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

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Abstract

Transforming growth factor- β (TGF- β) is an immunosuppressive cytokine that inhibits the pro-inflammatory functions of T cells, and it is a major factor in abrogating T cell activity against tumors. Canonical signaling results in the activation of Smad proteins, transcription factors that regulate target gene expression. Here, we found that the cell surface molecule platelet endothelial cell adhesion molecule-1 (PECAM-1) facilitates non-canonical (Smad-independent) TGF- β signaling in T cells. Subcutaneously injected tumor cells dependent on TGF- β -mediated suppression of immunity 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 re-expression 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 domain-containing tyrosine phosphatase-2 (SHP-2). Such stimulatory conditions also induced the co-localization of PECAM-1 with the TGF- β receptor complex as identified by co-immunoprecipitation, 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

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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 anti-tumor responses of T cells within the tumor microenvironment and interfere with tumor eradication (2). Immune checkpoint therapies block interactions between immune checkpoint receptors and their ligands to enhance anti-tumor responses (1, 2). Although immune checkpoint therapy has emerged as a potent means to enhance the anti-tumor 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- β RI) and TGF- β RII 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 anti-tumor 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 anti-tumor 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 type I 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 trans-endothelial migration (11). Phosphorylation of the cytoplasmic ITIMs of PECAM-1 depends upon a series of serine and threonine (S/T) and tyrosine (Y) 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 resultant 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 anti-tumor 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- β RI/II complex and 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 tumor 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 a derivative cell line EL4-ova, engineered to constitutively express the immunogenic protein ovalbumin (18). For example, short hairpin RNA (shRNA)-mediated decreases in TGF β abundance within EL4 tumor cells, or expression of dominant-negative forms of TGF- β RI/II in T cells, result in tumor rejection. Although PECAM-1 exerts immunosuppressive activity in T cells (19–22), it is not known whether the immunosuppressive properties of PECAM-1 are sufficient to effect 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 seven-week old PECAM-1^{+/+} or PECAM-1^{-/-} mice were inoculated in the flank, and tumor growth was assessed three 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 transendothelial 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 (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 function

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 , PECAM-1^{+/+} and PECAM-1^{-/-} CD8⁺ T cells did not differ with respect to the amount of 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 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 that by PECAM-1^{-/-} CD8⁺ T cells, and the difference in cytokine production by stimulated PECAM-1^{+/+} and PECAM-1^{-/-} CD8⁺ T cells was not statistically significant (fig. S2). Together, these results indicate 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 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 mab-ahTGF- β , which blocks the binding of TGF- β to the TGF- β RI/II complex, and SB-505124, which blocks the kinase activity of TGF- β RI/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 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 Smad, 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- β RI/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 co-immunoprecipitation, confocal immunofluorescence microscopy, and in situ proximity ligation assay (PLA) to evaluate the extent to which PECAM-1 formed a complex with TGF- β RI/II in human T cells cultured in the presence or absence of OKT3 and TGF- β . We found that both TGF- β RI and TGF- β RII co-immunoprecipitated 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 co-localization of PECAM-1 with TGF- β RI and TGF- β RII 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- β RI/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- β RI in T cells stimulated with OKT3 and TGF- β was inhibited by the TGF- β blocking antibody maba-hTGF- β and the TGF- β RI/II kinase inhibitor SB-505124, which demonstrated that this interaction requires TGF- β receptor signaling (Fig. S6).

Note that signals associated with TGF- β RI and TGF- β RII, 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- β RI and TGF- β RII in the T cells might change depending upon the stimulation conditions, we evaluated the amounts of TGF- β RI and TGF- β RII by Western blotting and flow cytometry. We found that the amounts of TGF- β RI and TGF- β RII in or on T cells did not change as a function of the different stimulation conditions (Fig. 6A). Based on these findings, we conclude that the appearance of more intense TGF- β RI and TGF- β RII staining on those T cells cultured in the presence of TGF- β (Fig. 6B) was likely a result of the co-localization of TGF- β RI and TGF- β RII 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- β RI and TGF- β RII.

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 mAb-hTGF- β completely blocked PECAM-1 ITIM phosphorylation and SHP-2 binding in T cells in response to TGF- β and (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 fully inhibited (Fig. S5D). Together, these data suggest that PECAM-1 and TGF- β RI/II cooperatively inhibit T cell responses through the TGF- β -dependent formation of a PECAM-1/TGF- β RI/II 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 ITIM (27), however, a physiologic role for the ITIM in T cells has not previously been identified. A role for PECAM-1 in dampening T cell activity in vivo was postulated based on the enhanced phenotype of T cell-mediated diseases, such as experimental autoimmune encephalitis (EAE) 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 effects of TGF- β , a potent inhibitor of T cells that plays a major role in suppressing anti-tumor responses in both 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- β -1 in EL4 cells delays tumor growth in recipient mice, and over-expression of soluble TGF- β RII by EL4 cells reduces tumor growth by 80% and restores CD8+ T cell-mediated cytotoxicity (31). Additionally, expression of dominant negative forms of TGF- β RII 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 were 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 over 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 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 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 in either humans or mice 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. Indeed, 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 non-canonical TGF- β signaling in T cells. In the most proximal events of TGF- β signaling, TGF- β binds to TGF- β RI, which results in phosphorylation of TGF- β RII and the formation of a tetrameric complex consisting of two

subunits each of TGF- β RI and TGF- β RII. In canonical signaling, which is required for efficient TGF- β signaling in all cell types, TGF- β RII phosphorylates Smad2 and Smad3, which together with Smad4 translocate to the nucleus to effect activation or repression of target genes (39, 40). In contrast, non-canonical signaling involves the direct phosphorylation by TGF- β RI/II of poorly defined mediators that facilitate the activation or inhibition of numerous signaling mediators such as phosphoinositide 3-kinase (PI(3)K), AKT, Ras, mitogen-activated protein kinase (MAPK), MAPK kinase (MEK), extracellular signal-regulated kinase (Erk), nuclear factor κ B (NF- κ B), p38 MAPK, and many others, in a cell type-specific manner (24). As in other cells, non-canonical 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 co-localizes with both TGF- β RI and TGF- β RII 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 RI/II 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- β RI/II-mediated phosphorylation events, it is unlikely that the PECAM-1 ITIMs are directly phosphorylated by TGF- β RI/II. Instead, initial phosphorylation of serine residues in PECAM-1 by TGF- β RI/II likely makes the ITIMs accessible to other kinases for phosphorylation. This prediction is consistent with our previous findings that, in resting cells, the C-terminal PECAM-1 ITIM is cryptic 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- β RII phosphorylates PECAM-1 Ser⁷⁰², which conforms to a consensus TGF- β RII 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 underway to determine whether PECAM-1 is a direct substrate of TGF- β RII, and to delineate the sequence of phosphorylation events that occur on PECAM-1 secondary to the activation of TGF- β RI/II.

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 anti-

tumor 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 limits the utility of its systemic inhibition (44). Our results suggest that targeting of the interaction between PECAM-1 and the TGF- β RI/II 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 anti-tumor 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 within exon 7 (5'-CTGCCAGTCCGAAAATGGAAC-3') and reverse primers that hybridize with either a sequence within the neomycin cassette (5'-TGCTCCTGCCGAGAAAGTATCC-3'), which amplifies a 626-bp band from the disrupted allele, or a sequence within intron 7 (5'-GGTCAAAAAGGGTTACAACGGC-3'), which amplifies a 387-bp band from the wild-type (WT) 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. 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^{+/+} (WT) 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

2.5×10^5 EL4-ova cells were injected subcutaneously into the flanks of WT 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 β RI antibody (V-22, Santa Cruz Biotechnology); anti-TGF β -RII antibody (L-21, Santa Cruz Biotechnology); Alexa Fluor 488-conjugated AffiniPure goat anti-mouse IgG (H+L, Jackson ImmunoResearch); Alexa Fluor 594-conjugated AffiniPure goat anti-rabbit IgG (H+L, Jackson ImmunoResearch).

Mouse T cell purification and functional assays

Mouse splenic CD4⁺ and CD8⁺ T cells were purified with the CD4⁺ T cell Isolation Kit II (Miltenyi Biotec) and the EasySep Mouse CD8⁺ T Cell Isolation Kit (StemCell), respectively. After purification, cells were resuspended in T cell medium [RPMI-1640 complete medium containing penicillin (100 μ g/ml), streptomycin (100 μ g/ml), 2 mM L-glutamine, 10 mM HEPES (pH 7.0), 10 mM nonessential amino acids, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol, 10% fetal bovine serum (FBS)] at 10×10^6 cells/ml and incubated at 37°C for 25 min with an equal volume of 8 μ M carboxyfluorescein succinimidyl ester (CFSE) from the CellTrace CFSE Cell Proliferation Kit (Life Technologies), which was diluted in phosphate-buffered saline (PBS). Cells were then washed twice with 20 $\times$ volume of cold T cell medium and divided into aliquots of 1.5 to 2×10^5 cells/well in a 96-well tissue culture plate (Midwest Scientific) that had been pre-coated with anti-mouse CD3e antibody clone 145–2C11 (BD Bioscience) at a concentration of 2.5 μ g/ml for CD4⁺ cells and 1 μ g/ml for CD8⁺ cells. Cells cultured in uncoated wells served as a control. Recombinant mouse TGF- β 1 protein (R&D Systems) was added at the concentrations indicated in the figure legends, and cells were cultured at 37°C for 72 hours. Culture medium was collected for measurement of IFN- γ secretion with a previously described assay (42). Flow cytometry was used to measure the extent of CFSE dilution and intracellular staining of granzyme B with a phycoerythrin (PE)-conjugated anti-granzyme B antibody, clone GB11 (Invitrogen/Life Technologies). The isolation of T cell nuclei after stimulation with TGF β and subsequent Western blotting analysis for phosphorylated Smad2 and DNA polymerase γ in cell lysates were performed as previously described (42).

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 blood donors were diluted with 45 ml of Hanks balanced salt solution (HBSS), 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 10^7 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 using Dynabeads Untouched Human CD8 T Cells Kit (Invitrogen/Life Technologies), and PECAM-1-positive and PECAM-1-negative T cells were separated from the purified CD4⁺ and CD8⁺ T cells using Dynabeads CD31 Endothelial Cell Kit (Invitrogen/Life Technologies).

Human T cell functional assays

CD4⁺ and CD8⁺ T cells were labeled with CFSE as described earlier and divided into aliquots of 2×10^5 cells/well into a 96-well tissue culture plate pre-coated with anti-human CD3, clone OKT3 (2.5 µg/ml, eBioscience) or with PBS as a control. Recombinant TGF-β-1 (R&D Systems) diluted in PBS was added at 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 been previously described (49, 50) and was generated for these studies by the Viral Vector Core Lab at the Blood Research Institute of the BloodCenter of Wisconsin. PECAM-1-negative CD4⁺ and CD8⁺ T cells, purified as described earlier, were divided into aliquots of 2×10^6 cells/well in a 12-well tissue culture plate that had been pre-coated with OKT3 (2.5 µg/ml). After 24 hours at 37°C, the medium was removed and replaced with viral supernatant (3.62×10^8 transduction units/ml) containing polybrene (Santa Cruz) at a final concentration of 4 µg/ml. After an additional 6–8 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 blot analysis

Purified human CD4⁺ T cells were divided into aliquots of 2×10^6 cells/well in a 12-well tissue culture plate that was pre-coated 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 TGFβ (4.5 ng/ml). Where indicated in the figures, the activin receptor-like kinase 5 (ALK) inhibitor SB-505124 (Sigma Aldrich; 5 µg/ml) or the anti-hTGF-β-IgA neutralizing monoclonal antibody (InvivoGen; 5 ng/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 Hepes, 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 pre-cleared with 20 µl of Protein A/G Plus-Agarose beads (Santa Cruz) in the presence of normal mouse IgG (NMIgG, Jackson ImmunoResearch) for 1 hour at room temperature. Pre-cleared 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-PAGE on 7.5% acrylamide gels and transferred to Immobilon-P PVDF membranes (EMD Millipore) for Western blotting analysis. Membranes were blocked in 3% non-fat 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 horse radish 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).

Immunofluorescent staining

Human CD4⁺ 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, 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 of 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 Vecta-Shield 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⁺ T cells were treated as described earlier for immunofluorescent 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 [10mM Tris-acetate, 10mM magnesium acetate, 50 mM potassium acetate, (pH 7.5)]. The soaking buffer was removed, 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 were incubated with detection solution for 30 min at 37°C in the dark. Slides were again washed, 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 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 Material

Refer to Web version on PubMed Central for supplementary material.

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Summary

TGF- β uses PECAM-1 to enhance inhibition of T cell responses and tumor clearance by inducing formation of a complex between PECAM-1 and TGF- β receptor subunits that results in PECAM-1 phosphorylation and SHP-2 binding.

Figure 1. EL4-ova tumors grow more slowly in PECAM-1^{-/-} than in PECAM-1^{+/+} mice (A and B) PECAM-1^{+/+} mice (closed symbols) and PECAM-1^{-/-} mice (open symbols) were inoculated with EL4-ova lymphoma cells (2.5×10^5 cells) by subcutaneous flank injection. (A) Tumor mass was determined after tumor excision from euthanized mice fourteen days later. Data are means $\pm$ SD from three pooled experiments. (B) Tumor cell suspensions were prepared from tumors excised from the indicated mice. CD4⁺ (top) and CD8⁺ (bottom) tumor infiltrating lymphocytes (TILs) were identified by flow cytometry after gating for live cell populations. Results are presented as the percentage of total cells within the tumor cell suspension that were TILs. Data are means $\pm$ SD pooled from three independent experiments. n.s., not significant.

Figure 2. PECAM-1 enhances 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 ELISA to determine the concentration of secreted IFN-γ. Data are mean ± 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. 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 independent experiments.

P* < 0.05, *P* < 0.01, ****P* < 0.001, *****P* < 0.0001

Figure 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 $\pm$ 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 $\pm$ 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 $\pm$ SD of four to six independent experiments. * P <0.05, ** P <0.01, *** P <0.001, **** P <0.0001

Figure 4. PECAM-1 enhances the sensitivity of human T cells to TGF- β -mediated inhibition (A) Human CD8⁺ (top) and CD4⁺ (bottom) were left unseparated (left), enriched for PECAM-1-negative cells (middle) or were enriched for PECAM-1 negativity before being transduced with a virus encoding human PECAM-1 (right). Each group of cells was then analyzed by flow cytometry after incubation with isotype control (dashed lines) or anti-PECAM-1 antibodies (solid lines). Histograms are representative of four independent experiments. **(B and C)** PECAM-1-reconstituted (hatched bars) and PECAM-1-negative (open bars) CD8⁺ (B) and CD4⁺ T cells were stimulated for 72 hours with plate-bound OKT3 in the presence of the indicated concentrations of TGF- β . Cell culture medium was analyzed by ELISA to determine the concentration of secreted IFN- γ . Data are means $\pm$ SD of ten independent experiments. * P <0.01, ** P <0.001, *** P <0.0001, **** P <0.00001

Figure 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 genotype were left untreated or were incubated for 1 hour with TGF β (10 ng/mL) and then were either harvested or were stimulated for an additional 30 min with soluble anti-CD3 antibody (2.5 μ g/ml). Cells were then lysed and fractionated and nuclear fractions were analyzed by Western blotting with antibody against phosphorylated Smad2. DNA polymerase γ was used as a loading control. Western blots are from one experiment and are representative of three independent experiments.

Figure 6. TGF-β induces the formation of a PECAM-1/TGF-βRI/II complex on the surfaces of T cells

(A) Human CD4⁺ T cells were cultured in the presence or absence of plate-bound OKT3 and TGF-β as indicated for 18 hours. (Left) Cell lysates were prepared, precleared, and subjected to immunoprecipitation (IP) with a PECAM-1-specific antibody (PECAM-1.2), after which total cell lysates and immunoprecipitates were analyzed by Western blotting with antibodies specific for PECAM-1, TGF-βRI, and TGF-βRII. Results are representative of four independent experiments. (Right) The cell surface abundances of TGF-βRI and TGF-βRII under the different stimulation conditions were assessed by flow cytometric analysis. Results are representative of four independent experiments. (B) Human CD4⁺ T cells were cultured in the presence or absence of plate-bound OKT3 and TGF-β as indicated for 6 hours. Cells were fixed and stained with fluorescently tagged antibodies specific for PECAM-1 (green, PECAM-1.3) and either TGF-βRI (red, top) or TGF-βRII (red, bottom). Yellow indicates co-localized green and red fluorescent tags. Results are representative of three independent experiments. (C) Human CD4⁺ T cells were cultured in the presence or absence of plate-bound OKT3 and TGF-β as indicated for 6 hours, fixed, and subjected to PLA with oligonucleotide-tagged antibodies specific for PECAM-1 (PECAM-1.3) and either TGF-βRI (top) or TGF-βRII (bottom). Red indicates PECAM-1- and TGF-βR-specific antibodies within 40 nm of each other. Results are representative of four independent experiments.

Figure 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- β R1/II kinase activity
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- β R1/II (SB-505124) or a neutralizing antibody specific for human TGF- β (maba-hTGF- β). Cell lysates were pre-cleared with normal mouse IgG and subjected to immunoprecipitation (IP) 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, PECAM-1 phosphorylated at Tyr⁶⁸⁶ (PECAM-1-pY⁶⁸⁶), and SHP-2. Results are representative of three independent experiments.