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TGF- β : a mobile purveyor of immune privilege

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Summary: Functionally barricaded immune responses or sites of immune privilege are no longer considered dependent on specific anatomical considerations, but rather, they can develop in any location where immunoregulatory cells congregate and express or release products capable of deviating the host response to foreign antigens. Among the pivotal molecules involved in orchestrating these ectopic sites of immune suppression is transforming growth factor- β (TGF- β), a secreted and cell-associated polypeptide with a multiplicity of actions in innate and adaptive immunity. While beneficial in initiating and controlling immune responses and maintaining immune homeostasis, immunosuppressive pathways mediated by TGF- β may obscure immune surveillance mechanisms, resulting in failure to recognize or respond adequately to self, foreign, or tumor-associated antigens. CD4⁺CD25⁺Foxp3⁺ regulatory T cells represent a dominant purveyor of TGF- β -mediated suppression and are found in infiltrating tumors and other sites of immune privilege, where they influence CD8⁺ T cells; CD4⁺ T-helper (Th)1, Th2, and Th17 cells; natural killer cells; and cells of myeloid lineage to choreograph and/or muck up host defense. Defining the cellular sources, mechanisms of action, and networking that distinguish the dynamic establishment of localized immune privilege is vital for developing strategic approaches to diminish or to embellish these tolerogenic events for therapeutic benefit.

Keywords: immune privilege, TGF- β , macrophages, tolerance, tumor, immune surveillance

Introduction

Immune privilege, historically defined by anatomical sequestration in which access to antigen-recognition pathways and induction of a host immune response are marginalized (1), has become recognized as a much more complex and still nebulous sequence of events associated with restriction of immune-mediated inflammation and/or allograft rejection. Although these adaptations for minimizing risk of immune-mediated injury to innocent bystander cells can be afforded by unique immunological and anatomical features, such as those in the eye, brain, and gonads (2–4), the concept of immune privilege has evolved to recognize that a micromilieu of immune privilege or tolerance can develop in any locale and can be both transient and mobile. Reining in the immune response without

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compromising host anti-microbial defense is critical for the successful outcome of immune mechanisms associated with tissue repair, pregnancy, and transplantation. Nonetheless, representing obstacles to immune surveillance, sites of immune privilege that may emerge through confluence of cells, cellular factors, and architectural barriers within a tissue may also be associated with significant pathogenesis.

With the realization that non-traditional sites may become loci of immune privilege, most notably areas of tissue injury and tumor evolution (5, 6), independent of systemic considerations, efforts have focused on defining the context-dependent cellular and molecular mechanisms underlying obstruction of immune surveillance, barring evidence of anatomic compartmentalization. That sites of immune privilege could be transiently established implicated an inducible and/or a mobile basis for evoking immune suppression and tolerance. Consequently, development of immune privilege represents a dynamic process that can be sustained without dependence on anatomical features and/or lack of lymphatic drainage.

Tumors as a model of a local site of immune privilege

As a model for subversion of immune surveillance outside unique anatomical configurations, tumor cells can establish a foothold and grow, often unimpeded, in sites readily accessible to cells of the innate and adaptive immune systems; yet, the inhibitory disequilibrium in the tumor milieu shares features with those characterized in classical fortresses of immune privilege (7). Despite the primary function of the immune system in surveillance, recognition, and elimination of invading or infectious pathogens, damaged cells, and/or other foreign antigens, cancer cells shrewdly evade this fate. In the context of a tumor site, a flotilla of infiltrating mononuclear cells may arrive on the scene, yet be wholly inadequate to recognize and respond to tumor antigens in a productive manner. Why these newly recruited cells appear incompetent remains uncertain, but it may stem from tumor release of deviant immune mediators, mesenchymal production of inhibitors, or resistant resident immune cells. More recently, it has been appreciated that along with the infiltrating antigen-responsive T cells, a platoon of cells with immunoregulatory properties accumulates around the tumor. It is also known that neoplastic cells themselves generate immunosuppressive molecules (8), including transforming growth factor- β (TGF- β) (9), and hijack host defense mechanisms for their own benefit (10). Thus, local sources of TGF- β and migrant populations invading the region, which serve as couriers, may set up a restricted site of immune tolerance as a sanctuary for the tumor to grow and expand.

While not minimizing the multiple other anti-inflammatory and immunosuppressive factors contributing to the network involved in induction and maintenance of an immunoprivileged state (11), TGF- β is one of the salient features underlying tolerance and is the focus of this review.

TGF- β : the super mediator

Although a panoply of structural, cellular, and molecular mechanisms restrain immune surveillance, TGF- β has emerged as a key regulator of host defense, straddling both innate and adaptive immune pathways and orchestrating the subsequent healing response (12–17). Beyond its disparate roles in development, differentiation, and tumorigenesis, TGF- β clearly plays a defining role as a switch factor in locoregional immune suppression. TGF- β 1, TGF- β 2, and TGF- β 3 are mammalian members of a superfamily of structurally and functionally related multifunctional polypeptides that serve as positive and negative control devices for a diverse set of cellular processes, including cell proliferation, differentiation, apoptosis, and cytokine generation (18), with TGF- β 1 being the foremost in leukocyte populations. Among its renowned properties, TGF- β 1 is most appreciated for its ability to instigate and maintain immune tolerance. As a dominant immunoregulatory member of this superfamily of secreted signaling molecules (9, 12, 19), TGF- β 1 binds to its cognate cell surface receptors to activate intracellular signaling pathways and to evoke a context-dependent cellular response (20). Under normal circumstances, TGF- β is secreted in a latent or inactive form that requires proteolytic, conformational, and/or acidic conditions to remove the 80-kDa latency-associated peptide, liberating the biologically active mature TGF- β recognized by transmembrane serine/threonine kinase TGF- β receptors (21). Once the ligand engages TGF- β type II receptors (TGF β RII), the TGF- β type I receptors (TGF β RI) (activin-receptor-like kinase 5) are recruited into a heteromeric receptor complex, inducing phosphorylation of the kinase domain of TGF β RI (18, 22). In the signaling cascade, these kinases phosphorylate downstream signaling molecules to propagate the signal through the intracellular Smad pathway (23–25).

In hematopoietic cells, major targets of the TGF β RI kinases are the receptor-regulated cytoplasmic Smad2 and Smad3, which are recruited to the TGF β RI through an interaction with a membrane-associated, lipid-binding FYVE domain protein, Smad anchor for receptor activation, and which form a heterooligomeric complex incorporating the common Smad4, enabling translocation to the nucleus and formation of transcription factor complexes (26). Although Smad4 is considered pivotal, in that it controls nuclear trafficking necessary for

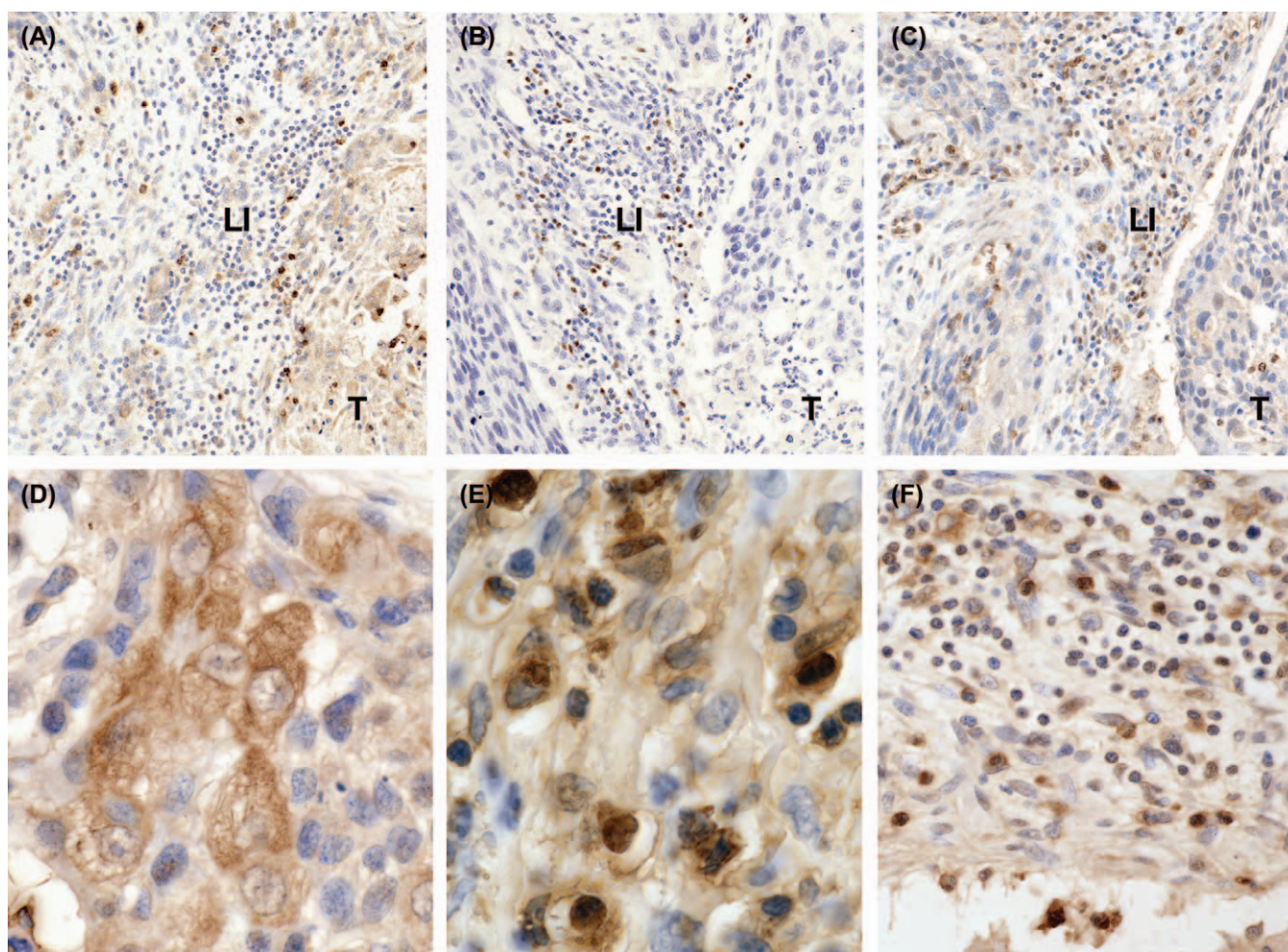


Fig. 1. TGF- β and Tregs in tumor and lymphoid cell infiltrates. Tissue sections of oral SCC were stained with antibodies to TGF- β (A, D–F), Foxp3 (B), and phospho-Smad2 (C). TGF- β is present in the tumor site (A, original magnification $\times 20$), tumor cells (D, $\times 63$), and infiltrating populations of macrophages (E, $\times 100$); some of which have

phagocytosed apoptotic cells. Tumor-infiltrating lymphocytes may be TGF- β^+ (F, $\times 40$), and Foxp3 $^+$ Tregs are found only in the LI (B, $\times 20$). (C) Evidence of phospho-Smad2 staining is consistent with TGF- β signal transduction in the infiltrating cell populations. LI, lymphoid infiltrate. T, tumor.

regulating transcriptional activity of the Smad complexes, certain TGF- β -mediated activities can occur independently of Smad4, including cell cycle arrest in some cell populations (27, 28). Once within the nucleus, the Smad complexes not only regulate transcription of a specific set of genes through Smad binding elements but also network through p300/CBP, TFE3, Ski, and c-Jun (29) to influence transcriptional pathways. In this regard, TGF- β signaling cascades extend beyond Smad proteins to include phosphatidylinositol 3-kinase/Akt, p38 mitogen-activated protein kinase, Rho proteins, extracellular signal-regulated kinase, and stress-activated kinases (30, 31) in a coordinated cell-context-specific response. Broadening the influence of TGF- β , recent evidence indicates that the Smad pathway intersects with the Wnt signaling pathway, the interferon- γ (IFN γ) signal transducer and activator of transcription pathway, and pathways that engage activator protein

complexes (19, 32). In addition to forward driving Smads, there are antagonistic or inhibitory Smads, Smad6 and Smad7, which antagonize TGF- β signaling to exert control over this potent mediator (33). A third receptor, TGF- β type III receptors, although non-signaling, enhances TGF- β interactions with TGF β RII (34).

Through gene knockout experiments, TGF- β and its component signaling pathways have emerged as requisite bifunctional regulators of inflammation and host responses to pathogens and foreign agents (35–37). While initiating early leukocyte chemotaxis and potentiating T-helper (Th) cell responses, TGF- β also participates in resolution and aberrant immune events. Gradients of secreted TGF- β trigger recruitment of inflammatory cells to a target site (38), wherein TGF- β further influences the accumulating cells by modifying their response to inflammatory stimuli. Development of CD4 $^+$ Th

cell(Th1, Th2, and Th17) behavior involves TGF- β not only as a soluble mediator but also as a membrane-associated molecule exploiting contact-dependent interactions. Mechanistically, TGF- β inhibition of the transcription factor, T-bet, blocks Th1 differentiation (39, 40), whereas interruption of interleukin (IL)-4-driven GATA-3 by TGF- β deviates Th2 differentiation (41–43). Whether TGF- β sways differentiation of a putative Th25 lineage (44) remains unexplored, but a role for TGF- β in Th17 lineage commitment has been recently uncovered (45, 46) in a newly emerging and clearly more complex paradigm of T-cell lineage development. When TGF- β is absent, the imbalance results in profound alterations in immune system development, homeostasis, and host defense (47–51). Furthermore, in the absence of TGF- β or in models in which TGF- β signaling is disrupted (37, 52), immune privilege and tolerogenic mechanisms are overtly compromised, documenting a causative link between TGF- β and immune deviation.

Local sources of TGF- β thwart immune surveillance

Within a tumor site, TGF- β may derive from multiple sources: it may be tethered to the extracellular matrix or newly secreted by mesenchymal cells, resident leukocytes, and tumor cells, and can be transported in by recruited populations of cells, characteristic of squamous cell carcinoma (SCC) (Fig. 1A,D–F). Local stromal cells are a ready source of TGF- β (53), which has direct regulatory effects on malignant transformation and may cause an imbalance of signals favoring tumor progression and evasion of immune surveillance. Local TGF- β production by and direct control of tumor cell growth, invasion, and metastatic events are often paradoxical (9), in that TGF- β and its signaling partners act as tumor suppressors for many cancer types, including SCC and adenocarcinomas, but in others, they support tumor growth and metastasis. The complexity of mechanisms by which TGF- β influences the onset and progression of cancer (54) represents a challenge at multiple levels associated with cell proliferation, DNA damage repair, transcription, and apoptosis and not within the scope of this review. Inactivating mutations or loss of expression of TGF- β receptors and Smad signaling components in human cancers suggest that these intrinsic pathway disruptions, selected for during tumor formation, form an escape route from TGF- β signaling and cell cycle arrest (55, 56). Beyond cyclin-associated proteins (32), genes associated with apoptosis and differentiation may be transcriptionally regulated by TGF- β (19, 57) to further fuel growth and survival characteristics. In the microcosm of a tumor bed, introduction of TGF- β is often linked with a negative outcome through its facilitation of the

burgeoning growth of malignant cells, coupled with shielding of the tumor from the adaptive immune response. In this regard, as advances in the understanding of TGF- β and its signaling consequences come to light, TGF- β antagonists are being developed for potential anti-tumor therapy (58).

Mobile sources of TGF- β

Monocytes and macrophages

Secretion of TGF- β by the tumor cells (Fig. 1A,D) supports the evolution of a localized sanctuary through recruitment of innate and adaptive immune cells (38, 59, 60) and then by governing their functional repertoire (61). Among the populations contributing to the accumulation of TGF- β within a tumor sanctuary are constituents of the innate immune system, including neutrophils, natural killer (NK) cells, monocytes, dendritic cells (DCs), and monocyte-derived macrophages (Fig. 1A,E). These cells, particularly DCs, macrophages, and mast cells, may exist and function as pathogen- and antigen-sensing sentinels interspersed within the tissue, but new inflammatory cells are constantly on the move and can be rapidly recruited from the circulation on evidence of local distress. TGF- β itself, even at extremely low concentrations, guides leukocyte transendothelial migration to the site under siege (38). This memory-independent swift response results in the release of multiple cytokines, inflammatory mediators, cyclooxygenase-2, reactive oxygen and nitrogen intermediates, matrix metalloproteinases, and other proteases, amplifying the response, promoting angiogenesis, and orchestrating tissue repair. Failure to restore homeostasis can drive chronic, tissue-damaging pathologic sequelae, which may in some circumstances drive malignant transformation (10, 62).

Once thought to be a favorable prognosticator of tumor resolution due to their participation as antigen-presenting cells (APCs), sources of proinflammatory mediators, and foraging of necrotic and apoptotic tumor cells, the tumor-infiltrating macrophages have more recently been incriminated as perpetrators of tumor progression and metastasis (63). In some tumors, macrophages journeying into the tissue have been correlated with increased angiogenesis and malignancy (64–66). Timing and location are crucial, in that evidence suggests that early in tumor development, macrophages may be beneficial, but in later stages, they may exacerbate rather than mitigate tumorigenesis (63, 67). A link between chronic inflammation, nuclear factor κ B (NF κ B) activation, and susceptibility to cancer promotion has long been suspected (68, 69), and macrophage products may be contributory. Further assessment of the nature of the relationship between

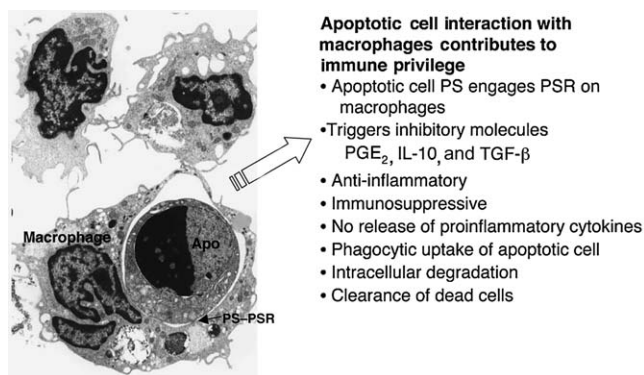


Fig. 2. Generation of immunoregulatory molecules during apoptotic clearance. Cells (T cells, tumor cells, leukocytes) undergoing apoptosis express PS on their outer membrane, which is recognized by PSRs on phagocytic macrophages. This interaction is instrumental in phagocytic uptake of the apoptotic cell along with triggering of the macrophages to release immunosuppressive molecules including TGF- β . Suicidal T cells are also a source of TGF- β , adding to the immunosuppressive milieu. Apo, apoptotic cell.

tumor cells and tumor-infiltrating macrophages elucidate the complex molecular mechanisms responsible for their opposing activities. However, it is plausible that embedded in this complex and dynamic cellular and molecular network, macrophage generation, transport, release, and/or activation of TGF- β may influence their seemingly incompatible contributions (70) in tumor progression.

In addition to a plethora of cascading signals triggering TGF- β generation by newly recruited blood monocytes and tissue macrophages, a major instigator of TGF- β production is their interaction with apoptotic cells (Fig. 2). Within and around the tumor, a variety of cells, including malignant cells, immune cells, and inflammatory cells, commence irreversible pathways, leading to apoptotic cell death. Typically, adaptive immune responses involve clonal expansion of antigen-specific T cells, followed by contraction, underwritten by activation-induced cell death, and clearance of the no longer needed T cells. Contributing to the life and death decisions of T cells through membrane receptor events, TGF- β also functions as an intracellular intermediary in the mitochondria (50, 61, 71, J. Swisher and S. Wahl, unpublished observations). Once apoptotic pathways become engaged, flipping of phosphatidylserine (PS) from the inner membrane leaflet to the outer membrane enables recognition of these apoptotic cells by PS receptors (PSRs) on phagocytic cells, uptake, and degradation. Inherent in these sequelae of events is the lack of triggering of inflammatory stimuli, mediated in part by induction of immunosuppressive factors. In this regard, PS⁺ apoptotic cell binding to PSRs on macrophages triggers the release of prostaglandin E₂, IL-10, and TGF- β (Fig. 2), which are intended to extinguish potentially

injurious immune-based inflammatory responses (72). In a perversion of this response, cancer cells themselves express PS and PSRs, and shed PS interacting with tumor PSRs further crushes any host response, averting anti-tumor immunity (73). This confluence of PS and TGF- β also disrupts immature DCs, impairing their tumor antigen-presenting functions and contributing to tumor acceptance (73).

Members of the tumor necrosis factor (TNF) family, including TNF α , and the membrane proteins, Fas ligand (FasL) (CD95L) and TNF-related apoptosis-inducing ligand (TRAIL), are major players in the regulation of apoptotic sequelae. The apoptosis-inducing molecule FasL expressed on many cells, not the least of which are tumor cells, triggers death and destruction of Fas-receptor-bearing cells that approach too closely, notably potential anti-tumor T lymphocytes (74). Similarly, TRAIL, expressed on many of the same cells that are decorated with FasL, is a potent inducer of suicidal tendencies in cells expressing the TRAIL receptors, TRAIL-R1 and TRAIL-R2, including inflammatory cells and tumor cells (75). By whatever initiation event, once engaged in an irreversible apoptotic trajectory, cell membrane exposure of PS results in triggering of an accumulation of anti-inflammatory mediators, further enriching the local levels of TGF- β , which acts in an autocrine and/or in a paracrine fashion to downregulate immune sequelae (76). Soluble TGF- β , by engaging TGF β R on tumor-infiltrating lymphocytes, be they CD4⁺, CD8⁺, or NK cells, governs their differentiation, proliferation, and effector cytokine generation (12).

Dendritic cells

Carrying a cargo of TGF- β , infiltrating DCs are a family of professional APCs with the power to influence T-cell lineage commitment (77). While critical in priming protective CD4⁺ Th1 and CD8⁺ cytotoxic T lymphocyte (CTL)-mediated anti-tumor responses, these motile DCs not only contribute TGF- β but also are swayed by the presence of TGF- β (78, 79). Detrimentally, TGF- β has been shown to immobilize DCs *ex vivo* and within murine skin tumors, inhibiting DC migration and antigen transport to draining lymph nodes (LNs), effectively obstructing T-cell activation (80). Consistent with these observations, reduced DC numbers have been detected in draining LNs in patients with breast cancer (81), and decreased circulating DCs in colorectal cancer reflect increased serum TGF- β levels (82). In addition to immobilization, TGF- β may also decrease DCs by escalating apoptosis (83) and warp their function by inhibiting maturation and expression of major histocompatibility complex (MHC) class II and costimulatory molecules (12).

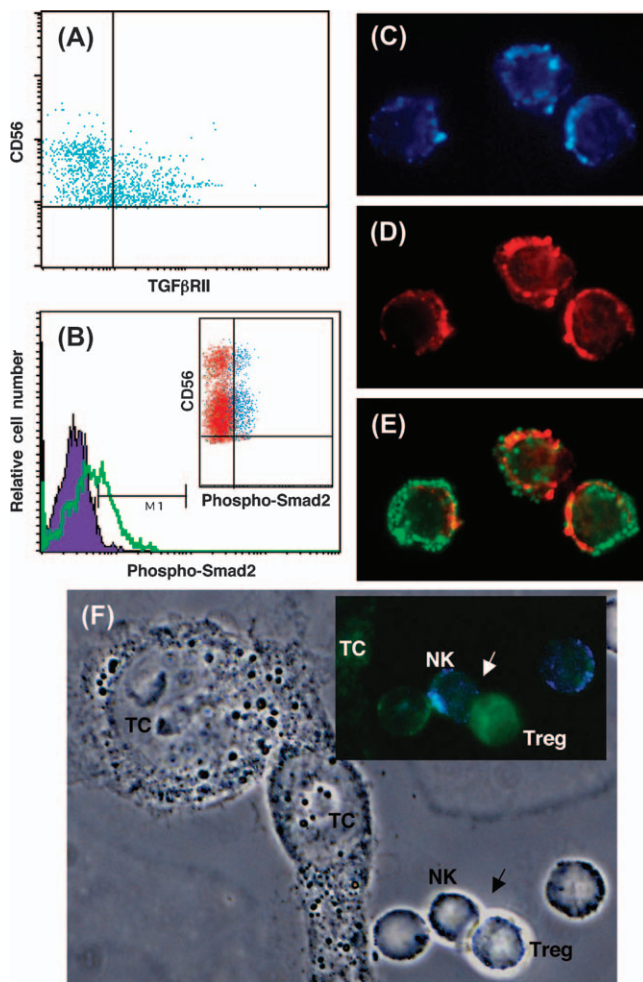


Fig. 3. Ligand interactions with NK cell TGF- β receptors trigger Smad signaling. (A) By dual color fluorescence with FITC-labeled TGF β RII antibody and an Alexa-647-conjugated antibody to CD56 (NK), a subset of unstimulated CD56⁺ PBMCs was shown by flow cytometry to constitutively express TGF β RII. (B) Following exposure to ligand (TGF- β 1, 1 ng/ml), increased intracellular phosphorylation of Smad2 was evident within 30 min, as detected with a rabbit anti-phospho-Smad2 antibody and an Alexa-488-tagged secondary antibody (green), compared with isotype control antibody (purple). Inset: Co-culture of purified CD56⁺ cells and Tregs (45 min) resulted in increased phospho-Smad2 consistent with a TGF- β -dependent signal. (C–E) By confocal microscopy, CD56⁺ cells (blue, C) were positive for TGF β RII expression (red, D), and in response to ligand, the TGF β RII⁺ cells (red) showed phospho-Smad2 (green; overlay, E). (F) PBMCs were stained with monoclonal antibodies to CD56 (blue) and CD25 (FITC) and sorted by MO-FLOW. The isolated CD56⁺ NK cells⁺ (5×10^5) were added to oral SCC tumor cell line (HN12; 2×10^4 /well) monolayer (TC) for 30 min, followed by CD25^{high} Tregs (3×10^5) for 30 min. After a brief fixation with 3% formaldehyde, the cell interactions were examined by phase and fluorescence (inset) microscopy (original magnification $\times 63$). Arrow indicates cell contact between NK and Tregs. FITC, fluorescein isothiocyanate; PBMC, peripheral blood mononuclear cells; TC, tumor cell.

Further skewing the tumor ecosystem, DC subsets (CD4⁻CD8⁻ DCs) producing TGF- β have been linked with the generation of CD4⁺ regulatory T cells (Tr1), which are responsible for faulty tumor immune responses (84). Regula-

tory T cell differentiation can be driven by DCs, when they are in an immature or 'tolerogenic' state (85). Depending on the triggering signal, DCs can also polarize cells toward the CD4⁺CD25⁺ Treg lineage, in support of reduced immune surveillance (86). However, this influence is not a one-way street, and activated Tregs also inhibit Toll-like-receptor (TLR)-triggered myeloid DC maturation, while sparing plasmacytoid DC maturation and function (78, 87). In an unlikely coalition, CD4⁺CD25⁺ Tregs or Tr1 cells expressing CTL antigen (CTLA)-4 engage DC or monocyte CD80/CD86 to induce expression of the enzyme indoleamine 2,3 dioxygenase, a potent inhibitor of T-cell responses (88), in a cascade of suppression. Through these direct and indirect pathways, TGF- β can also negatively affect DC-based tumor vaccine strategies (89, 90).

Regulatory T cells as a vehicle for TGF- β transport and action Pivotal convoys for transport of TGF- β into the tumor site and for enforcement of immune suppression are the regulatory T cells (Figs. 1, 3, and 4). If a fully functional adaptive anti-tumor immune response was operative, it should eradicate emergent tumor cells, but such a response is handicapped by the presence of these counteracting regulatory T cells. Several classes of regulatory T cells have been identified, including adaptive Tr1 and Th3 lymphocytes and naturally occurring CD4⁺CD25⁺ Tregs (91), all of which, to a greater or lesser degree, may involve TGF- β in development and/or in function (15, 92, 93). Another immunoregulatory T-cell population, CD1d-dependent NKT cells (94), may infiltrate and suppress tumor surveillance, but this activity occurs independently of TGF- β (95). These multiple regulatory populations act locally to block deleterious pathways or actively stimulate tolerance, functioning as a brake to avoid potential injurious effects of immune excess. Increasing their sphere of influence, one tolerant population may tolerize additional cells through a process of 'infectious tolerance' (96). Why these pathways of suppression, essential in reversing normal innate and adaptive immune responses, become fanatical in the context of a tumor is uncertain but may be relentlessly driven by the local buildup of inductive TGF- β .

Immune privilege orchestrated by TGF- β and Tregs

Among the mechanisms for improvising niches of immune privilege is the recruitment of a cohort of powerful immunosuppressive cells that are dependent on TGF- β . CD4⁺ Tregs represent a unique subpopulation of CD4⁺ T cells that constitutively express the high-affinity IL-2 receptor α -chain (CD25), comprise $\leq 10\%$ of CD4⁺ T cells, and coordinate the

maintenance of peripheral immune tolerance (97, 98) through inhibition of the cornerstones of adaptive immunity, CD4⁺ effector Th1 and Th2 cells. One of the defining characteristics of Tregs is their expression of the transcription factor, forkhead box protein 3 (Foxp3), which acts as their cell lineage specification factor or master switch (99). Critically, Foxp3, through its interaction with nuclear factor of activated T cells and NF κ B, represses Th cell cytokine gene expression and effector functions (100). Foxp3 expression in developing thymocytes is not dependent on IL-2 nor does IL-2 mediate inhibitory activity of Tregs, but its support of their survival and expansion in the periphery (99, 101, 102) may include enhancing levels of TGF- β .

Through propagation of tolerogenic pathways, Tregs bias the immune system against autoimmunity and promote tolerance of allogeneic organ grafts and successful pregnancy (96, 103–108). By a distinctive approach requiring direct cell-to-cell contact, Tregs deliver inhibitory signals to CD4⁺, CD8⁺, and NK cells to extinguish spontaneous emergence of organ-specific autoimmune diseases and contribute to dominant tolerance in infection, allergy, and transplantation (50, 109–112). On a more sinister side, Tregs can also obscure the host response to tumors (112–115). Increased proportions of Tregs have been detected in tumor-bearing mice (112, 114, 116–118) and in patient tumor sites (Foxp3⁺; Fig. 1B) (114, 119, 120, J. Wen and S. Wahl manuscript in preparation), although conceivably, both increased frequency and amplified activity may dominate failed anti-tumor responses.

In this microcosm of immunity, Treg touch leaves a telling effect on effector T cells. Although no longer debated that cell-cell contact is essential for Tregs to perform their duty, the mystery remains as to what occurs during that intimate encounter. Nonetheless, considerable evidence now portrays a TGF- β -dependent pathway by which Tregs, recruited to a point of inflammation, infection, or tumorigenesis, influence the fate and function of antigen-responder T cells and NK cells (12, 15, 50, 114, 115). Initial studies showing latent TGF- β on the surface of Tregs (121) were rapidly followed by evidence for the active form of TGF- β and increased expression of TGF β RII on their outer membrane (15, 50). These and subsequent studies (114, 122–124) identifying membrane-bound TGF- β on Tregs are consistent with *in vivo* studies indicative of TGF- β -dependent peripheral tolerance (125).

Dependent upon antigen to implement tolerance, upon T-cell receptor (TCR) stimulation, Tregs boost their levels of cell surface TGF- β , propelling them toward suppressive encounters (15). Once the switch for suppression is on, Tregs inhibit in an antigen-non-specific fashion (126), as would be the case in a TGF- β -driven mode of suppression. Neutralization of TGF- β

terminates the inhibitory phenotype of Tregs (15, 121), which can be reintroduced with soluble TGF- β . However, since resting CD4⁺ and CD8⁺ effector T cells typically lack demonstrable TGF β RII, how Treg membrane-associated TGF- β could influence these cells through a contact-dependent mechanism remained puzzling until it was shown that these cells become cloaked in TGF β RII postactivation and thus receptive to engagement through TGF- β on the surface of Tregs (15). Such a scenario is seemingly logical, in that a resting cell does not require suppression, whereas on activation, emergence of TGF- β receptors provides a bridge for transmittal of TGF- β signals, essential to reintroduce some semblance of control leading to resolution of immune responses. Confirming this link, contact between Tregs and T responder/effector T cells culminates in activation of the TGF- β signaling cascade with phosphorylation of intracellular Smad proteins (15, 50, 127) (Figs. 1C and 3). In this cell-cell conjugate, Smad activation occurs in the receptive effector T cell, endorsing downstream disruption of proliferation and cytokine production. In this regard, T cells that cannot respond to TGF- β reportedly escape control by Tregs (128).

Now, admittedly, some controversy remains as to the absoluteness of this TGF- β -linked pathway to suppression (128–130), and because Tregs represent an essential instrument of protection against self-reactive T cells, it is reasonable to assume that they would not rely on a single pathway. Nonetheless, despite years of effort, an alternative mechanism remains elusive. Comparable severe lymphoproliferative autoimmune syndromes develop in mice deficient in TGF- β , Foxp3, or CTLA-4, in which escalating cytokine production by progressively expanding activated autoimmune T-cell populations without proper control becomes lethal (131–134), thereby inexorably linking these three molecules with Treg-mediated suppression. Similarly, in humans, Foxp3 mutations are linked to severe autoimmune disease (131, 135, 136). Phenotypically, Tregs express CTLA-4 on their membranes and also intracellularly, and whereas non-activating antibodies to CTLA-4 abrogate their suppressor activity (137), cross-linking and activation of CTLA-4 engages a pathway associated with generation of TGF- β (138–140). CD69 cross-linking in both CD4⁺ and CD8⁺ T cells is also reported to trigger expression of TGF- β , which is disruptive to anti-tumor immunity (141). TGF- β clearly appears to take center stage in the orchestration of suppression to achieve restoration of immune homeostasis, since in its absence, the requisite tightly controlled feedback loops that regulate Treg generation and function are discombobulated.

Within the context of a tumor ecosystem, antigen-driven Tregs may be recruited from the circulating pool (142), in part

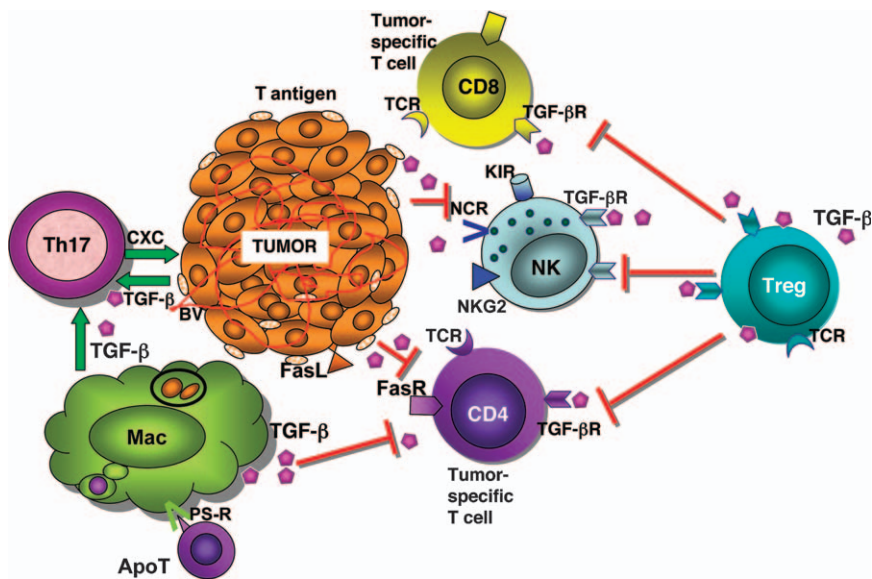


Fig. 4. Multiple sources of secreted and cell-associated TGF- β in a tumor bias toward immune privilege. Within a tumor site, the tumor cells themselves and the components of innate and adaptive immune pathways generate TGF- β . In addition, CD4⁺CD25⁺Foxp3⁺ Tregs influence the immune response through membrane-bound TGF- β in a contact-dependent pathway. Tregs interact with and block CD4⁺ and CD8⁺ tumor-specific T-cell activation. Through a similar pathway involving touch, Tregs also impair NK-mediated anti-tumor activity to block effective tumor immune surveillance. Cell-associated and secreted TGF- β drives naive T cells into Th17 cells that promote new BV formation for tumor growth and survival. BV, blood vessel; TCR, T-cell receptor; Mac, macrophage; NCR, natural cytotoxic receptors; Apo T, apoptotic T cell; KIR, killer cell immunoglobulin-like receptors; CXC, cxc motif chemokines.

through macrophage-derived CCL22 (CCR4 ligand) (120), and/or generated locally. There was a time when Tregs were considered to be an exclusive product of the thymus, representing a distinct lineage emerging in limited numbers in response to self-antigen during intrathymic incubation, but not inducible in the periphery (98, 143). However, accumulating evidence finds that these cells are inducible in the periphery, by both expansion and conversion, thereby enhancing their accumulation in peripheral lymphoid compartments or sites of immune activity (144). Conversion of peripheral CD4⁺CD25⁻ T cells into Foxp3⁺ cells can be effected through antigen activation in the presence of TGF- β , a facilitator of Treg-lineage expansion through induction of the transcription factor Foxp3 both *in vitro* and *in vivo* (50, 145–153). A milieu rich in TGF- β and antigen promotes the conversion of antigen-activated CD4⁺ T cells into CD4⁺CD25⁺ T cells, with all the characteristics of Tregs, including Foxp3, membrane-bound TGF- β , and elevated CTLA-4 and glucocorticoid-induced TNF receptor (15, 150).

Treg migration and retention further exaggerate their numbers within a targeted tissue, and this strength in numbers fosters a dominant state of immune privilege. In this regard, Tregs, which may account for $\geq 20\%$ of the tumor T-cell infiltrate (119, 120, J. Wen *et al.*, manuscript in preparation) (Fig. 1B), have the power to extinguish immune surveillance. While their normal goal is to target and maintain a modicum of control over T cells responding to environmental and self-antigens, in this context, their goal may be perverted in alignment with the overpowering survival objectives of the tumor cells. Enhanced numbers of Tregs have been correlated with poor tumor prognosis (114, 119, 120). While Tregs

typically regress on antigen depletion, the persistence of driving forces such as TGF- β and tumor antigens may sustain their high numbers and inhibitory network in a targeted tissue microenvironment, as exemplified in a site rife with neoplastic activity (154). Why these cells that are programmed to protect us from autoimmunity switch to the dark side and block protection from carcinogenesis remains unclear, but it is further obfuscated by recent evidence suggesting, at least in one model, that Tregs can also delay tumor growth through release of IL-10, which undermines the pro-tumor inflammatory activity of macrophages and/or neutrophils (155). Once again, timing and location may be critical determinants of these context-specific immunoregulatory pathways.

Targets of Treg suppression

CD4⁺ effector T cells

The first identified targets of direct Treg suppression were CD4⁺CD25⁻ antigen-specific responder T cells. Activated through antigen-loaded APCs, these cells respond by proliferating and secreting cytokines to recruit and activate an appropriate host response, depending on Th1- or Th2-type antigens (43, 156). In the context of a malignancy, such T cells recognizing tumor-specific antigens infiltrate the tumor site (157) to eliminate these offensive cells, but all too often fail miserably. Along with activation comes expression of surface TGF β RII and vulnerability to Tregs, accumulating TGF- β and other immunosuppressive modalities surrounding the tumor. Succumbing to the expanded platoon of Tregs, they become ineffective instruments of tumor rejection with escalating tumor growth, dissemination, and frequent lethal outcome.

CD8⁺ T cells

CD8⁺ T-cell responses retard tumor growth and, if not coerced into ineffective anti-tumor behavior by Tregs, participate in eradicating neoplastic cells. CD8⁺ T cells represent an important arm of the adaptive anti-tumor response, exerting their effector functions through the production of inflammatory cytokines such as IFN γ and through cytolytic activities mediated by perforin/granzyme and death receptor (Fas/FasL) pathways (158). In a two-pronged approach, both free TGF- β and Treg-associated TGF- β influence CD8⁺ CTL proliferation, differentiation, and acquisition of effector molecules, albeit at differing levels of sensitivity (113, 159). TGF- β subdues not only the generation of CTL responses but also their existing lytic activity (160), and dominant negative TGF β RII transgenic mice with TGF- β non-responsive T cells exhibit unimpeded generation of tumor-specific CTLs and resistance to tumor growth (161). As a corollary, in human patients with melanoma, antigen-specific CD8⁺ T-cell phenotypic and effector function *in vitro* is inhibited by the addition of TGF- β (162). Through binding of TGF- β -activated Smad and ATF1 transcription factors to the promoter regions of granzyme B and IFN γ , TGF- β directly inhibits naive or activated CD8⁺ T-cell expression of these instruments of cytotoxicity (163).

NK cells

Tregs clearly influence the evolution and outcome of adaptive immune responses, which are characterized by being T-cell dependent, antigen specific, and possessing memory, through their ability to blunt antigen-specific CD4⁺ and CD8⁺ T-cell activation. However, the concept of a demarcation between Treg control of adaptive immunity and lack of effect on innate events has been blurred by the recent identification of a role for Tregs and TGF- β in manipulating innate responses, through a direct interaction with NK cells (114, 115), and influencing Th17 cells (45, 46). Incompatible with tumor development, NK cells have anti-angiogenic and cytolytic properties (114, 164, 165). As NK cells arrive within a tumor niche, in part, through a chemotactic response to TGF- β (60), they are programmed to perform these vital anti-tumor functions, which are unfortunately thwarted not only by accumulating levels of soluble TGF- β but also by Treg-associated TGF- β .

By comparison with antigen-responsive CD4⁺ T cells, which increase their TGF β RII when stimulated through their T-cell receptor (15, 150), non-activated CD56⁺ NK cells express a functional complement of surface TGF β RII (Fig. 3A). Consistent with the need for rapid mobilization and response to neoplastic cells and pathogens, NK killing of tumor cells or virus-infected cells does not require presensitization nor is it

restricted by MHC. Nonetheless, malignant cells can even outsmart NK surveillance, at least partially, by releasing TGF- β (Figs. 1A,D and 4). These cytolytic cells are susceptible to control by soluble TGF- β (166), and their membrane TGF β RII provides a bridge across which Tregs deliver a TGF- β signal (114, 115, 167) (Fig. 3). When exposed to TGF- β or on NK cell close encounters with Tregs, an intracellular Smad signal is engaged with phosphorylation of Smad2 (Figs. 1C and 3B,E). The consequence of engaging TGF- β signal transduction in NK cells is to suppress lytic and secretory activities and to downregulate NKG2D tumor cell recognition receptors (114), all of which foil NK anti-tumor activity.

Treg control of NK cells, in some circumstances, may be a boon rather than a bane. In addition to tumor sites, another immune-privileged area, the pregnant uterus, may be infiltrated by lymphocytes, up to 70% of which are NK cells (168), and fortuitously, they may also be held in check by Tregs and TGF- β (169–172), thereby enabling tolerance to and persistence of the fetus. However, in pathologic situations, an imbalance of Tregs to responder T or NK cells may need to be reset to restore homeostasis. Whereas in autoimmunity it may be beneficial to augment Tregs to downplay the host response, in tumors, depleting or incapacitating Tregs may be the plan of choice. In this regard, targeting CD25⁺ cells with cytotoxic anti-CD25 antibodies, CD25-immunotoxins (173), or other more specific approaches may restore immune surveillance. Consistent with the multiple aspects of Treg participation in establishing a mobile landscape of immune privilege, depletion of these cells has been shown to bolster CD4⁺ T-cell- and NK-cell-dependent tumor clearance and also DC-based tumor therapy (114, 174). Although immunopharmacological modulation of Tregs may benefit tumor immunity, it must be considered in the context of potential autoimmune deviation.

TGF- β -linked differentiation of Th17 lymphocytes in tumorigenesis

While considerable focus has been directed on Tregs, secreted and cell-free TGF- β in the tissues surrounding tumors, wounds, and at the maternal-fetal interface dramatically frames the immunomilieu. The biological effects of TGF- β are complex and governed by cell type and state and may be Smad dependent and independent, with evolving concepts of TGF- β signaling integrated with pathways activated by other factors. Among the cells that may mobilize to a tumor site is a population of CD4⁺ T cells that expresses the cytokine IL-17, which in turn drives leukocyte recruitment and activation (175–177) to bridge innate and adaptive immunity. In a new turn of events, TGF- β

has been linked to the lineage-specific differentiation of these Th17 lymphocytes (45, 46). Although the contribution of Th17 cells to tumorigenesis is complex, with both pro- and anti-tumorigenic properties reported, in primary non-small-cell lung cancer, high expression of IL-17 in infiltrating inflammatory cells was associated with increased angiogenesis and tumor growth (178), and in cervical cancer, overexpression of IL-17 increased macrophage recruitment (179). Cutaneous T-cell lymphomas secrete IL-17 (180), and increased IL-17 in ovarian carcinomas also correlates with increased vascularity (181). Critical to tumor growth is sustained new vessel generation and arborization (182), and increased IL-17 may tip the balance to accelerate endothelial cell proliferation, migration, and tubular morphogenesis (Fig. 4) through its induction of angiogenic CXC motif chemokines interacting with CXC chemokine receptor (CXCR)-2 (178), rather than having a direct effect on tumor cell growth.

Both IL-15 and IL-23 were implicated initially as upstream regulators of IL-17 (183–185), but their role was relegated to one of promoting survival with the discovery that TGF- β is the driver of IL-17 (45, 46) and the appreciation that these IL-17-producing cells (Th17) represent a unique population of CD4⁺ Th cells, distinct from cells of Th1 and Th2 lineages (185–187). Whether originating from Tregs, tumors, APCs, or other purveyors, TGF- β confers polarization toward a Th17 lineage commitment with increased expression of IL-23R, making them more responsive to DC and macrophage-derived IL-23 (45, 46, 188). TGF- β , perhaps collaboratively with IL-6 (46, 189), not only directly instructs Th17 effector cell differentiation but also dismisses Th1 (IFN γ) and/or Th2 (IL-4) cell differentiation, shifting lineage commitment toward Th17 (45).

Dysregulation of IL-17 may escalate malignant transformation, either as an outcome of infection or inflammation-mediated carcinogenesis (68, 190) or related to control by TGF- β . Although TGF- β has been associated with neovascularization *in vivo*, it was proposed many years ago that it may occur through an indirect effect on inflammatory cells (191). Of the six IL-17 family members, A–F, the prototypic IL-17A, originally identified as CTLA-8 (192), is secreted as a disulfide-linked, homodimeric, 35-kDa glycoprotein (193), as is the highly homologous IL-17F. By triggering production of CXCL8 (IL-8), other chemokines, IL-6, and colony-stimulating factors (175, 193, 194), IL-17 impacts neutrophil and macrophage migration and retention (176, 186), harkening back to the potential link between infection/inflammation and carcinogenesis. IL-17E (IL-25), with overlapping activity, is a product of Th2 lymphocytes and mast cells (195). IL-17 also targets

other single transmembrane IL-17-receptor-bearing cells, including fibroblasts, epithelial cells, and endothelial cells, to trigger CXC chemokine release, together with vascular endothelial growth factor-A (176, 196). Since IL-17 augments local proteolytic activity (186), tumorigenesis and metastatic activity may be altered. Continuing the cycle, evidence indicates that IL-17F can induce TGF- β expression (196). Clearly, questions abound regarding the interplay of newly recognized links between TGF- β , Th17, and immune regulation, but as increasing evidence implicates IL-17 family members not only in inflammatory and autoimmune diseases but also in cancer, IL-17 and its receptors may emerge as targets for future pharmacotherapy.

Conclusions

Proper functioning of innate and adaptive immune responses is vital for survival; yet, control of these powerful responses is equally critical. Without a means of resolving immune events, autoimmunity, transplant rejection, and response to infection/sepsis can have devastating consequences, including promotion of carcinogenesis. As the immune system is geared to protect the host from foreign bodies, innate and adaptive immune pathways are likely initially engaged, setting out on a course to eradicate the neoplastic cells. However, with so many mechanisms colluding against anti-tumor immunity (Fig. 4), it is no wonder that these attempts to eradicate neoplastic cells are frustrated and that immunotherapeutic approaches are often not overwhelmingly successful.

The basis for this immune privilege no longer remains shrouded in mystery, with the identification of TGF- β as the main choreographer. Concurrent with the accumulation of TGF- β through local and traveling sources (Figs. 1–4), an inhibitory disequilibrium is established to dampen host defense. Nonetheless, such inherent strategies of suppression, having evolved to guarantee self-tolerance, can be misguided and compromise immune surveillance, resulting in a safe haven for malignancies and infections. Conversely, loss or inadequacies of immune privilege in otherwise privileged sites can have dire consequences and have been associated with diseases, such as multiple sclerosis and uveitis, graft rejection, and possibly immune abortion (11, 96). Tregs have evoked considerable interest as potential therapeutic entities, in that they can be induced and transferred to affect immune suppression, or alternatively, deletion or antagonism of this population in provisional sites of immune privilege, depending on context, may provide therapeutic relief from unwanted immune suppression. Abolishing the immunological tolerance

of a solid tumor could reverse its resistance to eradication. Continued efforts to understand the confluence of architectural, cellular, and molecular cues associated with immune privilege are fundamental to devising strategies to harness tolerance mechanisms.

Where do we go from here? Partial understanding drives the quest for further exploration of the underlying mechanisms responsible for generation of fortresses of immune privilege. Knowing that there are mobile, transient, and powerful camps of tolerance provides us with strategies geared toward blocking transport of the troops, largely Tregs with their cargo of TGF- β , into the privileged camp. Tumor vaccines, tumor-targeted T cells, and adoptive transfer of autologous anti-cancer T cells (197) may be emboldened in their attack, should TGF- β and/or Tregs be depleted and/or defeated. Can Treg trafficking be diverted by targeting specific adhesion molecules (CD103, α_E) (198) or chemotactic factor recognition receptors (CCR4) (96)? Is there a way to dissipate the link

between DCs and Tregs? Will interruption of TGF- β signaling be beneficial, or perhaps the transfer of the TGF- β signal from Tregs to T effector cells can be intercepted? Will transient systemic depletion of Tregs (antibody ablation) reverse local enclaves of immune privilege without compromising protection against autoimmune responses? Adoptive transfer of Tregs generated *in vitro* mitigates rambunctious immune pathogenesis (50), so perhaps, the converse will address overzealous suppression and its attendant pathology. Because of the ubiquitous nature of TGF- β and its receptors, it may not be wise to systemically deplete or block it, as we are aware from animal models that total ablation can be lethal. However, local delivery of antagonists (soluble decoy receptors, short interfering RNA, antibodies, antisense oligodeoxynucleotides) may be appropriate. To this end, as suggested in recent clinical trials (199), application of local antagonists of TGF- β may restore immune surveillance and promote tumor eradication.

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