Published in final edited form as:

Genesis. 2009 June ; 47(6): 423–431. doi:10.1002/dvg.20516.

Generation of Mice With a Conditional Allele for Transforming Growth Factor beta 1 Gene

Mohamad Azhar^{1,2,*}, Moying Yin³, Ramireddy Bommireddy^{1,4}, John J. Duffy^{3,†}, Junqi Yang⁵, Sharon A. Pawlowski³, Gregory P Boivin⁶, Sandra J. Engle⁷, L.P. Sanford¹, Christina Grisham³, Ram R. Singh⁸, George F. Babcock^{9,10}, and Thomas Doetschman^{1,2,*}

¹ BIO5 Institute, University of Arizona, Tucson, Arizona

² Department of Cell Biology, University of Arizona, Tucson, Arizona

³ Department of Molecular Genetics, University of Cincinnati, Cincinnati, Ohio

⁴ Department of Immunobiology, University of Arizona, Tucson, Arizona

⁵ Department of Cancer & Cell Biology, University of Cincinnati, Cincinnati, Ohio

⁶ Department of Pathology and Laboratory Medicine, University of Cincinnati, Cincinnati, Ohio

⁷ Pfizer, Inc., Groton, Connecticut

⁸ David Geffen School of Medicine, University of California, Los Angeles California

⁹ Department of Surgery, University of Cincinnati, Cincinnati, Ohio

¹⁰ Shriners Hospital for Children, Cincinnati, Ohio

Summary

Transforming growth factor β 1 (TGF β 1) is a multifunctional growth factor involved in wound healing, tissue fibrosis, and in the pathogenesis of many syndromic diseases (e.g., Marfan syndrome, Camurati-Engelmann disease) and muscular, neurological, ophthalmic, cardiovascular and immunological disorders, and cancer. Since the generation of *Tgfb1* knockout mice, there has been extraordinary progress in understanding its physiological and pathophysiological function. Here, we report the generation of a conditional knockout allele for *Tgfb1* in which its exon 6 is flanked with *LoxP* sites. As proof of principle, we crossed these mice to *LckCre* transgenic mice and specifically disrupted *Tgfb1* in T cells. The results indicate that T-cell-produced TGF β 1 is required for normal in vivo regulation of peripheral T-cell activation, maintenance of T-cell homeostasis, and suppression of autoimmunity.

Keywords

transforming growth factor β ; conditional knockout mice

Transforming growth factor *betas* (TGF β s) are regulatory cytokines that function in embryonic development and adults and in the pathogenesis of various diseases and cancer (Derynck and Akhurst, 2007; McLennan and Koishi, 2002; Sporn, 2006). In mammals, there are three TGF β ligands which express differently in time and space (Azhar *et al.*, 2003; Millan *et al.*,

*Correspondence to: Mohamad Azhar and Thomas Doetschman, 1656 E Mabel St, PO Box 245217, Tucson, AZ 85724-5217., E-mail: azharm@email.arizona.edu and tdoetsch@u.arizona.edu.

[†]Deceased

1991; Pelton *et al.*, 1991). Activated forms of TGF β s usually interact with TGF β R1 and TGF β R2 receptors in conjunction with the TGF β R3 or endoglin and induce phosphorylation of SMADs (Massague and Gomis, 2006). TGF β signaling can also occur through SMAD-independent pathways (e.g., RAS-MAPK, Ca²⁺/calcineurin and a non-transcriptional pathway such as platelet aggregation) (Azhar *et al.*, 2003; Bommireddy *et al.*, 2003a).

Tgfb knockout mice display distinct phenotypes (Kaartinen *et al.*, 1995; Kulkarni *et al.*, 1993; Proetzel *et al.*, 1995; Sanford *et al.*, 1997; Shull *et al.*, 1992; Yang *et al.*, 2007). *Tgfb1* is expressed in numerous tissues during embryonic development and its expression is dysregulated under pathophysiological conditions in many tissues in adults (Azhar *et al.*, 2003; Kehrl *et al.*, 1986; Pelton *et al.*, 1991; Saxena *et al.*, 2008; Thompson *et al.*, 1989). *Tgfb1*^{-/-} mice develop severe multifocal inflammatory disease (Kulkarni *et al.*, 1993; Shull *et al.*, 1992), yolk sac defects (Dickson *et al.*, 1995), and colon cancer (Engle *et al.*, 1999). It is thought that TGF β 1 can act in an autocrine or paracrine/juxtacrine manner (Bommireddy and Doetschman, 2007; Longenecker *et al.*, 2002). Therefore, mouse models that could precisely delete *Tgfb1* in a cell-type or tissue-specific fashion will be a highly useful tool for determining in vivo functions of TGF β 1.

Here we have generated mice harboring a conditional knockout allele for *Tgfb1*. Targeting vectors were designed in which exon 6 of *Tgfb1* was flanked with *LoxP* sites in introns 5 and 6 to produce conditional deletion of *Tgfb1* in mice using a *Cre-LoxP* strategy (Fig. 1a). Exon 6 was chosen because we previously showed that its disruption results in TGF β 1-deficient mice (Shull *et al.*, 1992). *Tgfb1*^{+/flox-ex 6} mice were intercrossed, and the resulting *Tgfb1*^{flox-ex 6/flox-ex 6} mice were born in a normal (1:2:1) Mendelian ratio (Fig. 1e). Both male and female *Tgfb1*^{flox-ex 6/flox-ex 6} animals had normal fertility and fecundity, and their longevity was identical to that of wild type mice. Morphological and histological examination of tissues from various organs in age- and sex-matched littermates (*n* = 6) of *Tgfb1*^{flox-ex 6/flox-ex 6} mice indicated normal phenotype.

To test the ability of *Tgfb1*^{flox-ex 6/flox-ex 6} mice to produce *Tgfb1* conditional deletion and gain more specific information about the role of *Tgfb1* in T-cell-mediated immune function, these mice were intercrossed to *LckCre* transgenic (Tg) mice which have been successfully used to conditionally delete genes in T cells. The *LckCre* transgene first comes on in immature CD4⁻ CD8⁻ T cells in thymus and is later expressed in mature CD4⁺CD8⁻ and CD4⁻CD8⁺ T cells in thymus. It is expressed in T cells that have migrated to the lymphoid organs, including spleen (Orban *et al.*, 1992). PCR analysis of thymic genomic DNA from 1-month-old *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice indicated significant but incomplete conditional deletion of *Tgfb1* (Fig. 1f). Since the PCR was done on whole organ which contained both T cells and non-T cells (e.g., thymic epithelial cells), and since *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice are on a mixed genetic background (129/Black-Swiss/C57BL/6), it is possible that these factors have contributed to the partial Cre-mediated excision seen in these mice. *Tgfb1*^{flox-ex 6/flox-ex 6} mice are currently being crossed to other Cre transgenic lines to further investigate conditional *Tgfb1* ablation in other tissues. Q-RT-PCR data indicated that this partial conditional deletion of *Tgfb1* in T cells is sufficient to significantly reduce *Tgfb1* expression (Fig. 1g).

FACS analyses using markers of T cells (i.e., CD3, CD4, and CD8) indicated reduced CD3 expression, a substantial increase in percent of CD3⁺ CD8⁺ T cells, and a concomitant decrease in the percent of CD3⁺ CD4⁺ T cells in 5-month-old *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice (see Fig. 2). These data suggest that TGF β 1 is required for normal T-cell homeostasis. By using CD44 and CD62L as specific markers of T-cell activation and function, this study also revealed significant induction of T-cell activation in 5-month-old *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice (see Fig. 3). This enhanced T-cell activation is seen as early as 1 month of age when these mice

were genetically combined with a *Tgfb1*⁻ allele (*Tgfb1*^{flox-ex 6/-}/*LckCre* Tg) (not shown). Together, these data indicate that T-cell-produced TGFβ1 is required for normal activation of peripheral T cells and that juxtacrine production of TGFβ1 by other cell types is not sufficient to rescue the T-cell defect.

Tgfb1^{flox-ex 6/flox-ex 6}/*LckCre* Tg adult mice exhibit significant weight loss and decreased cellularity of thymus and spleen as compared with the control mice (see Fig. 4), indicating signs of inflammation and wasting (see Fig. 4). Histological examination of tissues from various organs, including lungs and salivary glands, revealed modest inflammation in 5-month-old *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice (Fig. 5a, b, d, e), which became more severe when *Tgfb1* expression was further decreased in *Tgfb1*^{flox-ex 6/-}/*LckCre* Tg mice (Fig. 5a–f). Finally, analysis of cytokine profiles of splenic T cells revealed that production of both interferon-γ (IFN-γ) and interleukin-2 (IL-2) was significantly increased in *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice (Fig. 5f, g). TGFβ1 has been shown to be required for the maintenance of a diverse and self-tolerant T-cell repertoire and for appropriate T-cell responses necessary for an effective adaptive immune system (Li and Flavell, 2008). Complete absence of TGFβ1 in mice leads to a breakdown in T-cell tolerance, which consequently results in multiorgan inflammation, organ failure, and death at weaning age (Bommireddy and Doetschman, 2007; Shull *et al.*, 1992). Several studies have indicated that reduced TGFβ1 can cause immune disorders such as lupus (Saxena *et al.*, 2008) and inflammation-induced cancer (Engle *et al.*, 1999; Sporn, 2006). Thus, the data presented in this study is novel by indicating that T-cell-produced TGFβ1 is required for suppression of autoimmunity and inflammation.

Several lines of evidence indicate the importance of the findings presented in this study. First, TGFβ1 is produced by T cells (Thompson *et al.*, 1989) and *Tgfb1* knockout mice have activated T cells (Bommireddy *et al.*, 2003b; Robinson and Gorham, 2007), hyper-responsive immature T cells (Bommireddy *et al.*, 2003a; Marie *et al.*, 2005) and TGFβRII-deficient T cells have increased expansion of CD8⁺ T cells that results in defective T-cell homeostasis (Leveen *et al.*, 2005; Lucas *et al.*, 2000), all of which are consistent with the requirement of an autocrine role of TGFβ1 in T-cell homeostasis and activation. Secondly, endocrine expression of the active form of TGFβ1 in *Tgfb1*^{-/-} mice fails to ameliorate the lethal inflammatory phenotype (Longenecker *et al.*, 2002). Thirdly, IFN-γ is a proinflammatory cytokine which is produced by Th1-effector cells and that higher levels of INF-γ and IL-2 in *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice is in concordance with enhanced Th1-cell differentiation and severe inflammation (Bommireddy *et al.*, 2003b; Saxena *et al.*, 2008). Finally, a mouse model with a floxed *Tgfb1* allele has been recently described in which exon 1 of *Tgfb1* and exon 4 of the B9 protein domain 2 gene (*B9d2* or *LOC232987*) are both flanked with *LoxP* sites (Li *et al.*, 2007). The exon 4 of *B9d2* resides 340-bp upstream of the transcriptional start site of *Tgfb1*. The *B9d2* gene is highly expressed in thymus, and *CD4Cre*-mediated excision of floxed *Tgfb1/B9d2* alleles results in complete loss of expression of both *Tgfb1* and *B9d2*. Since it is not possible to conditionally delete *Tgfb1* using this mouse model without the accidental deletion of one or both alleles of the *B9d2* gene, *Tgfb1*^{flox-ex 6/flox-ex 6} mice provide a unique mouse model to conditionally delete *Tgfb1* in a cell or tissue-specific fashion without the accidental deletion of neighboring genes.

In conclusion, this study reports the generation of an exon 6-specific conditional knockout allele for the *Tgfb1* gene in mice. The *Tgfb1*^{flox-ex 6/flox-ex 6} mice have been tested as a proof of principle for their ability to produce *Tgfb1* conditional deletion in T cells and have also provided initial information which indicates important roles of T-cell-produced *Tgfb1* in proper in vivo regulation of peripheral T-cell activation, maintenance of T-cell homeostasis, and suppression of autoimmunity.

METHODS

Conditional Gene Targeting of *Tgfb1* Locus and Generation of Mice Carrying Floxed-*Tgfb1*^{ex 6} Allele

We used a mouse genomic library clone (129/J) to produce a targeting vector for the *Tgfb1*^{flox-ex 6} mice. The targeting strategy was designed to flank exon 6 of *Tgfb1* by two *LoxP* sites to effect the disruption of the mature peptide, while leaving the precursor pro-region and neighboring *B9d2* gene (i.e., *LOC232987*) intact. A similar approach had been successfully used to disrupt *Tgfb1* in targeting a *Neo* gene into exon 6 of the *Tgfb1* locus, thereby producing *Tgfb1*^{-/-} mice (Shull *et al.*, 1992). A 7.4-kb *ApaI*-digested genomic DNA fragment of *Tgfb1* was subcloned in a modified Bluescript plasmid (Stratagene) and used for building the targeting vector (Fig. 1a). A floxed *PMCI-Neo* gene was placed in intron 5 of *Tgfb1*. A unique *AscI* diagnostic restriction enzyme site was introduced into the upstream *LoxP* sequence of this floxed-*Neo* gene. A third *LoxP* site was added in intron 6 of *Tgfb1* and this *LoxP* sequence contained *SgfI* as a unique diagnostic restriction enzyme site. Diphtheria toxin (*PGK-DT-A*) gene was used to kill random ES cell recombinants by automatic negative selection. The *NotI*-linearized targeting vector was used for electroporation of 129-derived KG1 embryonic stem (ES) cells according to the previously published procedure (Azhar *et al.*, 2008). Southern blot analysis revealed that five out of 80 G418-resistant ES cells colonies had a correctly targeted *Tgfb1* locus (Fig. 1b). One of these clones (#17) was used to remove the *Neo* in ES cells by Cre-mediated excision of the floxed-*Neo* gene. Both southern blotting and PCR screening identified several clones in which *Neo* was completely removed (Fig. 1c). Two of these clones which contain *Tgfb1*^{flox-ex 6} allele were microinjected into C57BL/6 mouse blastocysts. Both southern blotting and PCR screening identified many chimeric mice. Germline transmission of *Tgfb1*^{flox-ex 6} allele was established by breeding the male ES cell chimeric mice to Black-Swiss females according to previously described methods (Azhar *et al.*, 2008). PCR analysis on tail clip DNA was used for genotyping mice. *Tgfb1*^{+/flox-ex 6} mice were intercrossed, and the resulting *Tgfb1*^{flox-ex 6/flox-ex 6} mice were maintained on a mixed genetic background (129 and Black-Swiss, 50:50). *Tgfb1*^{flox-ex 6/flox-ex 6/LckCre Tg mice (129/J/Black-Swiss/C57BL/6) were generated by crossing *Tgfb1*^{flox-ex 6} mice (C57BL/6) (Jax Lab, Bar harbor, ME) to *LckCre* mice. *Tgfb1*^{flox/-} mice were produced by crossing *Tgfb1*^{flox-ex 6/flox-ex 6} mice to *Tgfb1*^{+/-} (BALB/c), and breeding of *Tgfb1*^{flox/-} to *Tgfb1*^{flox-ex 6/flox-ex 6/LckCre} Tg mice resulted in *Tgfb1*^{flox-ex 6/-/LckCre} Tg mice (129/Black Swiss, C57BL/6, BALB/c). Nontransgenic littermates (*Tgfb1*^{flox-ex 6/flox-ex 6/LckCre} nTg) mice were used as control animals.}

Southern Blot, DNA Sequencing and PCR Analyses

For Southern blotting, genomic DNA from ES cells or mouse tail-clips was digested with *StuI*, *ApaI*, and *AscI/EcoRV*, resolved on 0.8% agarose gels, transferred to nylon membranes (GE Healthcare USA), hybridized to ³²P-labeled probes and autoradiographed using Biomax (USA) X-ray films. *StuI* cuts outside at the 3' end of the targeting vector. *EcoRV* cuts outside the targeting vector. *AscI* was introduced as a unique cutter in the 5' *LoxP* sequence of the floxed-*Neo* gene in the targeting vector. Probe A was used as an outside probe in hybridization with *AscI/EcoRV* genomic DNA digest to confirm the homologous recombination at both ends of the targeting vector. Probe B was an internal probe and it was used in conjunction with *StuI* in southern blot hybridization to further confirm the correct integration of the 3' end of the targeting vector. The *Neo* primers (forward, CCATT-GAACAAGATGGATTGCACGCAG; reverse, AGAAGAAC TCGTCAAGAAGGCGATAGAAGG) were used in an automated DNA sequencing of a PCR amplified product from the targeted (*Neo*, exon 6) ES cells to confirm the sequences of both *LoxP* sites in the floxed-*Neo* gene. Similarly, *Tgfb1* exon 6-specific primer (forward, CCTA

TATTTGGAGCCTGGACAC) was used to sequence the *LoxP* site situated in the intron 6 of the targeted floxed (*Neo*, exon 6) ES cells.

Southern blotting was also performed to confirm the removal of floxed-*Neo* gene from the *Tgfb1* targeted floxed (*Neo*, exon 6) allele in the targeted ES cells and to identify germline chimeras. PCR analysis was used to reconfirm the loss of the *Neo* gene in the targeted (*Tgfb1*^{flox-ex 6}) ES cells and screen the chimeric mice and their germline offspring using p1 and p2 primers. Primer p1 is located in exon 5 just outside the 5' end of the targeting vector. PCR analysis was also used for routine genotyping of mice with wild type and *Tgfb1*^{flox-ex 6} alleles (primer set p4/p5). Genotyping of *Tgfb1*^{+/+}, *Tgfb1*^{+/-} and *Tgfb1*^{-/-} animals was done as described previously (Shull *et al.*, 1992). In addition, PCR was used for detecting the Cre-mediated excision of the *Tgfb1* targeted floxed (*Neo*, exon 6) allele in ES cells and the *Tgfb1*^{flox-ex 6} allele in *Tgfb1*^{flox-ex 6}/*LckCre* Tg mice (primer set p3/p2). Efficiency of Cre-mediated excision of the *Tgfb1*^{flox-ex 6} allele was determined by densitometric analysis of floxed (ex 6) and floxed deleted (ex 6) PCR bands in PCR amplified products from the *Tgfb1*^{flox-ex 6}/*LckCre* Tg mice. Densitometry was done by using the program ONE-DSscan (Scanalytics, VA). Specific primers that were used for the PCR amplification included (all sequences in 5'-3' directions): p1 (forward), GCACCATC-CATGACATGAAC; p2 (reverse), GGAGCAGTTGTCCAA-CATGAT; p3 (forward), TACAAAGTGAGCTGGCGCGC-CATAACTT; p4 (forward), AAGAC CTGGGTTGGAAGTG; p5 (reverse), CTTCTCCGTTTC TCTGTCACCCTAT.

Quantitative Real-Time RT-PCR (Q-RT-PCR) Analysis

Total RNA from T cells was extracted by RNeasy Mini kit (Cat # 74704: Qiagen, Valencia, CA). Three *Tgfb1*^{flox-ex 6}/*LckCre* nTg and *Tgfb1*^{flox-ex 6}/*LckCre* Tg mice were assessed (each in triplicate) by Q-RT-PCR. Q-RT-PCR was performed using an Opticon 2 real-time PCR machine (Biorad, Hercules, CA) according to the published procedure (Rajan *et al.*, 2006). Primers that were used for the Q-RT-PCR amplification of *Tgfb1* included (all sequences in 5'-3' directions): *Tgfb1* forward, ATCCTGTCCAACTAAGGCTCG; *Tgfb1* reverse, ACCTCTTTAGCATAGTAGTCCGC; *b-actin* forward, GGC TGTATTCCCCTCCATCG; *b-actin* reverse, CCAGTTGG TAACAATGCCATGT.

Flow Cytometry

Isolation of thymocytes and splenocytes and cell counting, and FACS analysis were done according to the previously described methods (Bommireddy *et al.*, 2003b). Freshly prepared T cells from spleen from at least three animals per genotype were incubated with anti-CD16/32 (2.4G2) to block FcγII/III, followed by staining with fluorochrome-conjugated mAbs as indicated in figure legends. All antibodies were purchased from BD-Biosciences (USA). Stained cells were analyzed using a Becton Dickinson (Mountain View, CA) LSR or FACSCalibur flow cytometer and CellQuest software as described elsewhere (Bommireddy *et al.*, 2003b). Absolute cell numbers of each cell subset was calculated by percentage of staining cells × total cell number.

Cytokine Assay

T cells from spleen (1.5×10^6 /ml) were stimulated with Con A (Sigma, 1 μg/ml), plate-bound anti-CD3 mAb (PharMingen, 5 μg/ml), and PMA (Sigma, 25 ng/ml) plus ionomycin (Sigma, 250 ng/ml) (Bommireddy *et al.*, 2003b; Saxena *et al.*, 2008). The supernatants were collected after 72 h and tested for cytokines by ELISA using mAb pairs and recombinant standards from PharMingen. IFNγ and IL-2 were assayed by sandwich ELISA with PharMingen's paired rat anti-mouse cytokine mAbs (purified mAb for capture, biotinylated mAb for detection) according to the manufacture's protocol. These antibodies were R4 6A2 and XMG1.2 for IFNγ, and JES6-1A12 and JES6-5H4 for IL-2. Alkaline phosphatase-conjugated streptavidin

(Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was used in the procedure. Plates were developed with *p*-nitrophenyl phosphate substrate (Sigma) and OD was determined at 405 nm using Multiskan (Thermo Labsystems, Vantaa, Finland). The cytokines were quantified by reference to the standard curves derived from recombinant cytokines (PharMingen; R&D Systems, Inc.).

Histology

Transverse tissue sections (5 μ m) of paraformaldehyde-(4%) fixed and paraffin-embedded organs including lungs and salivary gland from adult *Tgfb1*^{+/+}, *Tgfb1*^{flox-ex 6/flox-ex 6}, *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* nTg, and *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg and *Tgfb1*^{flox-ex 6/-}/*LckCre* Tg mice were cut and analyzed for tissue inflammation as described earlier (Bommireddy *et al.*, 2003b). Histological examination was done on hematoxylin and Eosin (H&E)-stained serial sections. All sections were analyzed using bright-field optics with a Zeiss Axio Imager M1 microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) equipped with a AxioVision 4.6.3 imaging software.

Statistical Analysis

Microsoft Excel was used for managing the data. Findings are reported as means $\pm$ SD of the mean, and two-tailed Student's *t*-test (SigmaPlot, Systat Software, Inc., Richmond, CA) was used for comparing groups. *P*-values were calculated, and a *P* < 0.05 was considered significant.

Acknowledgments

Contract grant sponsor: National Institutes of Health Grant, Contract grant numbers: CA084291, AI067903; Contract grant sponsor: Arizona Biomedical Research Commission, Contract grant number: ABRC #0901; Contract grant sponsors: Steven M. Gootter Foundation; BIO5 Institute of the University of Arizona

We thank Dillihan Gumus, Sandy Schwemberger, Fauzia Nazir, Nabeel Al Moamen, and Dr. Carlos Hidalgo for providing technical assistance.

LITERATURE CITED

- Azhar M, Schultz JE, Grupp I, Dorn GW, Meneton P, Molin DG, Gitten-berger-de Groot AC, Doetschman T. Transforming growth factor beta in cardiovascular development and function. *Cytokine Growth Factor Rev* 2003;14:391–407. [PubMed: 12948523]
- Azhar M, Yin M, Zhou M, Li H, Mustafa M, Nusayr E, Keenan JB, Chen H, Pawlosky S, Gard C, Grisham C, Sanford LP, Doetschman T. Gene targeted ablation of high molecular weight fibroblast growth factor-2. *Dev Dyn* 2008;238:351–357. [PubMed: 19105223]
- Bommireddy R, Doetschman T. TGFbeta1 and T(reg) cells: Alliance for tolerance. *Trends Mol Med* 2007;13:492–501. [PubMed: 17977791]
- Bommireddy R, Ormsby I, Yin M, Boivin GP, Babcock GF, Doetschman T. TGFbeta1 inhibits Ca²⁺-Calcineurin-mediated activation in thymocytes. *J Immunol* 2003a;170:3645–3652. [PubMed: 12646629]
- Bommireddy R, Saxena V, Ormsby I, Yin M, Boivin GP, Babcock GF, Singh RR, Doetschman T. TGF-beta1 regulates lymphocyte homeostasis by preventing activation and subsequent apoptosis of peripheral lymphocytes. *J Immunol* 2003b;170:4612–4622. [PubMed: 12707339]
- Derynck R, Akhurst RJ. Differentiation plasticity regulated by TGF-beta family proteins in development and disease. *Nat Cell Biol* 2007;9:1000–1004. [PubMed: 17762890]
- Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. *Development* 1995;121:1845–1854. [PubMed: 7600998]

- Engle SJ, Hoying JB, Boivin GP, Ormsby I, Gartside PS, Doetschman T. Transforming growth factor beta1 suppresses nonmetastatic colon cancer at an early stage of tumorigenesis. *Cancer Res* 1999;59:3379–3386. [PubMed: 10416598]
- Kaartinen V, Voncken JW, Shuler C, Warburton D, Bu D, Heisterkamp N, Groffen J. Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat Genet* 1995;11:415–421. [PubMed: 7493022]
- Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, Sporn MB, Fauci AS. Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J Exp Med* 1986;163:1037–1050. [PubMed: 2871125]
- Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. *Proc Natl Acad Sci USA* 1993;90:770–774. [PubMed: 8421714]
- Leveen P, Carlsen M, White-Makowska A, Oddsson S, Larsson J, Goumans MJ, Cilio CM, Karlsson S. TGF- β type II receptor deficient thymocytes develop normally but demonstrate increased CD81 proliferation in vivo. *Blood* 2005;106:4234–4240. [PubMed: 16131565]
- Li MO, Flavell RA. TGF-beta: A master of all T cell trades. *Cell* 2008;134:392–404. [PubMed: 18692464]
- Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* 2007;26:579–591. [PubMed: 17481928]
- Longenecker G, Thyagarajan T, Nagineni CN, Flanders KC, Factor V, Miller G, Ward JM, Nalca A, Rangnekar VM, Thorgeirsson S, Kulkarni AB. Endocrine expression of the active form of TGF-beta1 in the TGF-beta1 null mice fails to ameliorate lethal phenotype. *Cytokine* 2002;18:43–50. [PubMed: 12090759]
- Lucas PJ, Kim SJ, Melby SJ, Gress RE. Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. *J Exp Med* 2000;191:1187–1196. [PubMed: 10748236]
- Marie JC, Letterio JJ, Gavin M, Rudensky AY. TGF- β 1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. *J Exp Med* 2005;201:1061–1067. [PubMed: 15809351]
- Massague J, Gomis RR. The logic of TGFbeta signaling. *FEBS Lett* 2006;580:2811–2820. [PubMed: 16678165]
- McLennan IS, Koishi K. The transforming growth factor-betas: Multifaceted regulators of the development and maintenance of skeletal muscles, motoneurons and Schwann cells. *Int J Dev Biol* 2002;46:559–567. [PubMed: 12141444]
- Millan FA, Denhez F, Kondaiah P, Akhurst RJ. Embryonic gene expression patterns of TGF beta 1, beta 2 and beta 3 suggest different developmental functions in vivo. *Development* 1991;111:131–143. [PubMed: 1707784]
- Orban PC, Chui D, Marth JD. Tissue- and site-specific DNA recombination in transgenic mice. *Proc Natl Acad Sci USA* 1992;89:6861–6865. [PubMed: 1495975]
- Pelton RW, Saxena B, Jones M, Moses HL, Gold LI. Immunohisto-chemical localization of TGF beta 1, TGF beta 2, and TGF beta 3 in the mouse embryo: Expression patterns suggest multiple roles during embryonic development. *J Cell Biol* 1991;115:1091–1105. [PubMed: 1955457]
- Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN, Ding J, Ferguson MW, Doetschman T. Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* 1995;11:409–414. [PubMed: 7493021]
- Rajan S, Williams SS, Jagatheesan G, Ahmed RP, Fuller-Bicer G, Schwartz A, Aronow BJ, Wieczorek DF. Microarray analysis of gene expression during early stages of mild and severe cardiac hypertrophy. *Physiol Genom* 2006;27:309–317.
- Robinson RT, Gorham JD. TGF-beta 1 regulates antigen-specific CD41 T cell responses in the periphery. *J Immunol* 2007;179:71–79. [PubMed: 17579023]
- Sanford LP, Ormsby I, Gittenberger-de GA, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 1997;124:2659–2670. [PubMed: 9217007]

- Saxena V, Lienesch DW, Zhou M, Bommireddy R, Azhar M, Doetschman T, Singh RR. Dual roles of immunoregulatory cytokine TGF- β in the pathogenesis of autoimmunity-mediated organ damage. *J Immunol* 2008;180:1903–1912. [PubMed: 18209088]
- Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, Doetschman T. Targeted disruption of the mouse transforming growth factor- β 1 gene results in multifocal inflammatory disease. *Nature* 1992;359:693–699. [PubMed: 1436033]
- Sporn MB. The early history of TGF- β , and a brief glimpse of its future. *Cytokine Growth Factor Rev* 2006;17:3–7. [PubMed: 16290110]
- Thompson NL, Flanders KC, Smith JM, Ellingsworth LR, Roberts AB, Sporn MB. Expression of transforming growth factor- β 1 in specific cells and tissues of adult and neonatal mice. *J Cell Biol* 1989;108:661–669. [PubMed: 2645303]
- Yang Z, Mu Z, Dabovic B, Jurukovski V, Yu D, Sung J, Xiong X, Munger JS. Absence of integrin-mediated TGF β 1 activation in vivo recapitulates the phenotype of TGF β 1-null mice. *J Cell Biol* 2007;176:787–793. [PubMed: 17353357]

FIG. 1.

Generation of *Tgfb1*^{flox-ex 6} mice. **a:** A Cre-*LoxP* based conditional gene targeting scheme depicting the relevant piece of *Tgfb1* genomic locus, the *Tgfb1*^{flox-ex 6} targeting vector and the *Tgfb1*^{flox-ex 6} targeted and deleted locus. PCR primers and southern blot probes are indicated. E, EcoRV; St, StuI; A, ApaI; S, SacI; As, AscI; Sg, SgfI. **b:** Southern blot screening. StuI cuts both outside and inside and EcoRV cuts only outside at both ends of the targeting vector. AscI and SgfI are only present in the *LoxP*. Probe A is an outside side probe. Only two (clone #1 and #17) of the five originally targeted clones are shown. StuI, ApaI, and AscI/EcoRV blots confirm the presence of a targeted floxed (exon 6, neo) allele. Positive clones were identified by 3.0 kb (StuI blot), 8.9 kb (ApaI blot), and 10.1 kb (AscI/EcoRV blot). A positive targeted

floxed (exon 6, neo) clone (#17) was treated with Cre recombinase and the floxed-*neo* was deleted in these ES cells. **c:** Long PCR shows the presence of an expected 8.9-kb band before the Cre-excision and a 7.8-kb band after the Cre-mediated excision in #17 clone, indicating a successful removal of floxed-*neo* from the targeted floxed (exon 6) allele in ES cells. **d:** ApaI blot confirms the presence of *Tgfb1*^{flox-ex 6} allele in germline chimera. Wild type (+/+) mice and targeted floxed (exon 6, neo) ES cells are used as controls. **e:** PCR genotyping of wild type, *Tgfb1* floxed heterozygous (+/flox (exon 6)) and *Tgfb1* floxed homozygous (flox-ex 6/flox-ex 6) mice. **f:** PCR amplification indicating partial but specific Cre-mediated excision in thymus of *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice. PCR primer p3 is a hybrid primer with sequences from both the *Tgfb1* and *LoxP* and detects specifically *Tgfb1*^{flox-ex 6} allele but not the wild-type allele under conditions described in the Materials and Methods. There is no floxed deleted allele (1.23 kb) in control mice which is seen in *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice. Note that no deletion product(s) at the *Tgfb1* locus in liver, heart, lung, colon and brain tissue of *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* nTg or *Tgfb1*^{+/+}/*LckCre* Tg mice was found (not shown), indicating that Cre-mediated excision occurred only in the thymus of *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice. **g:** Q-RT-PCR analysis showing significantly decreased *Tgfb1* expression (**P* = 3.5747e-3) in T cells from *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice as compared to the control non-transgenic mice. Each control value is normalized to 1.0.

FIG. 2.

T cell-specific deletion of *Tgfb1* affects T cell homeostasis in *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice. Splenocytes are prepared from 5-month-old control and *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice and immunostained for surface expression of CD4, CD8, and CD3 and analyzed by flow cytometry. **a:** CD3 expression and percent of CD3⁺ splenocytes. Both the mean fluorescence intensity (MFI) and the percent of CD3⁺-T cells are decreased in *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice. **b:** Analysis of CD4⁺ and CD8⁺ cells. **c:** CD3⁺-gated splenocytes indicating distribution of CD4⁺ and CD8⁺ T cells. The % of cells positive for each marker is shown in the quadrants in (a, b). The % of CD4⁺ T cells is reduced and percent of CD8⁺ T cells is increased in both non-gated and CD3-gated splenocytes. **d:** CD8 expression

and percent of CD8⁺ splenocytes. The percent of CD8⁺-T cells is increased but their MFI is slightly reduced in *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice.

FIG. 3.

T cell-specific deletion of *Tgfb1* results in increased T cell activation in *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice. Splenocytes from 5-month-old control and *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice are analyzed for CD4, CD8, CD44 and CD62L by flow cytometry. Expression of CD44 and CD62L is analyzed on CD4⁺ or CD8⁺ T cells by gating on these subsets. The percent of cells positive for each marker is shown in the quadrants. **a:** FACS analyses showing an increased CD44 and reduced CD62L on CD4⁺ cells in *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice. **b:** CD44 and CD62L expression on CD8⁺ T cells. CD44 expression is increased, whereas CD62L expression is reduced on CD8⁺ T cells in *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice.

FIG. 4.

Analysis of body weight and thymus and spleen weight and number of thymocytes and splenocytes in $Tgfb1^{flox-ex\ 6/flox-ex\ 6}/LckCre\ Tg$ mice. **a:** Body weight at 5 month. **b:** Thymic weight at 2 month. **c:** Spleen weight at 2 month. **d:** Number of thymocytes at 1 month. **e:** Number of splenocytes at 5 months. $Tgfb1^{flox-ex\ 6/flox-ex\ 6}/LckCre\ Tg$ mice exhibit significant reduction in body weight ($n = 4$, $*P = 8.9415e-4$) and thymic ($n = 3$, $*P = 0.0166$) and splenic weight ($n = 4$ for control and $n = 5$ for $Tgfb1^{flox-ex\ 6}/LckCre\ Tg$, $*P = 0.0165$), and number of total thymocytes ($n = 3$, $*P = 2.4698e-3$) and splenocytes ($n = 3$, $*P = 0.0236$).

FIG. 5.

T cell-specific deletion of *Tgfb1* results in increased organ inflammation and Th1-effector cytokine production. **a–f**: Hematoxylin and eosin staining of serial sections of lung (a–c) and salivary gland (d–f) from 5-month-old *Tgfb1*^{flox-ex 6/flox-ex 6} control (a, d), *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg (b, e) and *Tgfb1*^{flox-ex 6/-}/*LckCre* Tg (c, f) mice. These histopathology data are representative results of at least three mice per genotype analyzed. All photomicrographs are taken at equal magnification. *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice exhibit moderate inflammation in both the lungs (arrows in b) and the salivary gland (arrows in e) which becomes severe in both organs (arrows in c, f) in *Tgfb1*^{flox-ex 6/-}/*LckCre* Tg mice. **g**: INF- γ production. **h**: IL-2 production. Splenic T cells (1.5×10^6 /ml) from 2-month-old control and

Tgfb1^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice are stimulated for 72 h with T cell receptor-dependent (plate-bound anti-CD3 mAb (5 µg/ml) and Con A (1 µg/ml)) and receptor-independent (PMA (25 ng/ml) plus ionomycin (250 ng/ml)) mitogens. The cytokines in the culture supernatants are assayed using ELISA as described in the Materials and Methods section. The mitogen-stimulated T cells of *Tgfb1*^{flox-ex 6/flox-ex 6}/*LckCre* Tg mice exhibit significant increase in the levels (mean ±SD) of IFN γ (G) (**P* < 0.0001 for anti-CD3, **P* < 0.0001 for Con A, **P* = 0.0017 for PMA+ionomycin; and IL-2 (H) (**P* < 0.0001 for anti-CD3, **P* < 0.0001 for Con A, **P* < 0.0001 (PMA+ionomycin).