The adhesion molecule PECAM-1 enhances the TGF-β–mediated inhibition of T cell function

Debra K. Newman,1,2,3* Guoping Fu,1 Tamara Adams,1 Weiguo Cui,1,3 Vidhyalakshmi Arumugam,1 Theresa Bluemn,1 Matthew J. Riese1,3,4*

Transforming growth factor-β (TGF-β) is an immunosuppressive cytokine that inhibits the proinflammatory functions of T cells, and it is a major factor in abrogating T cell activity against tumors. Canonical TGF-β signaling results in the activation of Smad proteins, which are transcription factors that regulate target gene expression. We found that the cell surface molecule platelet endothelial cell adhesion molecule-1 (PECAM-1) facilitated noncanonical (Smad-independent) TGF-β signaling in T cells. Subcutaneously injected tumor cells that are dependent on TGF-β–mediated suppression of immunity for growth grew more slowly in PECAM-1−/− mice than in their wild-type counterparts. T cells isolated from PECAM-1−/− mice demonstrated relative insensitivity to the TGF-β–dependent inhibition of interferon-γ (IFN-γ) production, granzyme B synthesis, and cellular proliferation. Similarly, human T cells lacking PECAM-1 demonstrated decreased sensitivity to TGF-β in a manner that was partially restored by reexpression of PECAM-1. Co-incubation of T cells with TGF-β and a T cell–activating antibody resulted in PECAM-1 phosphorylation on an immunoreceptor tyrosine–based inhibitory motif (ITIM) and the recruitment of the inhibitory Src homology 2 (SH2) domain–containing tyrosine phosphatase-2 (SHP-2). Such conditions also induced the colocalization of PECAM-1 with the TGF-β receptor complex as identified by coimmunoprecipitation, confocal microscopy, and proximity ligation assays. These studies indicate a role for PECAM-1 in enhancing the inhibitory functions of TGF-β in T cells and suggest that therapeutic targeting of the PECAM-1–TGF-β inhibitory axis represents a means to overcome TGF-β–dependent immunosuppression within the tumor microenvironment.

INTRODUCTION

Immune checkpoint receptors, which are expressed by T cells upon activation to prevent excess inflammation (1), limit the antitumor responses of T cells within the tumor microenvironment and interfere with tumor eradication (2). Immune checkpoint strategies block interactions between immune checkpoint receptors and their ligands to enhance antitumor responses (1, 2). Although immune checkpoint therapy has emerged as a potent means to enhance the antitumor responses of T cells, it elicits durable clinical responses in only a fraction of cancer patients. Inhibitory molecules produced by tumor cells, stroma, T regulatory (Treg) cells, and myeloid-derived suppressor cells in the tumor microenvironment represent barriers that must be overcome for immune checkpoint therapies to become universally effective.

Transforming growth factor–β (TGF-β) is a potent soluble inhibitor of T cell responsiveness (3). Deficiency in TGF-β in mice results in early death because of a multifocal hyper-inflammatory response (4, 5), and this phenotype can be recapitulated through the expression of a dominant-negative form of one of the subunits of the complex formed between TGF-β receptor I (TGF-βRI) and TGF-βRII (TGF-βRII/II) specifically in T cells (6). Secretion of large amounts of TGF-β helps tumors evade clearance by tumor reactive T cells, and tumors that secrete large amounts of TGF-β have proven resistant to immune checkpoint therapy (7, 8). These findings have led to the development and use of TGF-β–blocking agents to enhance antitumor immune responses in cancer patients with TGF-β–rich tumor microenvironments (9); however, TGF-β is a pleiotropic cytokine that has both positive and negative effects on many different cell types (10). Consequently, the efficacy of TGF-β–targeting antitumor therapies is limited by off-target effects. Strategies that specifically block the effects of TGF-β on T cells would be expected to improve the efficacy of TGF-β blockade.

Platelet endothelial cell adhesion molecule-1 (PECAM-1), also known as CD31, is a transmembrane glycoprotein member of the immunoglobulin (Ig) gene superfamily, which contains six extracellular Ig domains and two cytoplasmic immunoreceptor tyrosine–based inhibitory motifs (ITIMs) (11). PECAM-1 is restricted to endothelial cells and cells of the hematopoietic system (12). In mice, PECAM-1 is present on all hematopoietic cells, whereas in humans, it does not appear on mature B lymphocytes or on certain subsets of T lymphocytes (13, 14). The two most membrane-distal Ig domains of PECAM-1 support homophilic interactions that facilitate maintenance of endothelial barrier integrity and leukocyte transendothelial migration (11). Phosphorylation of the cytoplasmic ITIMs of PECAM-1 depends upon a series of serine and threonine (Ser/Thr) and tyrosine (Tyr) phosphorylation events that support the recruitment and activation of tandem Src homology 2 (SH2) domain–containing phosphatases, such as SHP-2 (15, 16). The phosphorylation of ITIMs in PECAM-1 and the resulting binding of SHP-2 interfere with signal transduction by immunoreceptor tyrosine–based activation motif (ITAM)–coupled receptors in platelets and lymphocytes (12). PECAM-1–deficient mice exhibit a hyper-inflammatory phenotype that, although milder than that associated with TGF-β deficiency, is nevertheless also a result of loss of the inhibitory action of PECAM-1 on T cell responsiveness (17). Whether loss of the inhibitory effects of PECAM-1 can enhance the antitumor activity of T cells is not known.

We sought to determine whether the immunosuppressive properties of PECAM-1 are manifest in the presence of the more potent immunosuppressive cytokine TGF-β in vivo and in vitro. Using an established tumor model
dependent upon TGF-β-mediated disabling of T cell responses for growth, we found that loss of PECAM-1 suppressed the growth of EL4-ova tumors in vivo. Furthermore, PECAM-1 markedly enhanced the inhibitory effects of TGF-β in a multitude of responses of both mouse and human T cells to antigen receptor stimulation in vitro. Mechanistically, exposure of T cells to TGF-β stimulated the formation of a PECAM-1−TGF-βRII/II complex, phosphorylation of PECAM-1 ITIMs, and binding of SHP-2 to PECAM-1. These results suggest that PECAM-1 and TGF-β function together to inhibit T cell function. Furthermore, our findings suggest that strategies designed to target PECAM-1 interactions with TGF-β receptors would selectively interfere with the TGF-β-mediated inhibition of T cells, but leave its regulation of most other cells intact, resulting in enhanced immune activity against tumors with minimal off-target effects.

RESULTS

EL4-ova tumors grow more slowly in PECAM-1−/− mice than in PECAM-1−/+ mice

TGF-β plays a major role in tumor-mediated suppression of the immune system in mice and humans. It has been most rigorously studied with a mouse model system involving subcutaneous inoculation with the murine lymphoma cell line EL4, or the derivative cell line EL4-ova, which is engineered to constitutively express the immunogenic protein ovalbumin (ova) (18). For example, short hairpin RNA (shRNA)–mediated decreases in TGF-β abundance within tumor cells or expression of dominant-negative forms of TGF-βRII in T cells result in tumor rejection. Although PECAM-1 exerts immunosuppressive activity in T cells (19–22), it is not known whether its immunosuppressive properties are sufficient to affect tumor clearance in a TGF-β-rich environment. To address this question, we evaluated the effect of PECAM-1 deficiency on the growth of EL4-ova lymphomas in mice. Five- to 7-week-old PECAM-1−/− or PECAM-1−/+ mice were inoculated in the flank, and tumor growth was assessed 2 weeks later. We observed that EL4-ova cells grew substantially more slowly in PECAM-1−/− mice than in PECAM-1−/+ mice (Fig. 1A). Because loss of PECAM-1 reduces the transepithelial migration of leukocytes (23), we also examined the number of tumor-infiltrating lymphocytes (TILs) in the two strains of mice. After processing tumor samples and staining for the presence of T cell markers, we determined that there was no statistically significant difference in the numbers of CD4+ and CD8+ TILs between PECAM-1−/− mice and PECAM-1−/+ mice; however, there was a trend toward increased numbers of TILs in the PECAM-1−/+ mice (Fig. 1B). These data suggest that PECAM-1, perhaps through its ability to inhibit T cell function, plays a role in facilitating tumor growth in a TGF-β-rich environment in mice.

PECAM-1 enhances the ability of TGF-β to inhibit mouse T cell functions

To determine whether TGF-β and PECAM-1 functioned alone or together to inhibit T cell responses, we assessed the ability of cultured T cells isolated from PECAM-1−/+ mice and PECAM-1−/− mice to respond to stimulation with antibody against CD3 [anti-CD3; which stimulates T cell receptor (TCR) signaling] in the absence or presence of TGF-β. In the absence of TGF-β, anti-CD3–stimulated PECAM-1−/+ and PECAM-1−/− CD8+ T cells did not differ with respect to the amount of interferon-γ (IFN-γ) that they produced (Fig. 2A) or the percentage of cells that were positive for granzyme B (Fig. 2, B and C). However, PECAM-1−/+ and PECAM-1−/− CD8+ T cells differed with respect to their sensitivity to inhibition by TGF-β. Thus, lower concentrations of TGF-β were required to inhibit IFN-γ production (Fig. 2A) and granzyme B synthesis (Fig. 2, B and C) by PECAM-1−/− CD8+ T cells, and PECAM-1−/+ CD8+ T cells produced substantially more of these factors than did PECAM-1−/+ CD8+ T cells at all concentrations of TGF-β. Similar results were obtained from experiments with CD4+ T cells, such that anti-CD3–stimulated PECAM-1−/+ and PECAM-1−/− CD4+ T cells did not differ with respect to the amount of IFN-γ that they produced (Fig. 2D) or the extent to which they proliferated (Fig. 2, E and F) in the absence of TGF-β. However, the concentrations of TGF-β that inhibited IFN-γ production (Fig. 2D) and proliferation (Fig. 2, E and F) by PECAM-1−/+ CD4+ T cells were less than those required to produce the same degree of inhibition in PECAM-1−/+ CD4+ T cells. Furthermore, PECAM-1−/+ CD4+ T cells produced substantially more IFN-γ and proliferated to a greater extent than did PECAM-1−/+ CD4+ T cells at all inhibitory concentrations of TGF-β. Similar results were obtained from experiments with PECAM-1−/+ and PECAM-1−/− CD8+ and CD4+ T cells stimulated with anti-CD3 and anti-CD28 in the absence or presence of TGF-β (fig. S1). At very high concentrations of TGF-β, granzyme B and IFN-γ production by PECAM-1−/+ CD8+ T cells was more inhibited than was by PECAM-1−/+ CD8+ T cells, and the difference in cytokine production by stimulated PECAM-1−/+ and PECAM-1−/− CD8+ T cells in the absence of TGF-β was not statistically significant (fig. S2). Together, these results suggest that PECAM-1 potentiates the TGF-β–mediated inhibition of T cell function in mice.

PECAM-1 enhances the ability of TGF-β to inhibit the function of human T cells in a Smad2-independent manner

In humans, PECAM-1 is present on early hematopoietic progenitor cells, but is lost from the surfaces of B cells and a subset of T cells upon maturation, such that naïve, but not activated, T cells express PECAM-1 (13, 14). To determine whether human PECAM-1–positive and PECAM-1–negative T cells exhibited similar differential sensitivity to inhibition by TGF-β as that observed in mice, we isolated PECAM-1–positive and PECAM-1–negative CD8+ and CD4+ T cells from the peripheral blood of healthy adult human volunteers and assessed responsiveness to stimulation with OKT3 (anti-human CD3) in the presence and absence of TGF-β (Fig. 3). As was
Fig. 2. PECAM-1 enhances the TGF-β-mediated inhibition of mouse T cell function. (A to F) CD8+ T cells (A to C) and CD4+ T cells (D to F) purified from PECAM-1+/+ mice (closed bars) and PECAM-1−/− mice (open bars) were stimulated for 72 hours with plate-bound anti-CD3 antibody in the presence of the indicated concentrations of TGF-β. (A and D) Cell culture medium was analyzed by enzyme-linked immunosorbent assay (ELISA) to determine the concentration of secreted IFN-γ. Data are means ± SD of three independent experiments. (B and C) Granzyme B synthesis by CD8+ T cells from the indicated mice was determined by flow cytometry. Numbers in the histograms (B) indicate the percentages of cells that fell within the indicated gates. (C) Quantitative analysis of the percentages of cells that were positive for granzyme B. Data are means ± SD of four independent experiments. (E and F) The proliferation of CD4+ T cells was analyzed by flow cytometry. Numbers in the histograms (E) indicate the percentages of cells associated with the indicated peaks. (F) Quantitative analysis of the percentage of carboxyfluorescein diacetate succinimidyl ester (CFSE)–positive (undivided) cells. Data are means ± SD of four independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
Fig. 3. PECAM-1 enhances TGF-β–mediated inhibition of human T cell function. (A to F) CD8+ (A to C) and CD4+ (D to F) PECAM-1–positive T cells (filled bars) and PECAM-1–negative T cells (open bars) were purified from the peripheral blood of healthy adult volunteers and stimulated for 72 hours with plate-bound OKT3 in the presence of the indicated concentrations of TGF-β. (A and D) Cell culture medium was analyzed by ELISA to determine the concentration of secreted IFN-γ. Data are means ± SD of 10 independent experiments. (B and C) Granzyme B synthesis by CD8+ T cells was determined by flow cytometry. Numbers in the histograms (B) indicate the percentages of cells that fell within the indicated gates. (C) Quantitative analysis of the percentages of cells that were positive for granzyme B. Data are means ± SD of four independent experiments. (E and F) The proliferation of CD4+ T cells was analyzed by flow cytometry. Numbers in the histograms (E) indicate the percentages of cells associated with the indicated peaks. (F) Quantitative analysis of the percentage of CFSE-positive (undivided) cells. Data are means ± SD of four to six independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
observed with mouse T cells, in the absence of TGF-β, PECAM-1–positive and PECAM-1–negative CD8+ (Fig. 3, A to C) and CD4+ (Fig. 3, D to F) human T cells produced similar amounts of cytokines and proliferated to a similar extent when stimulated with OKT3. Also similar to mouse CD8+ T cells, lower concentrations of TGF-β were required to inhibit IFN-γ production (Fig. 3A) and granzyme B synthesis (Fig. 3, B and C) by PECAM-1–positive human CD8+ T cells than were required to exert similar effects on PECAM-1–negative CD8+ human T cells. Furthermore, relative to their PECAM-1–positive counterparts, PECAM-1–negative CD8+ human T cells produced substantially more IFN-γ (Fig. 3A) at all but the highest concentration of TGF-β tested, and they synthesized more granzyme B at all TGF-β concentrations tested (Fig. 3, B and C).

With respect to human PECAM-1–negative CD4+ T cells, the concentrations of TGF-β that were required to inhibit IFN-γ production (Fig. 3D) and proliferation (Fig. 3, E and F) were greater than those required to inhibit these functions in PECAM-1–positive cells. PECAM-1–negative CD4+ T cells proliferated to a greater extent than did PECAM-1–positive CD8+ T cells at all but the lowest concentrations of TGF-β tested (Fig. 3, E and F). However, perhaps because of the large variability in the amounts of IFN-γ secreted by human CD4+ T cells, PECAM-1–negative CD4+ human T cells produced substantially more IFN-γ than did their PECAM-1–positive counterparts at only one of the concentrations of TGF-β tested (Fig. 3D). Similar results were obtained from experiments with PECAM-1–positive and PECAM-1–negative human CD8+ and CD4+ T cells stimulated with OKT3 and anti-CD28 in the absence or presence of TGF-β (Fig. S3). Similar to findings from the mouse experiments, the highest concentrations of TGF-β had a greater inhibitory effect on granzyme B and IFN-γ production by PECAM-1–positive human CD8+ T cells than on that by PECAM-1–negative cells (Fig. S4). Furthermore, the difference in cytokine production between PECAM-1–positive and PECAM-1–negative CD8+ T cells stimulated in the absence of TGF-β was not statistically significant (Fig. S4).

To verify that the ability of TGF-β to elicit PECAM-1–dependent inhibition of T cell responses was dependent on TGF-β receptor signaling, we assessed the effects of maba–hTGF-β, which blocks the binding of TGF-β to the TGF-βRI/II complex, and SB-505124, which blocks the kinase activity of the TGF-βRI/II complex, on the responses of PECAM-1–positive and PECAM-1–negative human T cells to stimulation with anti-CD3 in the presence or absence of TGF-β. We found that TGF-β failed to inhibit cytokine synthesis by CD8+ T cells and failed to inhibit both IFN-γ production and proliferation by CD4+ T cells in the presence of either inhibitor. Furthermore, inhibitor-treated PECAM-1–positive and PECAM-1–negative T cells responded equivalently when stimulated with anti-CD3 in the presence of a high concentration of TGF-β (Fig. S5).

To determine whether the enhanced sensitivity of PECAM-1–positive human T cells to TGF-β was dependent on the presence of PECAM-1 on these cells or was instead attributable to properties acquired by PECAM-1–positive cells during their development and maturation in vivo, we used a lentiviral transduction system to restore PECAM-1 in PECAM-1–negative human T cells in vitro (Fig. 4A). Production of IFN-γ by PECAM-1–negative and PECAM-1–reconstituted human T cells was assessed after they were stimulated with OKT3 in the presence or absence of TGF-β. Relative to the PECAM-1–negative cells from which they were generated, human PECAM-1–reconstituted CD8+ T cells (Fig. 4B) and CD4+ T cells (Fig. 4C) produced similar amounts of IFN-γ in the absence of TGF-β but produced substantially less IFN-γ in the presence of almost all of the concentrations of TGF-β tested. Together, the data suggest that, as in mice, TGF-β is required for PECAM-1 to suppress the responses of human T cells to TCR stimulation, and that PECAM-1 markedly enhances the sensitivity of human T cells to TGF-β.

TGF-β signals both through canonical pathways that result from direct phosphorylation of effector Smad proteins by the TGF-β receptor and through Smad-independent signals that vary by cell type (24). To determine whether PECAM-1 influenced the TGF-β–mediated activation of Smads, we examined PECAM-1+/+ and PECAM-1−/− T cells for the nuclear accumulation of phosphorylated Smad2 after exposure to TGF-β, a process that requires phosphorylation of Smad2 and Smad3, complex formation with Smad4, and nuclear translocation (25). We observed equivalent amounts of phosphorylated Smad2 in the nuclei of PECAM-1+/+ and PECAM-1−/− T cells after exposure to TGF-β, in a manner that was not affected by additional stimulation with anti-CD3 (Fig. 5). This result suggests that PECAM-1 does not contribute to TGF-β signaling through the canonical activation of Smad complexes.

TGF-β stimulates the formation of a PECAM-1–TGF-βRII/II complex on the surfaces of T cells

To examine whether the cooperative inhibitory action of TGF-β and PECAM-1 was a result of an agonist-induced interaction between PECAM-1 and the TGF-β receptor complex in the plasma membrane, we used communoprecipitation, confocal immunofluorescence microscopy, and in situ proximity ligation assay (PLA) to evaluate the extent to which PECAM-1 formed a complex with TGF-βRII/II in human T cells cultured in the presence or absence of OKT3 and TGF-β. We found that both TGF-βRI and TGF-βRII communoprecipitated with PECAM-1 in lysates of T cells stimulated with OKT3 in the presence of TGF-β (Fig. 6A). Confocal immunofluorescence microscopy revealed the low extent of colocalization of PECAM-1 with TGF-βRI and TGF-βRII in T cells exposed to TGF-β alone, which was enhanced upon stimulation of T cells with OKT3 (Fig. 6B). PLAs revealed a weak interaction between TGF-βRII/II and PECAM-1 in OKT3-stimulated cells, a stronger interaction in the presence of TGF-β alone, and the strongest interaction when cells were activated by both OKT3 and TGF-β (Fig. 6C). The interaction between PECAM-1 and TGF-βRII in T cells stimulated with OKT3 and TGF-β was inhibited by the TGF-β blocking antibody maba–hTGF-β and the TGF-βRII kinase inhibitor SB-505124, which demonstrated that this interaction required TGF-β receptor signaling (Fig. S6).

Note that the signals associated with TGF-βRI and TGF-βRII, as measured by confocal microscopy, appeared to be more intense in cells treated with TGF-β than in unstimulated cells or in cells treated with OKT3 in the absence of TGF-β (Fig. 6B). To address the concern that the abundances of TGF-βRI and TGF-βRII in the T cells might change depending on the stimulation conditions, we evaluated the amounts of TGF-βRI and TGF-βRII by Western blotting and flow cytometry. We found that the amounts of TGF-βRI and TGF-βRII or β in or on T cells did not change as a function of the different stimulation conditions (Fig. 6A). On the basis of these findings, we conclude that the appearance of the more intense TGF-βRI and TGF-βRII staining on those T cells cultured in the presence of TGF-β (Fig. 6B) was likely a result of the colocalization of TGF-βRI and TGF-βRII with PECAM-1, which was stained with a fluorescently tagged antibody that apparently enhanced the intensity of the signal of the fluorescently tagged antibodies used to stain for TGF-βRI and TGF-βRII.

TGF-β elicits PECAM-1 ITIM tyrosine phosphorylation and SHP-2 binding in T cells

We previously demonstrated that the PECAM-1–mediated inhibition of signals transduced by antigen receptors in immortalized T and B cells requires the phosphorylation of the two ITIMs that surround the tyrosine (Y) residues at positions 663 and 686 of human PECAM-1 followed by the binding of SHP-2 to these sites (26, 27). To determine whether TGF-β stimulated PECAM-1 ITIM phosphorylation and SHP-2 recruitment, we cultured human T cells in the presence or absence of OKT3 and TGF-β.
for 1, 6, or 18 hours; after which, the cells were lysed and PECAM-1 was subjected to immunoprecipitation. Western blotting analysis of PECAM-1 immunoprecipitates revealed that ITIM phosphorylation and SHP-2 binding occurred in response to TGF-β and were enhanced by concurrent stimulation with OKT3 (Fig. 7A). Treatment with either SB-505124 or maba–hTGF-β completely blocked PECAM-1 ITIM phosphorylation and SHP-2 binding in T cells in response to TGF-β and OKT3 (Fig. 7B). Under these conditions, the TGF-β–mediated inhibition of OKT3-induced proliferation of both PECAM-1–positive and PECAM-1–negative T cells was blocked (fig. S5D). Together, these data suggest that PECAM-1 and TGF-β RI/II co-operatively inhibit T cell responses through the TGF-β–dependent formation of a PECAM-1–TGF-β RI/RII complex that induces the phosphorylation of PECAM-1 ITIMs and the recruitment of SHP-2.

**DISCUSSION**

The vascular cell adhesion molecule PECAM-1 has been extensively studied over the past two decades, and it is known to play a crucial role in several aspects of the biology of endothelial cells and cells of hematopoietic origin (11, 28). Whereas the best-described functions of PECAM-1 relate to extracellular Ig domains important in leukocyte transmigration and the integrity of endothelial cell junctions, PECAM-1 also contains cytoplasmic ITIMs that inhibit activating signals within hematopoietic cells (11). In T cells in vitro, the inhibitory phosphatase SHP-2 binds to the PECAM-1 ITIMs (27); however, a physiologic role for the ITIMs in T cells has not previously been identified. A role for PECAM-1 in dampening T cell activity in vivo was postulated on the basis of the enhanced phenotype of T cell–mediated diseases, such as experimental autoimmune encephalitis in PECAM-1−/− mice, but this was largely attributed to the impaired migration of leukocyte subsets (19). Moreover, PECAM-1−/− T cells have not demonstrated enhanced functional changes after stimulation of the TCR in vitro. One potential reason for observing inhibitory effects of PECAM-1 on T cell responsiveness in vivo, but not in vitro, could be that PECAM-1 functions as an enhancer of the inhibitory effects of another molecule that is present in vivo, but not in vitro.

The present investigation was designed, therefore, to examine the hypothesis that PECAM-1 contributes to the inhibitory function of TGF-β.
effects of TGF-β, a potent inhibitor of T cells that plays a major role in suppressing antitumor responses in murine models of cancer and human malignancy. To test this, we evaluated the growth patterns of subcutaneously injected EG.7 (EL4-ova) cells, because EL4 and EL4-ova cells represent a well-established model to assess TGF-β activity in vivo, and the presence of ovalbumin enables the tracking of tumor-specific T cells (7, 29, 30). The importance of TGF-β in the growth of EL4 cells in vivo has been extensively documented; the shRNA-mediated knockdown of TGF-β1 in EL4 cells delays tumor growth in recipient mice, and overexpression of soluble TGF-βRII by EL4 cells reduces tumor growth by 80% and restores CD8+ T cell–mediated cytotoxicity (31). Additionally, expression of dominant-negative forms of TGF-βRII in CD8+ T cells results in the eradication of EL4 tumors, indicating that the pro-tumor growth properties of TGF-β result from the generation of an immune-permissive microenvironment (7). We found that EL4-ova tumors grew more slowly in PECAM-1−/− mice than in PECAM-1+/+ mice, but that the transmigration of T cells into tumors was not substantially affected. These data are consistent with a defect in TGF-β signaling, but not leukocyte transmigration, in the PECAM-1−/− mice.

We next turned to in vitro assays to directly assess whether the effects of TGF-β signaling were affected by PECAM-1 in T cells. TGF-β has myriad effects on cytotoxic T cells and directly modulates the transcription of more than 100 genes (31). Some of the most marked changes induced by TGF-β, as assessed by previous mRNA microarray studies, included inhibition of the TCR-mediated increased production of IFN-γ and granzymes A and B in cytotoxic T cells, as well as inhibition of proliferation (18). We observed attenuation of the TGF-β–mediated inhibition of murine and human T cells that lacked PECAM-1. To evaluate TGF-β signaling in murine T cells, we compared cells obtained from wild-type mice to those isolated from mice with genetic deletion of PECAM-1. To examine TGF-β signaling in human cells, we compared naïve T cells, which bear PECAM-1, with antigen-experienced T cells, which lack PECAM-1 (13, 32).

Because this experimental approach could have been confounded by the previous exposure of the cells to antigen, we repeated our experiments

Fig. 5. The TGF-β–induced phosphorylation and nuclear translocation of Smad2 are not affected by loss of PECAM-1. Total T cells isolated from mice of the indicated genotypes were left untreated or were incubated for 1 hour with TGF-β (10 ng/ml) and then were either harvested or stimulated for an additional 30 min with soluble anti-CD3 antibody (2.5 μg/ml). Cells were then lysed and fractionated, and nuclear fractions were analyzed by Western blotting with antibody against phosphorylated Smad2 (pSmad2). DNA polymerase γ was used as a loading control. Western blots are from one experiment and are representative of three independent experiments.
in human cells by evaluating PECAM-1–negative cells that were transduced with a lentivirus engineered to express PECAM-1. Under all of the circumstances tested in T cells obtained from either mice or humans, we observed that PECAM-1 enhanced the sensitivity of the T cells to TGF-β–mediated inhibition of activity. Furthermore, a correlate to this finding is that PECAM-1 may represent a means by which human and mouse T cell subsets fine-tune the responsiveness to TGF-β. Thus, human memory T cells (which are characterized by the presence of the cell surface marker CD45RO and the absence of CD45RA) and highly activated T cells lose PECAM-1 (33–36). The extent to which memory cells in mice behave similarly to human memory T cells, and whether loss of PECAM-1 renders memory T cells either resistant or more resistant to TGF-β, is currently being explored in our laboratories. Irrespective of differences in PECAM-1 abundance between human and mouse memory T cell populations, it is likely that PECAM-1 plays an important role in facilitating TGF-β signaling in naïve T cells in both organisms. IFN-γ production is abrogated by TGF-β to a greater degree in naïve T cells than in memory T cells (37), and TGF-β signaling is required to limit inappropriate T cell activation during non-TCR–mediated proliferation, such as that observed during homeostatic proliferation (38). We are currently exploring a specific role for PECAM-1 in naïve T cells, which is likely to be conserved between mice and humans, in in vivo models.

The identification of PECAM-1 as an enhancer of cell sensitivity to TGF-β suggests that PECAM-1 may act to facilitate noncanonical TGF-β signaling in T cells. In the most proximal events of TGF-β signaling, TGF-β binds to TGF-βRII, which results in phosphorylation of TGF-βRI and the formation of a tetrameric complex consisting of two subunits each of TGF-βRII and TGF-βRII. In canonical signaling, which is required for efficient TGF-β signaling in all cell types, TGF-βRII phosphorylates Smad2 and Smad3, which together with Smad4 translocate to the nucleus to affect activation or repression of target genes (39, 40). In contrast, noncanonical signaling involves the direct phosphorylation by TGF-βRII of poorly defined mediators that facilitate the activation or inhibition of numerous signaling mediators, such as phosphoinositide 3-kinase (PI3K), AKT, Ras–mitogen-activated protein kinase (MAPK) kinase (MEK)–extracellular signal–regulated kinase (ERK), nuclear factor κB (NF-κB), p38 MAPK, and many others, in a cell type–specific manner (24). As in other cells, noncanonical TGF-β signaling in T cells has not been well defined, although data from others suggest that TGF-β inhibits proximal TCR signaling through an unknown mechanism (41), and data from our own studies indicate that the TGF-β–mediated inhibition of cytotoxic T cell responses can be relieved by increasing the strength of the TCR signal (42).

Using three independent biochemical methods, we provide evidence that PECAM-1 colocalizes with both TGF-βRI and TGF-βRII in T cells in a TGF-β–dependent manner that is enhanced by TCR stimulation. This finding provides biochemical support for a role for PECAM-1 in TGF-β signaling in T cells. Furthermore, it justifies efforts to define the nature of the interaction between PECAM-1 and the TGF-β receptor complex. Additionally, we evaluated the phosphorylation status of the C-terminal PECAM-1 ITIM, which encompasses Tyr686, and observed that it was phosphorylated after TGF-β bound to its receptor and activated receptor kinase activity, in a manner enhanced by co-incubation with anti-CD3. Phosphorylation of this ITIM led to the recruitment of SHP-2, a phosphatase that antagonizes TCR signaling.

Although these findings suggest that PECAM-1 ITIM phosphorylation and SHP-2 recruitment require TGF-βRII–mediated phosphorylation events, it is unlikely that the PECAM-1 ITIMs are directly phosphorylated by TGF-βRII. Instead, initial phosphorylation of serine residues in PECAM-1 is required for the recruitment of SHP-2, a phosphatase that antagonizes TCR signaling.

**Fig. 7.** TGF-β stimulates the tyrosine phosphorylation of PECAM-1 ITIMs and SHP-2 binding in T cells, which requires TGF-β binding to its receptor and activation of TGF-βRII kinase activity. (A and B) Human CD4+ T cells were cultured in the presence or absence of plate-bound OKT3 and TGF-β for the indicated times in the absence (A) or presence (B) of an inhibitor of the kinase activity of TGF-βRII (SB-505124) or a neutralizing antibody specific for human TGF-β (maba–hTGF-β). Cell lysates were precleared with normal mouse IgG and subjected to immunoprecipitation with an antibody specific for human PECAM-1 (PECAM-1.3). Proteins present in the immunoprecipitated samples were analyzed by Western blotting with antibodies specific for total PECAM-1 protein, PECAM-1 phosphorylated at Tyr686 (PECAM-1–pY686), and SHP-2. Results are representative of three independent experiments.
because of interactions with the plasma membrane that are relieved by the phosphorylation, in activated cells, of PECAM-1 on Ser702, thereby exposing Tyr686 for phosphorylation by Src family kinases (SFKs), which are activated upon TCR stimulation (15, 16). Thus, we hypothesize that TGF-βRII phosphorylates PECAM-1 Ser702, which conforms to a consensus TGF-βRII phosphorylation site, resulting in accessibility of Tyr686 to SFKs, which leads to weak phosphorylation in the absence, and strong phosphorylation in the presence, of TCR stimulation. Experiments are under way to determine whether PECAM-1 is a direct substrate of TGF-βRII and to delineate the sequence of phosphorylation events that occur on PECAM-1 secondary to the activation of TGF-βRII/RI.

Together, our data delineate a co-inhibitory axis in T cells that is induced by TGF-β and involves the cell surface inhibitory receptor PECAM-1. These findings may provide an opportunity to improve cytotoxic T cell activity within TGF-β-rich tumor microenvironments. Enhancing the activation state of cytotoxic T cells has become an increasingly important means by which to enhance tumor therapies in malignancy. Whereas the development of antibodies against immune checkpoint receptors, such as CTLA4 and PD-1, has demonstrated the potential for using immune checkpoint therapies to enhance antitumor responses, the success of anti-PD-1 remains confined to a minority of patients with solid malignancies, and it is clear that additional strategies will be required to improve efficacy (1, 2, 43). Although TGF-β has been intensively investigated as a target to improve cytotoxic T cell activity because of its potent ability to inhibit cytotoxic T cell responses within the tumor microenvironment, its ubiquitous and pleiotropic functions in nearly all mammalian organ systems limit the utility of its systemic inhibition (44). Our results suggest that targeting of the interaction between PECAM-1 and the TGF-βRII complex in T cells might enable dampening of the effects of TGF-β selectively in T cells, which would enhance the efficacy of T cell antitumor therapies in TGF-β-rich tumor microenvironments with minimal off-target complications. Ongoing studies in tumor models will ascertain the preclinical value of pursuing PECAM-1 as a target for immunotherapies.

MATERIALS AND METHODS

Mice

PECAM-1−/− mice, in which exon 7 of the gene encoding PECAM-1 was disrupted by the introduction of a neomycin cassette, were previously generated (45) and were backcrossed for >12 generations onto a C57BL/6J background. Homozygosity for the disrupted PECAM1 allele was confirmed for all mice by performing a polymerase chain reaction (PCR) assay with tail DNA using a forward primer that hybridizes with a sequence selectively in T cells, which amplified a 626-bp (base pair) band from the disrupted allele, or a sequence within intron 7 (5′-GCTTCTGCGAAGATGATCC-3′), which amplifies a 626-bp band from the wild-type allele and a 1546-bp band from the disrupted allele. The presence of a 626-bp band and absence of a 387-bp band in the PCR product was used as confirmation of homozygosity for the disrupted PECAM1 allele in PECAM-1−/− mice. The absence of PECAM-1 from the surfaces of T cells purified from PECAM-1−/− mice was used in some cases as further confirmation of PECAM-1 deficiency (fig. S7). PECAM-1−/− (wild-type) C57BL/6J mice were purchased from Jackson Laboratories. Mice were maintained in a pathogen-free facility under the supervision of the Biological Resource Center at the Medical College of Wisconsin (MCW). Animal protocols were approved by the MCW Institutional Animal Care and Use Committee.

In vivo EL4-ova growth assay

EL4-ova cells (2.5 × 10^5) were injected subcutaneously into the flanks of wild-type or PECAM-1−/− mice. Fourteen days later, mice were euthanized, and tumors were weighed and disrupted into single cells as previously described (46). Cells in tumor cell suspensions were stained with antibodies before being analyzed with an LSR II (BD) flow cytometer.

Antibodies

Domain-specific, mouse anti-human PECAM-1 monoclonal antibodies (mAbs) PECAM-1.2 (which is specific for PECAM-1 Ig domain 6), PECAM-1.3 (which is specific for PECAM-1 Ig domain 1), and PECAM-1–pY686 were previously generated and characterized (15, 47, 48). The following antibodies were purchased: anti-phosphotyrosine antibody, clone 4G10 (EMD Millipore); anti–SH-PTP2 antibody (C-18, Santa Cruz Biotechnology); anti–TGF-βRI antibody (V-22, Santa Cruz Biotechnology); anti–TGF-βRII antibody (L-21, Santa Cruz Biotechnology); Alexa Fluor 488-conjugated AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch); and Alexa Fluor 594-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch).

Purification of PECAM-1–positive and PECAM-1–negative CD4+ and CD8+ T cells from human peripheral blood

 Buffy coats of blood obtained from healthy adult human donors were diluted with 45 ml of Hanks’ balanced salt solution, mixed gently, layered above 15 ml of Ficoll-Paque PLUS (GE Healthcare Life Sciences), and centrifuged at 400g for 30 min with minimum deceleration. Mononuclear cells (MNCs) at the interface were collected, washed twice with PBS at 100g for 10 min, and resuspended in PBS containing 0.1% bovine serum albumin (BSA) at a concentration of 10^6 cells/ml. CD4+ T cells were isolated from MNCs with the Dynabeads CD4 Positive Isolation Kit (Invitrogen/Life Technologies), CD8+ T cells were isolated from CD4-depleted MNCs with the Dynabeads Untouched Human CD8+ T Cells Kit (Invitrogen/Life Sciences).
Human T cell functional assays

CD4<sup>+</sup> and CD8<sup>+</sup> T cells were labeled with CFSE as described earlier and divided into aliquots of 2 × 10<sup>5</sup> cells per well into a 96-well tissue culture plate precoated with anti-human CD3 antibody, clone OKT3 (2.5 μg/ml; eBioscience), or with PBS as a control. Reconstituted TGF-β1 (R&D Systems) in PBS was added in the concentrations indicated in the figure legends, and cells were cultured at 37°C for the times indicated in the legends. Cell culture medium was collected for the measurement of secreted IFN-γ with the human IFN-γ ELISA Ready-SET-Go! Kit (eBioscience). Flow cytometry was used to measure the extent of CFSE dilution and for intracellular staining of granzyme B as described earlier.

Retroviral transduction of human PECAM-1–negative T cells

A retrovirus encoding human PECAM-1 was previously described (49, 50) and was generated for these studies by the Viral Vector Core Lab at the Blood Research Institute of BloodCenter of Wisconsin. PECAM-1–negative CD4<sup>+</sup> and CD8<sup>+</sup> T cells, purified as described earlier, were divided into aliquots of 2 × 10<sup>5</sup> cells per well in a 12-well tissue culture plate that had been precoated with OKT3 (2.5 μg/ml). After 24 hours at 37°C, the medium was removed and replaced with viral supernatant (3.62 × 10<sup>6</sup> transduction units/ml) containing polybrene (Santa Cruz Biotechnology) at a final concentration of 4 μg/ml. After an additional 6 to 8 hours of culture, T cell medium (1 ml) was added. After a further 48 hours of culture, the PECAM-1–reconstituted T cells were purified as described earlier.

PECAM-1 immunoprecipitation and Western blotting analysis

Purified human CD4<sup>+</sup> T cells were divided into aliquots of 2 × 10<sup>6</sup> cells per well in a 12-well tissue culture plate that was precoated with OKT3 (2.5 μg/ml) or PBS as a control and cultured for 1, 6, and 18 hours at 37°C in the absence or presence of TGF-β (4.5 ng/ml). Where indicated in the figures, the activin receptor–like kinase 5 (ALK5) inhibitor SB-505124 (Sigma-Aldrich; 5 μg/ml) or the anti–hTGF-β–IgG neutralizing mAb (InvivoGen; 5 μg/ml) was added. After the cells were cultured for the times indicated in the figure legends, the cells were lysed in 250 μl of cell lysis buffer [150 mM NaCl, 30 mM Heps, 10 mM tris (pH 7.2), 0.1% SDS, 1% NP-40, 0.5% deoxycholate, 5 mM EDTA, with protease and phosphatase inhibitors (Calbiochem)] on ice for 10 min. Lysates were precleared with 20 μl of Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology) in the presence of normal mouse IgG (Jackson ImmunoResearch) for 1 hour at room temperature. Precleared lysates were incubated with 0.2 to 2 μg of PECAM-1.2 or PECAM-1.3 mAb for 1 hour at 4°C followed by incubation with 20 μl of Protein A/G PLUS-Agarose beads overnight at 4°C. Beads were pelleted by centrifugation for 5 min at 3500g at 4°C and washed four times with 1 ml of cell lysis buffer; after which, bound proteins were eluted into electrophoresis sample buffer by boiling for 10 min. Immunoprecipitated proteins were resolved by SDS–polyacrylamide gel electrophoresis on 7.5% acrylamide gels and transferred to Immobilon-P PVDF membranes (EMD Millipore) for Western blotting analysis. Membranes were blocked in 3% nonfat milk and 1 × tris-buffered saline, 0.1% Tween-20 (TBST) at room temperature for 1 hour and incubated with primary antibodies in blocking buffer overnight at 4°C. Membranes were washed three times for 5 min each with 1 × TBST, incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies in blocking buffer for 1 hour at room temperature, and then washed three times for 10 min each with 1 × TBST. HRP-conjugated antibodies were detected with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Immunofluorescence staining

Human CD4<sup>+</sup> T cells were treated with or without TGF-β in the presence or absence of immobilized OKT3 for 6 hours in a slide chamber, fixed with 4% paraformaldehyde for 1 hour at room temperature, washed twice with PBS, and blocked with 100 μl of blocking buffer (10% BSA in PBS) in a humidified chamber at room temperature for 1 hour. After the blocking buffer was drained off the slides, 100 μl of appropriately diluted primary antibody (1:100 in 0.5% BSA in PBS) was added to slide sections and incubated in a humidified chamber at room temperature for 2 hours. The slides were rinsed twice in PBS and then incubated with 100 μl of diluted secondary antibody (1:200 in PBS) in a humidified chamber at room temperature for 1 hour. Slides were rinsed twice in PBS before 100 μl of 28.6 μM 4’6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) in PBS was added to each well of the slides. The slides were rinsed three times in PBS and covered with a coverslip using VECTASHIELD HardSet Antifade Mounting Medium (Vector Laboratories). The mounted samples were analyzed with an Olympus multiphoton laser scanning confocal microscope (FV1000-MPE).

In situ PLA

The in situ PLA (Olink) was performed according to the manufacturer’s instructions. Human CD4<sup>+</sup> T cells were treated as described earlier for immunofluorescence staining. PLA probes were attached to primary antibodies specific for TGF-βRI or TGF-βRII and to PECAM-1, 3, according to the manufacturer’s instructions; after which, the antibodies indicated in the figure legends were added to cells and incubated for 1 hour at 37°C. The slides were washed three times with tris-buffered saline containing 0.05% Tween-20 (TBST) and soaked for 1 min in 1 × ligation buffer [10 mM tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate (pH 7.5)]. The soaking buffer was removed, and the slides were incubated with Duolink Ligase diluted in ligation buffer for 30 min at 37°C and then were washed three times with TBST. The polymerase mix was added, and the slides were incubated for 1.5 hours at 37°C, washed three times with TBST, and then incubated again with detection solution for 30 min at 37°C in the dark. Slides were washed again, stained with DAPI, and covered with a coverslip. The mounted samples were analyzed with an Olympus multiphoton laser scanning confocal microscope.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software (GraphPad Software). Data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparisons test. Multiple comparisons tests were only applied when a statistically significant difference was determined by ANOVA (P < 0.05).

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/9/418/ra27/DC1

Fig. S1. PECAM-1 enhances the TGF-β–mediated inhibition of mouse T cells stimulated to the manufacturer’s instructions; after which, the antibodies indicated in the figure legends were added to cells and incubated for 1 hour at 37°C. The slides were washed three times with tris-buffered saline containing 0.05% Tween-20 (TBST) and soaked for 1 min in 1 × ligation buffer [10 mM tris-acetate, 10 mM magnesium acetate, 50 mM potassium acetate (pH 7.5)].

The soaking buffer was removed, and the slides were incubated with Duolink Ligase diluted in ligation buffer for 30 min at 37°C and then were washed three times with TBST. The polymerase mix was added, and the slides were incubated for 1.5 hours at 37°C, washed three times with TBST, and then incubated again with detection solution for 30 min at 37°C in the dark. Slides were washed again, stained with DAPI, and covered with a coverslip. The mounted samples were analyzed with an Olympus multiphoton laser scanning confocal microscope.

Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software (GraphPad Software). Data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparisons test. Multiple comparisons tests were only applied when a statistically significant difference was determined by ANOVA (P < 0.05).

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/9/418/ra27/DC1

Fig. S1. PECAM-1 enhances the TGF-β–mediated inhibition of mouse T cells stimulated with anti-CD3 and anti-CD28 antibodies.

Fig. S2. PECAM-1 enhances the inhibition of mouse T cell function by low, but not high, concentrations of TGF-β.

Fig. S3. PECAM-1 enhances the TGF-β–mediated inhibition of mouse T cells stimulated with OKT3 and anti-CD28.

Fig. S4. PECAM-1 enhances the inhibition of mouse T cell function by low, but not high, concentrations of TGF-β.

Fig. S5. The ability of PECAM-1 to enhance the TGF-β–mediated inhibition of human T cells requires the binding of TGF-β to its receptor and activation of the kinase activity of TGF-βRII.
Fig. S7. PECAM-1 is expressed on the surface of T cells purified from wild-type, but not PECAM-1−/−, mice.

REFERENCES AND NOTES


**Acknowledgments:** We thank P. Newman (Blood Research Institute) and S. Albelda (University of Pennsylvania) for thoughtful insights and R. Silverstein (MCW) and his laboratory for technical advice regarding the PLA. **Funding:** The work was supported by grants from the NIH, P01-HL44612 (D.K.N.) and K08-CA151893 (M.J.R.); the BloodCenter Research Foundation (D.K.N.); the American Society for Hematology (D.K.N. and M.J.R.); the Kathy Duffy Fogarty Foundation for Breast Cancer Research (M.J.R.); and an institutional grant from the American Cancer Society (M.J.R.). **Author contributions:** D.K.N. and M.J.R. designed the study and wrote the manuscript. G.F. and T.A. performed the experiments with assistance from V.A. and T.B.; and W.C. provided advice and technical assistance. **Competing interests:** The authors declare that they have no competing interests.

Submitted 29 July 2015
Accepted 19 February 2016
Final Publication 8 March 2016
10.1126/scisignal.aad1242

Sticking it to T cells

Because of its immunosuppressive effects and abundance in tumor microenvironments, the cytokine transforming growth factor β (TGF-β) inhibits the antitumor activities of T cells. Targeting TGF-β systemically would interfere with other functions of TGF-β, thus, therapies that block the effects of TGF-β specifically on T cells are needed. Newman et al. found that T cells without the adhesion molecule PECAM-1 were less sensitive to the TGF-β–mediated inhibition of their function than were PECAM-1-positive cells. In a tumor model, PECAM-1–deficient mice exhibited reduced tumor size compared to wild-type mice. Thus, targeting PECAM-1 may specifically enhance the antitumor activity of T cells.