGF-β signaling pathways (Stavnezer, 1996; Horwitz *et al*., 1997; Letterio and Roberts, 1998). Production of TGF-β by specialized suppressor T cells has been implicated in controlling the susceptibility to colitis (Powrie *et al*., 1996; Strober *et al*., 1997), the establishment of oral tolerance in humans with autoimmune disorders (Fukaura *et al*., 1996), and in experimental models of autoimmunity (Miller *et al*., 1992; Chen *et al*., 1995). Not all targets of these effector cells may be compromised by mutations in SMAD3, as suggested by the normal susceptibility of $Smad3^{ex8/ex8}$ B lymphocytes to growth inhibitory effects of TGF-β. However, T lymphocytes differentiated toward a T helper 1 (Th1) profile and primed to secrete proinflammatory cytokines may be resistant to the suppressive effects of TGF-β or other TGF-β family ligands, including and especially activin (Mizuguchi *et al*., 1993; Tompkins *et al*., 1998). Recent studies suggest that TGF-β itself may be an important cytokine for priming T cells toward differentiation into the suppressor, Th2/Th3 phenotype (Seder *et al*., 1998). The lack of sensitivity of $Smad3^{ex8/ex8}$ T cells to TGF-β predicts that an imbalance between effector and suppressor T cells may be a mechanism contributing to the inflammatory phenotype in these mice. The concept that altered cytokine signaling may lead to an imbalance in regulatory T cells and defective mucosal immunity is supported by the development of similar forms of spontaneous colitis in T-cell receptor (TCR)-α chain-deficient mice (Mombaerts *et al*., 1993), IL-2-deficient mice (Sadlack *et al*., 1993) and IL-10-deficient mice (Kuhn *et al*., 1993).

SMAD3 mutation results in impaired neutrophil chemotaxis and susceptibility to invasive mucosal infections

Several distinctive features of the $Smad3^{ex8/ex8}$ phenotype suggest that additional components of the immune system may be disrupted. The periodontitis and periodontal abscesses observed in mutant mice are also features of a series of well-described human clinical disorders which are caused by abnormalities in one or more steps of phagocytic function, including chemotaxis, adhesion, ingestion, degranulation and oxidative metabolism. Many of these functions of neutrophils, macrophages and monocytes are stimulated by TGF-β (reviewed in Letterio and Roberts, 1998). This cytokine provides a chemotactic signal for neutrophils and monocytes (Wahl *et al*., 1987; Brandes *et al*., 1991), induces expression of several integrin receptors, and upregulates expression of FcγRIII, which recognizes bound IgG and is a key factor in immunophagocytosis. If these regulatory functions are mediated through a SMAD3-dependent pathway, one might predict problems with leukocyte adhesion, chemotaxis and ingestion of opsonized microbes.

Although we have not directly evaluated phagocytic activity or the regulation of adhesion molecule expression in Smad3-deficient neutrophils, the lack of a chemotactic response to TGF-β suggests one mechanism that may be directly responsible for the chronic mucosal infections in $Smad3^{ex8/ex8}$ mice. Transepithelial migration of neutrophils is an important component of the mucosal immune response, and is thought to be important in maintaining an equilibrium with normal bacterial flora. Our ability to isolate *Providencia* bacterial species only from formed abscesses indicates such a breakdown in normal mucosal defense mechanisms, as this is typically an isolate in immune-compromised hosts (Vatopoulos *et al*., 1996). The molecular mechanisms controlling mucosal neutrophil numbers are thought to include local production of cytokines and expression of adhesion molecules such as ICAM-1 (Tonetti, 1997). TGF-β has been demonstrated to promote neutrophil chemotaxis through its ability to promote the interaction of neutrophil integrins with fibronectin (Parekh *et al*., 1994). In humans, defects in neutrophil adhesion and chemotaxis are associated with persistent granulocytosis, severe gingivitis, periodontitis and frequent bacterial infections which invade either locally or systemically (Curnutte, 1993). The potential value of the $Smad3^{ex8/ex8}$ mouse as a model of these disorders will be determined by future studies focused on mechanisms of immune deficiency and altered leukocyte function in these mice.

Materials and methods

Targeting vector

Recombinant phage containing genomic DNA of the *Smad3* locus were isolated from a 129 mouse library (Stratagene) by using a full-length *Smad3* cDNA (DDBJ/EMBL/GenBank accession No. AFD16189) as a probe. To construct the targeting vector for the *Smad3* gene, a 3 kb *Not*I–*Hin*dIII fragment (the *Not*I site is from polylinker of the phage vector) that is 5′ to the eighth exon of the *Smad3* gene was subcloned into *Not*I and *Hpa*I sites of pLoxpneo (Yang *et al*., 1998). The resulting construct was cleaved with *Asp*718 followed by insertion of a 5 kb *Asp*718 fragment which is 3′ to the eighth exon of the *Smad3* gene. This targeting strategy deletes a 1 kb *Hin*dIII–*Asp*718 fragment that contains the eighth exon of the *Smad3* gene. The finished construct, *pSmad3neo*, is shown in Figure 1A.

Homologous recombination in ES cells and generation of germline chimeras

TC1 ES cells (Deng *et al*., 1996) were transfected with *Not*I-digested *pSmad3neo*, and selected with G418 and FIAU as described (Deng *et al*., 1994). ES cell colonies that were resistant to both G418 and FIAU were picked and analyzed by Southern blotting for homologous recombination events within the *Smad3* locus. Genomic DNAs from these clones and the parental TC1 cell line were digested with *Eco*RV and *Bam*HI, respectively, followed by Southern blots using a 1 kb *Sal*I–*Eco*RI fragment that is 5′ to the targeting vector (Figure 1B).

ES cells heterozygous for the targeted mutation were microinjected into C57BL/6 blastocysts to obtain germline transmission. The injected blastocysts were implanted into the uteri of pseudopregnant Swiss Webster (Taconic) foster mothers and allowed to develop to term. Male chimeras (identified by the presence of agouti coat color) were mated with C57BL/6 and NIH Black Swiss females (Taconic). Germline transmission was confirmed by agouti coat color in the F_1 animals, and all agouti offspring were tested for the presence of the mutated *Smad3* allele by Southern analysis using the same conditions for the detection of the homologous recombination event in the ES cells.

Genotype analysis

Genotypes were determined by Southern blotting or PCR. For PCR analysis, the wild-type *Smad3* allele was detected using primer 1 (5′-CCACTTCATTGCCATATGCCCTG-3′) and primer 2 (5′-CCCGAACA-GTTGGATTCACACA-3′). The primer 1 is located 5′ to the deletion and the primer 2 is located within the deletion. This primer pair amplifies a fragment of ~400 bp from wild-type and $Smad3^{ex8/+}$, but not from $Smad3^{ex8/ex8}$ mice. DNA was also amplified using the primer 1 and primer 3, which is located in the pLoxpneo (5′-CCAGACTGCCTTGGG-AAAAGC-3′) to detect the mutant *Smad3* allele. In this case, a 250 bp fragment was detected in mice heterozygous or homozygous for the mutant *Smad3* allele, while no signal was detected in wild-type mice.

Histology and antibody staining

Histological sections were prepared from selected tissues fixed in Bouin's solution and embedded in paraffin. Five-micrometer sections were either stained with hematoxylin and eosin (H&E), or subjected to immunohistochemistry with antibodies to IgA (Zymed Laboratories, South San Francisco, CA), CD3 (Dako Corp., Carpinteria, CA) and B220 (Pharmingen). Detection of primary antibodies was performed with Vectastain Elite ABC kits (Vector Laboratories).

Flow cytometry of lymphocytes

Thymus, spleen and lymph nodes were harvested from 6–8-week-old mice. Single-cell suspensions were subjected to hypotonic lysis of red blood cells by incubation with 0.144 M NH_4Cl + 0.017 M Tris pH 7.2, washed once in phosphate-buffered saline/bovine serum albumin, stained with fluorescein (FITC)-conjugated antibodies according to the standard protocols, and analyzed on a FACScan (Beckman Dickinson). Antibodies were as follows: anti-CD4-FITC, anti-CD8-PE, anti-β220-FITC, anti-CD3-PE, anti-CD62-PE, anti-IgM-biotin and anti-NK1.1-PE anti-NK1.1-PE (PharMingen Corp., San Diego, CA).

Lymphocyte purification and proliferation assays

Single-cell suspensions were prepared from the thymus and spleen of 6–8-week-old mice and subjected to hypotonic red cell lysis. Thy1 $(CD90)^+$ T lymphocytes and $B220^+$ B lymphocytes were selected from spleen cells using the midi-MACs magnetic separation system according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA). Cells were resuspended in RPMI 1640 (Biofluids, Rockville, MD) supplemented with 5% heat-inactivated fetal bovine serum, and 5×10^{-5} M β-mercaptoethanol for cell culture. Isolated B lymphocytes (1×10^5) were cultured in 96-well round bottom tissue culture plates (Falcon) in the presence of 20 μg/ml LPS (Sigma), with or without the addition of TGF-β1 (2.5 ng/ml) (R&D Systems). Thymocytes and isolated splenic T cells were cultured in 96-well plates pre-coated with anti-CD3 with or without anti-CD28 (30 μl of 1 μg/ml), and in the presence or absence of TGF-β1. Cultures were allowed to incubate for 72 h, with 1 μCi of [^{3}H]thymidine (Amersham) added for the final 6 h of culture. Cells were harvested onto Packard 96-well filter plates and counts determined on a Top Count Microplate Scintillation reader according to the manufacturer's instructions (Packard).

Bacterial culture and stains

Material for bacterial culture was obtained by aseptic technique from abscess cavities and by cardiac puncture at necropsy. Bacterial cultures were performed by the NCI Animal Health Diagnostic Laboratory in Frederick, MD. Sections (5 μm) of formalin-fixed abscess tissue were subjected to a Brown and Hopps stain for detection of bacterial colonies.

Neutrophil isolation and chemotaxis assays

Neutrophil isolation from bone marrow of $Smad3^{ex8/ex8}$ and wild-type mice was performed using the NIM/NIM-2 Cell Isolation, Purification, and Enrichment System (Cardinal Associates, Inc., Santa Fe, NM) according to the manufacturer's instructions. Marrow was removed from both femurs and tibias of each mouse. *In vitro* chemotaxis to TGF-β was examined using the under-agarose chemotaxis assay, as previously described (Lauffenburger *et al*., 1983). Neutrophils were plated in wells created with a 2 mm punch biopsy needle and allowed 3 h to migrate prior to assessment of chemotactic response toward wells containing either vehicle or TGF-β. For assessment of chemotactic response to TGF-β *in vivo*, 200 ng of carrier-free TGF-β1 was injected subcutaneously at a site marked by india ink prior to inoculation. Twelve hours following injection, a circular area of skin, 3–4 mm in diameter and surrounding the injection site, was removed, fixed in formalin, and processed for evaluation by routine H&E stain.

Transient transfection assays

Gal4–Smad3ΔC containing amino acids 1–335 of Smad3 encoded by the mutant RNA was created by PCR using primers 5′-GCGGATCCTGT-CGTCCATCCTGCCCTTC-3′ and 5′-GCGGTACCGCTGGTGGGAT-CTTGCAGACAG-3′, and cloned into the Gal4 fusion vector pSG424 (a gift from M.Ptashne). The clones were sequenced to make sure that no mismatched mutation was introduced. Gal4–Smad3 was a gift from S.-J.Kim. Indicated constructs along with the reporter pG5E1B-luc and pRSV-βGal for normalization were transiently transfected in NMuMg cells using lipofectamine following the manufacturer's directions. Total DNA content was kept constant with empty pSG424. Cells were treated with 5 ng/ml TGF-β1 or left untreated overnight in the absence of serum. Luciferase activity was determined 16 h after TGF-β treatment in a luminometer and values normalized for transfection efficiency by β-galactosidase activity. Values shown represent duplicate measurements of triplicate wells ± SEM and are representative of three experiments.

Acknowledgements

We thank Cuiling Li and Xiaoling Xu for their excellent technical assistance. We are grateful to members of the Deng laboratory for helpful discussion and critical reading of the manuscript. We thank Miriam Anver (SAIC, Frederick, MD) for her expertise and assistance with immunohistochemical studies.

References

Abdollah,S., Macias-Silva,M., Tsukazaki,T., Hayashi,H., Attisano,L. and Wrana,J.L. (1997) TGF-βRI phosphorylation of Smad2 on Ser465 and Ser467 is required for Smad2–Smad4 complex formation and signaling. *J. Biol. Chem.*, **22**, 27678–27685.

Arai,T., Akiyama,Y., Okabe,S., Ando,M., Endo,M. and Yuasa,Y. (1998) Genomic structure of the human *Smad3* gene and its infrequent alterations in colorectal cancers. *Cancer Lett.*, **122**, 157–163.

Baker,J.C. and Harland,R.M. (1996) A novel mesoderm inducer, Madr2, functions in the activin signal transduction pathway. *Genes Dev.*, **10**, 1880–1889.

Brandes,M.E., Mai,U.E., Ohura,K. and Wahl,S.M. (1991) Type I transforming growth factor-beta receptors on neutrophils mediate chemotaxis to transforming growth factor-beta. *J. Immunol.*, **147**, 1600–1606.

Chen,X., Rubock,M.J. and Whitman,M. (1996) A transcriptional partner for MAD proteins in TGF-β signalling. *Nature*, **383**, 691–696.

Chen,X., Weisberg,E., Fridmacher,V., Watanabe,M., Naco,G. and Whitman,M. (1997) Smad4 and FAST-1 in the assembly of activin-responsive factor. *Nature*, **389**, 85–89.

Chen,Y., Inobe,J., Marks,R., Gonnella,P., Kuchroo,V.K. and Weiner,H.L. (1995) Peripheral deletion of antigen-reactive T cells in oral tolerance. *Nature*, **376**, 177–180.

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

Curnutte,J. (1993) Disorders of granulocyte function and granulopoisis. In Nathan,D and Oski,F. (eds), *Hematology of Infancy and Childhood*. W.B.Saunders, PA, pp. 904–977.

Deng,C., Wynshaw-Boris,A., Shen,M.M., Daugherty,C., Ornitz,D.M. and Leder,P. (1994) Murine FGFR-1 is required for early postimplantation growth and axial organization. *Genes Dev.*, **8**, 3045–3057.

Deng,C., Wynshaw-Boris,A., Zhou,F., Kuo,A. and Leder,P. (1996) Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell*, **84**, 911–921.

Dennier,S., Itoh,S., Vivien,D., ten Dijke,P., Huet,S. and Gauthier,J.-M. (1998) Direct binding of Smad3 and Smad4 to critical TGFβ-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J.*, **17**, 3091–3100.

Derynck,R. and Feng,X.H. (1997) TGF-β receptor signaling. *Biochim. Biophys. Acta*, **1333**, F105–150.

Eppert,K. *et al.* (1996) MADR2 maps to 18q21 and encodes a TGF-β-regulated MAD-related protein that is functionally mutated in colorectal carcinoma. *Cell*, **86**, 543–552.

Fukaura,H., Kent,S.C., Pietrusewicz,M.J., Khoury,S.J., Weiner,H.L. and Hafler,D.A. (1996) Antigen-specific TGF-β1 secretion with bovine myelin oral tolerization in multiple sclerosis. *Ann. N.Y. Acad. Sci*, **778**, 251–257.

Hayashi,H. *et al.* (1997) The MAD-related protein Smad7 associates with the TGFβ receptor and functions as an antagonist of TGFβ signaling. *Cell*, **89**, 1165–1173.

Heldin,C.H., Miyazono,K. and ten Dijke,P. (1997) TGF-β signalling from cell membrane to nucleus through SMAD proteins. *Nature*, **390**, 465–471.

Horwitz,D.A., Gray,J.D., Ohtsuka,K., Hirokawa,M. and Takahashi,T. (1997) The immunoregulatory effects of NK cells: the role of TGF-β and implications for autoimmunity. *Immunol. Today*, **18**, 538–542.

Imai,S., Okuno,M., Moriwaki,H., Muto,Y., Murakami,K., Shudo,K., Suzuki,Y. and Kojima,S. (1997) 9,13-di-*cis*-Retinoic acid induces the production of tPA and activation of latent TGF-β via RARα in a human liver stellate cell line, LI90. *FEBS Lett.*, **411**, 102–106.

Kingsley,D.M. (1994) The TGF-β superfamily: new members, new receptors and new genetic tests of function in different organisms. *Genes Dev.*, **8**, 133–146.

Kuhn,R., Lohler,D., Rennick,K., Rajewsky,K. and Muller,W. (1993) Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*, **75**, 263–274.

Lagna,G., Hata,A., Hemmati-Brivanlou,A. and Massague,J. (1996) Partnership between DPC4 and SMAD proteins in TGF-β signalling pathways. *Nature*, **383**, 832–836.

Lauffenburger,D.C.R. and Zigmond,S.H. (1983) Measurement of leukocyte motility and chemotaxis parameters with a linear under-agarose migration assay. *J. Immunol.*, **131**, 940–947.

Letterio,J.J. and Roberts,A.B. (1998) Regulation of immune responses by TGF-β. *Annu. Rev. Immunol.*, **16**, 137–161.

Liu,F., Hata,A., Baker,J.C., Doody,J., Carcamo,J., Harland,R.M. and Massague,J. (1996) Dual role of the Smad4/DPC4 tumor suppressor in TGFβ-inducible transcriptional complexes. *Nature*, **381**, 620–623.

Liu,F., Pouponnot,C. and Massague,J. (1997) Transforming growth factor β-induced phosphorylation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. *Genes Dev.*, **11**, 3157–3167.

Liu,X., Sun,Y., Constantinescu,S.N., Karam,E., Weinberg,R.A. and Lodish,H.F. (1997) Transforming growth factor β-induced phosphorylation of Smad3 is required for growth inhibition and transcriptional induction in epithelial cells. *Proc. Natl Acad. Sci. USA*, **94**, 10669–10674.

Lo,R.S., Chen,Y.G., Shi,Y., Pavletich,N.P. and Massague,J. (1998) The L3 loop: a structural motif determining specific interactions between SMAD proteins and TGF-β receptors. *EMBO J.*, **17**, 996–1005.

Macias-Silva,M., Abdollah,S., Hoodless,P.A., Pirone,R., Attisano,L. and Wrana,J.L. (1996) MADR2 is a substrate of the TGF-β receptor and its phosphorylation is required for nuclear accumulation and signaling. *Cell*, **87**, 1215–1224.

Massague,J. (1998) TGF-β signal transduction. *Annu. Rev. Biochem.*, **67**, 753–791.

Miller,A., Lider,O., Roberts,A.B., Sporn,M.B. and Weiner,H.L. (1992) Suppressor T cells generated by oral tolerization to myelin basic protein suppress both *in vitro* and *in vivo* immune responses by the release of transforming growth factor β after antigen-specific triggering. *Proc. Natl Acad. Sci. USA*, **89**, 421–425.

Mizuguchi,T., Kosaka,M. and Saito,S. (1993) Activin A suppresses proliferation of interleukin-3-responsive granulocyte-macrophage colony-forming progenitors and stimulates proliferation and differentiation of interleukin-3-responsive erythroid burst-forming progenitors in the peripheral blood. *Blood*, **81**, 2891–2897.

Mombaerts,P., Mizoguchi,E., Grusby,M.J., Glimcher,L.H., Bhan,A.K. and Tonegawa,S. (1993) Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice. *Cell*, **75**, 274–282.

Nakao,A. *et al.* (1997a) TGF-β receptor-mediated signalling through Smad2, Smad3 and Smad4. *EMBO J.*, **16**, 5353–5362.

Nakao,A., Roijer,E., Imamura,T., Souchelnytskyi,S., Stenman,G., Heldin,C.H. and ten Dijke,P. (1997b) Identification of Smad2, a human Mad-related protein in the transforming growth factor β signaling pathway. *J. Biol. Chem.*, **272**, 2896–2900.

Newfeld,S.J., Chartoff,E.H., Graff,J.M., Melton,D.A. and Gelbart,W.M. (1996) Mothers against dpp encodes a conserved cytoplasmic protein required in DPP/TGF-β responsive cells. *Development*, **122**, 2099–2108.

Nomura,M. and Li,E. (1998) Smad2 role in mesoderm formation, left-right patterning and craniofacial development. *Nature*, **393**, 786–970.

Page,R.C., Sims,T.J., Geissler,F., Altman,L.C. and Baab,D.A. (1985) Defective neutrophil and monocyte motility in patients with early onset periodontitis. *Infect. Immun.*, **47**, 169–175.

Parekh,R., Saxena,B., Reibman,J., Cronstein,B.N. and Gold,L. (1994) Neutrophil chemotaxis in response to TGF-β isoforms (TGF-β1, TGF-β2, TGF-β3) is mediated by fibronectin. *J. Immunol.*, **152**, 2456–2466.

Powrie,F., Carlino,J., Leach,M.W., Mauze,S. and Coffman,R.L. (1996) A critical role for transforming growth factor-β but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB (low) CD4+ T cells. *J. Exp. Med.*, **183**, 2669–2674.

Riggins,G.J. *et al.* (1996) Mad-related genes in the human. *Nature Genet.*, **13**, 347–349.

Roberts,A.B. and Sporn,M.B. (1990) The transforming growth factors-β. In Sporn,M.B. and Roberts,A.B. (eds), *Handbook of Experimental Pharmacology*. Springer-Verlag, Heidelberg, Vol. 95, pp. 418–472.

Sadlack,B., Merz,H., Schorle,H., Schimpl,A., Feller,A.C. and Horak,I. (1993) Ulcerative colitis-like disease in mice with a disrupted interleukin-2 gene. *Cell*, **75**, 253–261.

Savage,C., Das,P., Finelli,A.L., Townsend,S.R., Sun,C.Y., Baird,S.E. and Padgett,R.W. (1996) *Caenorhabditis elegans* genes *sma-2*, *sma-3* and *sma-4* define a conserved family of transforming growth factor β pathway components. *Proc. Natl Acad. Sci. USA*, **93**, 790–794.

Seder,R., Marth,T., Sieve,M., Letterio,J., Strober,W. and Kelsall,B. (1998) Factors involved in the differentiation of TGF-βs producing cells from naive CD4+ T cells: IL-4 and IFN-γ have opposing effects, while TGF-β positively regulates its own production. *J. Immunol.*, **160**, 5719–5728.

Sirard,C. *et al.* (1998) The tumor suppressor gene Smad4/Dpc4 is required for gastrulation and later for anterior development of the mouse embryo. *Genes Dev.*, **12**, 107–119.

Souchelnytskyi,S., Tamaki,K., Engstrom,U., Wernstedt,C., ten Dijke,P. and Heldin,C.H. (1997) Phosphorylation of Ser465 and Ser467 in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-β signaling. *J. Biol. Chem.*, **272**, 28107–28115.

Stavnezer,J. (1996) Transforming growth factor β. In Snapper,C.M. (ed.), *Cytokine Regulation of Humoral Immunity: Basic and Clinical Aspects*. Wiley, New York, NY, pp. 904–977.

Strober,W., Kelsall,B., Fuss,I., Marth,T., Ludviksson,B., Ehrhardt,R. and Neurath,M. (1997) Reciprocal IFN-γ and TGF-β responses regulate the occurrence of mucosal inflammation. *Immunol. Today*, **18**, 61–64.

Tompkins,A.B., Hutchinson,P., de Kretser,D.M. and Hedger,M.P. (1998) Characterization of lymphocytes in the adult rat testis by flow

cytometry: effects of activin and transforming growth factor β on lymphocyte subsets *in vitro*. *Biol*. *Reprod*., **58**, 943–951.
Tonetti,M.S. (1997) Molecular factors associated with compartmentalization of gingival immune responses and transepithelial neutrophil migration. *J*. *Periodontal Res*., **32**, 104–109.
Vatopoulos,A.C., Kalapothaki,V. and Legakis,N.J. (1996) Risk factors for nosocomial infections caused by Gram-negative bacilli. The Hellenic Antibiotic Resistance Group. *J*. *Hosp*. *Infect*., **34**, 11–22.
Wahl,S.M., Hunt,D.A., Wakefield,L.M., McCartney-Frances,N., Wahl,L., Roberts,A. and Sporn,M.B. (1987) Transforming growth factor type β induces monocyte chemotaxis and growth factor production. *Proc*. *Natl Acad*. *Sci*. *USA*, **84**, 5788–5792.
Waldrip,W.R., Bikoff,E.K., Hoodless,P.A., Wrana,J.L. and Robertson,E.J. (1998) Smad2 signaling in extraembryonic tissues determines anterior–posterior polarity of the early mouse embryo. *Cell*, **92**, 797–808.
Weinstein,M., Yang,X., Li,C., Xu,X., Gotay,J. and Deng,C.X. (1998) Failure of egg cylinder elongation and mesoderm induction in mouse embryos lacking the tumor suppresser smad2. *Proc*. *Natl Acad*. *Sci*. *USA*, **95**, 9378–9383.
Wiersdorff,V., Lecuit,T., Cohen,S.M. and Mlodzik,M. (1996) Mad acts downstream of Dpp receptors, revealing a differential requirement for dpp signaling in initiation and propagation of morphogenesis in the *Drosophila* eye. *Development*, **122**, 2153–2162.
Yang,X., Li,C., Xu,X. and Deng,C. (1998) The tumor suppressor SMAD4/DPC4 is essential for epiblast proliferation and mesoderm induction in mice. *Proc*. *Natl Acad*. *Sci*. *USA*, **95**, 3667–3672.
Yingling,J.M., Datto,M.B., Wong,C., Frederick,J.P., Liberati,N.T. and Wang,X.F. (1997) Tumor suppressor Smad4 is a transforming growth factor β-inducible DNA binding protein. *Mol*. *Cell*. *Biol*, **17**, 7019–7028.
Zawel,L., Dai,J.L., Buckhaults,P., Zhou,S., Kinzler,K.W., Vogelstein,B. and Kern,S.E. (1998) Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol*. *Cell*, **1**, 611–617.
Zhang,Y., Feng,X., We,R. and Derynck,R. (1996) Receptor-associated Mad homologues synergize as effectors of the TGF-β response. *Nature*, **383**, 168–172.
Zhu,Y., Richardson,J.A., Parada,L.F. and Graff,J.M. (1998) Smad3 mutant mice develop metastatic colorectal cancer. *Cell*, **94**, 703–714.

Received November 3, 1998; revised and accepted January 7, 1999