Role of TGF-β3 in the regulation of immune responses

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ABSTRACT
Transforming growth factor-betas (TGF-βs) are multifunctional cytokines that have been implicated in the regulation of a broad range of biological processes, including cell proliferation, cell survival, and cell differentiation. The three isoforms identified in mammals, TGF-β1, TGF-β2, and TGF-β3, have high sequence homology, bind to the same receptors, and show similar biological functions in many in vitro studies. However, analysis of the in vivo functions of the three isoforms and mice deficient for each cytokine reveals striking differences, illustrating their unique biological importance and functional non-redundancy. Although increasing evidence suggests that TGF-β1 and, to a lesser extent, TGF-β2 play an integral role in maintaining immune tolerance, the immunological role of TGF-β3 has not been carefully evaluated. Recent studies have focused on the multifunctional role of TGF-β3. In this review, we provide an overview of the role of TGF-β3 in immunity, with comparison to TGF-β1 and -β2.

Introduction
Three structurally similar isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) have been identified in humans (1). TGF-β1 was first cloned from a human cDNA library (2) and human TGF-β2 and TGF-β3 cDNA were subsequently cloned (3, 4). TGF-β1 is highly homologous to the TGF-β2 and -β3 isoforms (5). TGF-β2 and -β3 share 71% (β2 vs. β1) and 76% (β3 vs. β1) sequence identity to TGF-β1 (6). Although TGF-β4 and -β5 were identified from cDNA libraries of chicken chondrocyte and frog oocyte, respectively (6, 7), mammalian TGF-β4 and -β5 have not yet been described. In vitro experiments indicated that TGF-β1, -β2, and -β3 often exhibit similar biological activities (8-11). Despite this high sequence homology, analysis of the in vivo functions of the three isoforms reveals marked differences, indicating their non-redundancy (12-16). The TGF-βs have opposite effects on tissue fibrosis. Wound-healing experiments revealed that TGF-β1 and TGF-β2 cause fibrotic scarring responses and that TGF-β3 induces a scar-free response (17). Both TGF-β1 and -β2 activate the collagen α2 (I) gene promoter, resulting in increased collagen synthesis (18). It was also reported that TGF-β1, but not TGF-β3, is a crucial factor in the development of pulmonary fibrosis (19). Most of the information on the immunological activity of TGF-βs derives from studies of TGF-β1 and, in part, TGF-β2, whereas recent investigations have begun to illuminate the importance of TGF-β3 in the immune system. In this review, we focus on recent novel insights into the immunological role of TGF-β3, with comparison to TGF-β1 and -β2.

TGF-β family synthesis
The three isoforms of TGF-β each utilise a unique activation mechanism from their latent forms (20). TGF-βs are initially synthesised as larger precursor polypeptides containing a hydrophobic signal sequence, latency-associated peptide (LAP), and mature peptide (pre-pro-TGF-β). Pre-pro-TGF-β monomers dimerise through disulphide bridges between cysteine residues in LAP and cysteine residues in the mature peptide (pro-TGF-β), and are cleaved by furin convertase but remain non-covalently associated with the mature peptide. LAP confers latency to the mature peptide by shielding the receptor-binding region of active TGF-β (21). Similar latent complexes have not yet been reported for other members of the TGF-β superfamily.

The activity of secreted TGF-β is primarily regulated by the conversion of its latent form to its active form. Although tissues contain significant quantities of latent TGF-β, the activation of only a small fraction of this latent TGF-β exerts maximal effects on...
target cells. In lymphoid tissues, LAP is thought to be removed by thrombospondin, plasmin, and acidification during the inflammatory process (22). The TGF-β-LAP complex, called the small latent complex (SLC), is further bound to latent TGF-β-binding protein (LTBP). There are four isoforms of LTBP in humans (LTBP-1, -2, -3, and -4). LTBP-2 is unique in the LTBP family as it is the only isoform that does not bind to latent TGF-β (23). TGF-β-LAP-LTBP is termed the large latent complex (LLC). The LTBP is not necessary for latency (24), but it does play a critical role in the assembly and secretion of TGF-β (25), as well as targeting the LLC for storage through interactions with fibronectin and fibrillin in the extracellular matrix (ECM) through non-covalent interactions (26). LTBPs covalently bind to the LAP portion of SLC through the third eight-cysteine type domain of LTBP. The third eight-cysteine type domain of LTBP-1 and LTBP-3 can associate efficiently with pro-TGF-β1, -β2, and -β3. Conversely, LTBP-4 binds to TGF-β1-LAP more weakly than the other two LTBPs, indicating that LTBP-1 and LTBP-3 might be the primary proteins responsible for binding to SLC (27). The C-termini of LTBP-1, -2, and -4 bind to a similar region in the N-terminus of fibrillin-1 and -2 through non-covalent interactions, whereas LTBP-3 reportedly does not (28). However, LTBP-3 co-localises with fibrillin fibres in tissues, and loss of fibrillin-1 prevents the incorporation of LTBP-3 into a matrix, indicating that LTBP-3 association with the matrix depends on fibrillin-1 microfibrils (29). As for secretion of LTBPs from cells, LTBP-1 and LTBP-4 do not require binding to SLC, whereas LTBP-3 is secreted only as LLC (30, 31).

TGF-β1-LAP and TGF-β3-LAP, but not TGF-β2-LAP, contain an Arg-Gly-Asp (RGD) integrin-binding motif, and several RGD-binding integrins, such as αvβ3, are able to activate latent TGF-β. Thus, the expression of αvβ3 and αvβ6 integrins is thought to be crucial for the activation of TGF-β1 and -β3. The expression of αvβ6 integrin is restricted to a subset of epithelial cells and is not expressed on immune cells, whereas αvβ6 integrin is expressed on most leukocytes. Leucocyte-specific conditional deletion of Itgb6 (which encodes integrin β6) causes severe inflammatory bowel disease and the development of high levels of auto-antibodies against double-stranded DNA (32). These findings indicate the importance of αvβ6 integrin-mediated TGF-β activation by lymphoid cells for preventing the immune dysfunction that results in inflammatory bowel disease and autoimmunity. The expression of αvβ6 integrin on dendritic cells and activated CD4+CD25+Foxp3+ regulatory T cells (Tregs) (32, 33) might play a key role in promoting immune regulation by activating TGF-β1. Consistent with these reports, Itgb6-deficient mice show perinatal lethality with profound defects in vascular development, particularly in the brain (34), and a similar phenotype is also seen in mice with mutation in Tgfb1 or deletion of the Tgfb1 gene (35).

TGF-β family receptors and signal transduction

TGF-βs are secreted as latent proteins. After activation, TGF-βs interact with the same receptor heterodimers, TGF-β receptor I (TGFBR1/ALK-5) and TGF-β receptor II (TGFBR2), to activate the canonical Smad2/3 signalling pathway. However, TGF-β2 requires binding of TGF-β receptor III (TGFBR3) prior to binding TGFBR2 (36). TGF-β3 transmits signals biologically similar to and more potently than those transmitted by TGF-β1 and -β2, depending on the cell type and assay used (10, 37-39). Phosphorylated Smad2 and Smad3 form heteromeric complexes with Smad4 and translocate to the nucleus where they activate target gene transcription. The function of TGF-β3 in palate fusion is mediated by Smad2, since the overexpression of a Smad2 transgene in TGF-β3-deficient mice rescues the cleft palate phenotype in TGF-β3-deficient mice (40). Intriguingly, TGF-β3 can also activate ALK-1, but only in the presence of ALK5 (41). ALK-1 was first identified as a receptor for BMP-9 and BMP-10, and was shown to mediate downstream signals associated with Smad1/5/8 in endothelial cells (42). TGF-β3 induces the activation of TGF-β receptor II kinase and phosphorylates ALK5, which recruits ALK1 into a TGF-β receptor complex. ALK5 kinase activity is required for optimal ALK1 activation. Activated ALK1 and ALK5 induce the phosphorylation of Smad1/5 and Smad2/3, respectively, whereas ALK1 directly inhibits ALK5/Smad3 signalling (41). Intriguingly, endoglin, a co-receptor for TGF-β1 and TGF-β3, functions as a modulator of the balance between TGF-β/ALK1 and TGF-β/ALK5 signalling. Endoglin, which stimulates TGF-β1/ALK1 signalling and indirectly inhibits TGF-β/ALK5 signalling, regulates endothelial cell proliferation (43).

These findings suggest that the ratio between ALK1 and ALK5 signalling may provide one explanation for the bifunctionality of the TGF-βs. Smad-independent non-canonical pathways mediated by TGF-β have also been reported. TGF-β1 induces profibrotic signalling by activating tyrosine-protein kinase c-Abl, which is a mediator for fibrotic responses (44). Several studies have suggested other possible non-canonical pathways, such as mitogen-activated protein kinases (MAPK), phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K), AKT, and the NF-κB pathway (45). More extensive studies of TGF-β signal transduction in immune cells are needed to elucidate the specific role of each TGF-β isoform in the immune system.

TGF-β-deficient animals

TGF-β1-deficient mice die by 4 weeks of age with massive infiltrations of lymphocytes and macrophages resulting in multi-organ inflammation, including the lungs (vasculitis, perivascular cuffing, and interstitial pneumonia) and heart (endocarditis and myocarditis) (46, 47). The progressive inflammatory process is accompanied by several autoimmune manifestations, such as production of antibodies to nuclear antigens (single-stranded DNA, double-stranded DNA, Sm, and RNP), immune complex deposition, and increased expression of both major histocompatibility complex (MHC) class I and class II antigens (48, 49).
perinatal mortality and show cardiac, lung, craniofacial, limb, spinal column, eye, inner ear, and urogenital defects. Around 23% of TGF-β2 mutant embryos on the 129 background, but not C57, displayed cleft palate (50). TGF-β3-deficient mice survive throughout the prenatal period, only to die within 20 hr of birth due to defective palatogenesis and delayed pulmonary development (51, 52). Although TGF-β2 and TGF-β3-deficient mice both develop cleft palates, the structural features of cleft palate are quite different between the mutants (50). Thus, there is little phenotypic overlap between TGF-β1-, β2- and β3-deficient mice, indicating non-compensated functions between the three isoforms of TGF-β. However, vascular pathology is a common feature of TGF-β1-, β2 and β3 deficiency. Around 50% of TGF-β1-deficient mice show prenatal lethality due to impaired development of the yolk sac vasculature (53). TGFβ2 and TGFβ3 mutations are both associated with aortic aneurysms and dissections in humans (54, 55). Intriguingly, a knock-in mouse in which the coding region of exon1 of the Tgb3 gene replaced the full-length Tgfb1 cDNA display a significant, but not complete, improvement in the cleft palate phenotype when compared to TGF-β3-deficient mice, indicating that an isoform-specific role for TGF-β3 in the palatal epithelium during palate formation can be at least partially compensated by TGF-β1 (56). One can argue that mice with TGF-β2 and -β3 deletion do not survive long enough to develop autoimmune disease due to their perinatal mortality. The neonatal mortality in TGF-β2- and -β3-deficient mice make it difficult to investigate the significance of these two TGF-β isoforms in the immune system. Hence, more detailed studies with conditional TGFβ2 and β3 deletion in immune cells are needed.

Pro-inflammatory role of TGF-β3

It is recognised that TGF-β is a multi-functional cytokine. Of particular interest, TGF-β1 is known to play a crucial role in immune responses. TGF-β1 has been shown to induce a distinct lineage of pro-inflammatory interleukin (IL-17)-producing CD4+ T cells, known as Th17 cells. The differentiation of Th17 cells in mice is initiated synergistically by TGF-β1 and IL-6 (57-59). Th17 cells induced by TGF-β1 plus IL-6 do not readily induce autoimmune disease unless they are further exposed to IL-23 (60). TGF-β1 is able to stimulate immunoglobulin A (IgA) production by lipopolysaccharide (LPS)-stimulated B cell (61). In humans, TGF-β1 and IL-21, produced by follicular helper T (TFH) cells, synergistically promote naïve B cell proliferation and differentiation to IgA-producing plasmablasts (62). The combination of IL-21 and TGF-β1 up-regulates the expression of CCR10, a common mucosal homing receptor, which mediates migration to and retention in the germinal centers, on B cells. Although a high serum concentration of TGF-β1 was found in patients with SLE, it did not correlate with Systemic lupus erythematosus (SLE) disease activity (63), and the role of TGF-β1 in lupus pathogenesis has yet to be elucidated.

In mammals, TGF-β1 is predominantly expressed by haematopoietic cells, such as CD4+CD25+ Tregs, TFH cells, dendritic cells, and B cells, whereas TGF-β2 and TGF-β3 are present in negligible amounts and have been thought to play an insignificant role in the immune system (64-66). In humans, TGF-β1 is the most abundant circulating isoform in plasma (67). However, several recent lines of evidence suggest a role for TGF-β3 in the pathogenesis of autoimmune diseases. In lymphoid tissue, TGF-β3 is expressed in CD4+ T cells, CD8+ T cells, γδ T cells and B cells, but not in myeloid cells (CD11b+ and/or CD11c+ cells). Kuchroo and colleagues reported that TGF-β3 is a key molecule expressed by pathogenic Th17 cells (68). Acute ablation of TGF-β3-expressing cells in MOG-immunised mice led to selective loss of IL-17+ cells with no obvious change in IFN-γ+ cells. TGF-β3-induced Th17 cells highly express Il22 and Il23r mRNA compared to TGF-β1-induced Th17 cells. The adoptive transfer of TGF-β3-induced Th17 cells, but not TGF-β1-induced Th17 cells, induced severe EAE. Intriguingly, in contrast to TGF-β1-induced Th17 cells, TGF-β3-induced Th17 cells induced higher expression of Smad1/5 and lower expression of Smad2/3. T-bet might have a crucial role for the induction of TGF-β3-induced Th17 cells, because Th17 cells from T-bet-deficient mice failed to induce severe EAE due to lower TGF-β3 expression, and exogenous administration of TGF-β3 overcomes the T-bet requirement for the induction of pathogenic Th17 cells. However, the mechanism for inducing TGF-β3 by T-bet remains to be elucidated. Chikuma et al. also reported that mice with T cell-specific deficiency in TRIM28 show derepression of TGF-β3, especially the TGF-β3 isoform, which contributed to the development and accumulation of autoreactive Th17 cells (69). These findings indicate a pro-inflammatory role for TGF-β3 in some inflammatory conditions.

Anti-inflammatory role of TGF-β3

Deletion of Tgfr2 in various leukocyte subsets clarified the immunoregulatory roles of the TGF-βs (70), and many studies have revealed immunoregulatory roles for TGF-β1. Naïve CD4+ T cells can differentiate into Th17 cells when TGF-β1 synergises with IL-6, while TGF-β1 induces CD4+CD25+Foxp3+ Treg, which is an anti-inflammatory subset of CD4+ T cells in humans and animals (71). It should be noted that TGF-β1 negatively regulates the differentiation of Th1 and Th2 cells by blocking the induction of T-bet and GATA3, respectively (72, 73). Systemic administration of TGF-β1 suppressed experimental autoimmune encephalitis (EAE) in mice (74) and the acute and chronic phases of streptococcal cell wall (SCW)-induced arthritis in rats (75). TGF-β1 potently inhibits both proliferation and Ig production by Staphylococcus aureus-activated human B cells (76). TGF-β1 suppresses B cells by inhibiting both the synthesis of Ig mRNA and the switch from the membrane form to the secreted forms of μ and γ mRNA (77). Consistent with these studies, the addition of TGF-β1 neutralising antibody partially abrogated the CD4+CD25+CD69 Treg-mediated suppression of B cell antibody production in vitro (78).
Unlike TGF-β1, the studies referred to above did not investigate the suppressive activity of TGF-β3. There are few studies examining the distinct expression of TGF-β3 in immune cells at the protein level. However, indirect evidence suggested that TGF-β3 plays an important role in immune regulation. In the mouse experimental autoimmune encephalomyelitis (EAE) model, the induction of EAE was associated with high expression of Tgfb1 mRNA and low expression of Tgfb3 mRNA in spinal cord tissue. The treatment of EAE with 17β-estradiol elevated the expression of TGF-β3, but lowered the expression of TGF-β1 (79). TGF-β2 expression was not altered by EAE induction or treatment. The inverse relationship between TGF-β1 and TGF-β3 indicates that the two TGF-β isoforms play opposing roles in the control of EAE.

We recently reported that CD4+CD25+LAG3+ Tregs (LAG3+ Treg) regulate humoral immunity and lupus disease in MRL-Fas+/- mice via TGF-β3 production (11). LAG3+ Treg characteristically express the transcriptional factor early growth response gene 2 (Egr2), which is required for T cell anergy induction (80), and were first identified as Foxp3-negative induced Treg that produced high amounts of IL-10 (81). Egr2 mediates IL-27-induced IL-10 production in CD4+ T cells via signal transducer and activator of transcription 3 (STAT3) (82). Ectopic expression of Egr2 on naïve CD4+ T cells induces not only LAG3 and B-lymphocyte-induced maturation protein-1 (Blimp-1) expression, but also IL-10 production, and confers in vivo suppressive activity (81), indicating that Egr2 plays a major role in the suppressive activity of LAG3+ Tregs. Subsequently, Gagliani et al. reported that co-expression of LAG3 and CD49b is specific for IL-10-producing type 1 T regulatory (Tr1) cells (83). Thus, LAG3 is considered to be one of the phenotypic markers of IL-10-producing Foxp3-independent CD4+ Tregs. We and our collaborators have shown that polymorphisms in EGR2 influence SLE susceptibility in humans (84). SLE is regarded as a prototypic autoimmune disease, in which autoimmune inflammation is responsible for multiple organ damage, and autoantibodies play a pivotal role in triggering the inflammation. It was also reported that lymphocyte-specific Egr2 conditional knockout mice develop a lupus-like autoimmune disease (85). We have recently revealed that Egr2-expressing wild type (WT) LAG3+ Tregs repress excessive humoral immunity in T cell-specific Egr2 conditional knockout mice (11). Intriguingly, LAG3+ Tregs, but not CD4+CD25+ Tregs, produce enormous amounts of TGF-β3 in an Egr2- and Fas-dependent manner. TGF-β3 effectively suppresses both B cell proliferation and antibody production by inhibiting several important pathways for B cell function, such as STAT6, Syk, and NF-κB p65. In lupus-prone MRL-Fas+/- mice with a Fas mutation, adoptive transfer of LAG3+ Treg from Fas-sufficient MRL-Fas+ mice effectively suppressed the progression of lupus in a TGF-β3-dependent manner. TGF-β3-mediated B cell suppression requires programmed cell death-1 (PD-1) expression on B cells. PD-1 is an immunoinhibitory receptor of the CD28/B7 family, and polymorphism of the PDCD1 gene is associated with a higher incidence of SLE (86). Intriguingly, PD-1, the ligand for PD-1, is highly expressed on the cell surface of LAG3+ Treg in the steady state (11). These findings revealed the previously unknown cellular and molecular basis of TGF-β3 for the physiological control of humoral immunity.

Summary and perspective

TGF-β isoforms have bi-functional roles in the immune system, whereby they regulate both pro-inflammatory and anti-inflammatory activities. The biological activity of the three isoforms of TGF-β is similar in most in vitro experiments (10), whereas accumulating evidence demonstrates differences in their in vivo capabilities. However, we should be cautious when evaluating the roles of TGF-β isoforms in the immune system. It has been reported that the levels of Tgfb1 mRNA do not correlate with the distribution of TGF-β1 protein as measured by immunohistochemical staining (87). Membrane-bound TGF-β1 is important for the suppressive activity of CD4+CD25+ Tregs in spite of the fact that Tgfb1 mRNA is not elevated in CD4+CD25+ Tregs (88, 89). These findings highlight the importance of evaluating TGF-β isoforms at the protein level. However, most of previous studies have examined TGF-β3 expression only at the mRNA level. Some studies have illustrated the importance of the concentration of TGF-β1 in regulating regulatory and pro-inflammatory T cells. TGF-β1 can induce both Treg and Th17 cell development, depending on whether pro-inflammatory cytokines such as IL-6 and IL-23 are present (58, 90). Further, the effect of the TGF-β family is affected by their concentration. Lower concentrations of TGF-β1 synergise with IL-6 and IL-21 to promote the initial differentiation of Th17 cells. Higher concentrations of TGF-β1 suppress Th17 cell differentiation by inducing Foxp3-mediated ROR-γt antagonism and repressing IL-23R expression (91, 92). The concentration of TGF-β1 also affects its ability to directly suppress B cells. Although TGF-β1 (1.0 ng/ml) selectively stimulates IgG2b secretion from LPS-stimulated B cells, higher concentrations of TGF-β1 typically suppress all Ig isotypes (93). These physiological complexities make it difficult to assess the isotype-specific roles of each TGF-β isoform in the immune system.

Of special interest are the possible side effects of TGF-β treatment. High concentrations of TGF-β1 may induce undesirable side effects. Systemic administration of TGF-β1 at >20 μg/day, but not 5 μg/day (75), increases the incidence of anaemia and thrombocytopenia in mice (94). These effects are transient and completely reversed two weeks after cessation of treatment. Other possible side effects of TGF-β1 treatment are fibrosis (17, 19), obesity, and impaired glucose tolerance (95). Intriguingly, TGF-β3 not only induces scar-free healing (17), but also improves glucose tolerance and phenotypic changes in adipocyte morphology (96), suggesting the potential therapeutic advantages of TGF-β3. Further
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References


50. SANFORD L, ORMSBY I, GARRETT N et al.: TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development* 1997; 124: 2659-70.


57. VELDOHEN M, HOCKING RJ, ATKINS CJ, LOCKSLEY RM, STOCKINGER B: TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006; 24: 179-89.


74. THOMPSON NL, FLANDERS KC, SMITH JM, ELLINGWORTH LR, ROBERTS AB, SPORN MB: Expression of transforming growth fac-