Tenasin-C Protects Cancer Stem-like Cells from Immune Surveillance by Arresting T-cell Activation

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Abstract

Precociously disseminated cancer cells may seed quiescent sites of future metastasis if they can protect themselves from immune surveillance. However, there is little knowledge about how such sites might be achieved. Here, we present evidence that prostate cancer stem–like cells (CSC) can be found in histopathologically negative prostate draining lymph nodes (PDLN) in mice harboring oncogene-driven prostate intraepithelial neoplasia (PIN). PDLN-derived CSCs were phenotypically and functionally identical to CSC obtained from mPIN lesions, but distinct from CSCs obtained from frank prostate tumors. CSC derived from either PDLN or mPIN used the extracellular matrix protein Tenasin-C (TNC) to inhibit T-cell receptor–dependent T-cell activation, proliferation, and cytokine production. Mechanistically, TNC interacted with α5β1 integrin on the cell surface of T cells, inhibiting reorganization of the actin-based cytoskeleton therein required for proper T-cell activation. CSC from both PDLN and mPIN lesions also expressed CXCR4 and migrated in response to its ligand CXCL12, which was overexpressed in PDLN upon mPIN development. CXCR4 was critical for the development of PDLN-derived CSC, as in vivo administration of CXCR4 inhibitors prevented establishment in PDLN of an immunosuppressive microenvironment. Taken together, our work establishes a pivotal role for TNC in tuning the local immune response to establish equilibrium between disseminated nodal CSC and the immune system. Cancer Res; 75(10); 2095–108. ©2015 AACR.

Introduction

Metastatic disease is a dreadful complication of primary cancer, and the principal cause of cancer-related death. Two models explain the process of systemic cancer progression (1). The linear progression paradigm posits that tumor ontogeny occurs in the primary tumor, and only thereafter, disseminated tumor cells (DTC) deploy to sites of metastasis. Thus, the larger is the size of the primary tumor, the higher is the chance of metastatic spread. Conversely, the parallel progression model claims that tumor cells abandon the primary lesion before the acquisition of full malignant phenotype, and seed secondary growths where DTC undergo a stepwise progression of morphologic abnormalities. This model predicts greater genetic and epigenetic disparities between primary tumor cells and metastasis founders. Indeed, in several solid tumors, DTC exhibit significantly fewer genetic abnormalities than primary tumor cells, and heterogeneous chromosomal rearrangements can be found in primary tumors and DTC from different sites. The concept of early dissemination of tumor cells is also supported by the demonstration that in breast cancer models shortly after expression of the oncogenic transgene, neoplastic epithelial cells from atypical ductal hyperplasia disseminate to the lungs and bone marrow (2). Also, in women, bone marrow dissemination can occur at the stage of ductal carcinoma in situ (2).

Recent evidence strongly supports the role of cancer stem–like cells [CSC (3)] in the metastatic process (4), and pancreatic epithelial cells with stem cell properties may invade the liver before frank malignancy is detected in the pancreas (5).

Once the DTC has reached the site of future metastasis, it has to survive and protect itself from immune surveillance (6). Indeed, in vivo CSC are killed by NK and T cells (7, 8), but also inhibit T-cell proliferation and effector function (9) through yet
undefined mechanisms. Nothing is known about CSC–T cell interactions in vivo.

The transgenic adenocarcinoma of the mouse prostate (TRAMP) model relies on the SV40 early genes (small and large T antigens; Tag) expressed under the control of the rat probasin regulatory element, so that Tag appears at puberty selectively on the prostate epithelium (10). TRAMP mice progressively develop mouse prostate intraepithelial neoplasia (mPIN; week 6–12), adenocarcinoma (week 12–18), and lymph node (LN) and lung metastases (week 18–30) resembling the human pathology (11).

CSCs obtained from prostates of 10- to 11-week-old TRAMP mice affected by mPIN [hereafter named TPIN-SC; (12)] express prostate-cancer–associated antigens, MHC I and MHC II molecules, and ligands for NK cells. Indeed, CSC can be targeted by NK and cytotoxic T lymphocytes (CTL) both in vitro and in vivo (13). Nonetheless, they generated tumors when injected in immune-competent mice (13), suggesting they possess mechanisms of immune evasion. Concomitantly, at that age, TRAMP males develop full CTL tolerance to Tag (14), which behaves in this model as a tissue-restricted tumor-associated antigen. We then asked whether TPIN-SC adopt strategies to overcome immune surveillance. Here, we show that TPIN-SC precociously disseminated to prostate draining LN (PDLN), and used Tenascin-C (TNC), an extracellular matrix (ECM) disulfide-linked hexameric glycoprotein (15) to inhibit T-cell proliferation and effector function.

Materials and Methods

Mice, cell lines, and reagents

C57BL/6, C57BL/6-Tg(TcraTcrb)1100Mjb/Crl (Charles River), and B6.129S7-Rag1tm1- C0 were housed in a pathogen-free animal facility. C57BL/6-Tg(TcraTcrb)1100Mjb/Crl (Charles River), and B6.129S7-Rag1tm1- C0 were housed in a pathogen-free animal facility. Materials and Methods

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In vivo proliferation

Procedures are reported in Supplementary Materials and Methods.

Stable isotope labeling with amino acids in cell culture, mass spectrometry, and data analysis

OTI cells were propagated as described above and cultured in SILAC (stable isotope labeling with amino acids in cell culture) medium containing light (12C and 14N) or heavy (13C 15N) labeled l-lysine and l-arginine, in the presence of irradiated prostate CSC. After 7 days of coculture, CD8+ T cells from either condition were sorted for activated (CD8+ CD62Llow) or inhibited (CD8+ CD62Lhigh) phenotype and lysed in RIPA buffer (Cell Signaling Technology). Heavy and light lysates were mixed at 1:1 for protein content; proteins were then resolved onto a 4% to 12% NuPAGE precast gel (Invitrogen) and stained by Coomassie colloidal blue. The gel lane was cut into 12 slices, each of which was reduced, alkylated, and digested with trypsin as reported previously (19). Mass spectrometry (MS) procedures, according to ref. 20, and analysis of differentially expressed proteins in T cells from SILAC cultures are described in Supplementary Material and Methods.

Microarray-based gene expression profiling

Procedures are reported in Supplementary Materials and Methods.

Silencing of TNC

TPIN-SC were stably infected with TNC shRNA Lentiviral Particles or with control shRNA Lentiviral Particles (Santa Cruz Biotechnology, Inc.) as described in Supplementary Materials and Methods.

Flow cytometry, immunohistochemistry, immunofluorescence, and Western blot analysis

Procedures are described in Supplementary Material and Methods.

Generation of CSC from LN

PDLN, or axillary LN as control, were isolated from cancer patients, labeled with CFSE, activated with concanavalin A (5 μg/mL), and cultured and analyzed as described above.

In vitro proliferation

Procedures are reported in Supplementary Materials and Methods.

Stable isotope labeling with amino acids in cell culture, mass spectrometry, and data analysis

OTI cells were propagated as described above and cultured in SILAC (stable isotope labeling with amino acids in cell culture) medium containing light (12C and 14N) or heavy (13C 15N) labeled l-lysine and l-arginine, in the presence of irradiated prostate CSC. After 7 days of coculture, CD8+ T cells from either condition were sorted for activated (CD8+ CD62Llow) or inhibited (CD8+ CD62Lhigh) phenotype and lysed in RIPA buffer (Cell Signaling Technology). Heavy and light lysates were mixed at 1:1 for protein content; proteins were then resolved onto a 4% to 12% NuPAGE precast gel (Invitrogen) and stained by Coomassie colloidal blue. The gel lane was cut into 12 slices, each of which was reduced, alkylated, and digested with trypsin as reported previously (19). Mass spectrometry (MS) procedures, according to ref. 20, and analysis of differentially expressed proteins in T cells from SILAC cultures are described in Supplementary Material and Methods.
FGF2, and cultures were split every 2 to 10 days, according to the stage of origin (12).

**Results**

**CSCs and Tenascin-C**

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**CSC from autochthonous mPIN lesions dampen T-cell activation**

To investigate potential interactions between CSC and the immune system in the early phases of neoplastic transformation, we took advantage of TPIN-SC, which were obtained from young TRAMP mice affected by mPIN, and TNE-SC isolated from neuroendocrine lesions developed in 30-week-old TRAMP mice (12). Both CSC types fulfilled the criteria of endless self-renewal ability, multi-lineage differentiation, and tumorigenic potential (12). However, gene expression analysis revealed that the two CSC types were remarkably different, resembling the stages of progression of human prostate cancer (12).

CD4<sup>+</sup>T cells (sorted for being CD62L<sup>hi</sup>) and from fully activated T cells in a cell-to-cell contact fashion (Supplementary Fig. S2A). As expected, nonirradiated TNE-SC had no effect on T-cell proliferation and cytokine production (Supplementary Fig. S2).

**Gene Ontology and KEGG databases** showed that proteins extracted from T cells inhibited by the presence of TPIN-SCs (sorted for being CD62L<sup>hi</sup>) and from fully activated T cells in the presence of TNE-SCs (sorted for being CD62L<sup>low</sup>; Supplementary Fig. S4B) were analyzed by high-resolution MS, allowing the reliable identification and quantification of about 1,000 proteins (Supplementary Fig. S4B). Statistical analysis by MaxQuant highlighted 100 of them to be differentially expressed (P < 0.05; Supplementary Table S1) and query of Gene Ontology and KEGG databases showed that proteins involved in cytoskeleton organization and glucose metabolism were significantly modified in T cells after coculture with TPIN-SCs (Supplementary Table S2). Interestingly, TPIN-SC–conditioned T cells maintained upregulated proteins involved in release of inhibitory molecules, we repeated the above-described experiments with nonirradiated CSC. In line with the results reported in Fig. 1, nonirradiated TPIN-SC inhibited proliferation (Supplementary Fig. S2A) and IFN<sub>γ</sub> production (Supplementary Fig. S2B and S2C) of both CD4<sup>+</sup> and CD8<sup>+</sup>T cells in a cell-to-cell contact fashion (Supplementary Fig. S2A). As expected, nonirradiated TNE-SC had no effect on T-cell proliferation and cytokine production (Supplementary Fig. S2).

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**Real-time PCR**

Real-time PCR was performed and analyzed using the ΔΔC<sub>T</sub> method as described in Supplementary Experimental Procedures.

**Statistical analyses**

Statistical analyses were performed using the Student t or one-way ANOVA followed by Tukey tests. Values were considered statistically significant for *P < 0.05; **P < 0.01; ***P < 0.001.
A

B

C

D

E

F

% Proliferation (relative to control)

% Proliferation on April 30, 2017. © 2015 American Association for Cancer Research. cancerres.aacrjournals.org Downloaded from Published OnlineFirst March 25, 2015; DOI: 10.1158/0008-5472.CAN-14-2346
Cells producing IFN were primed in vitro with OVA257-264 and OVA323-339, respectively, alone (Nihil) or in the presence of irradiated TPIN-SCs (ratio, 1:10), and analyzed after 3 (OTI) or 5 (OTII) days for proliferation (left), expression of activation markers (middle), IFN production (right; A), or apoptosis (B). C, C57BL/6J mice bearing s.c. B16-OVA melanoma (at least 4 × 4 mm diameter) were inoculated intra-spleen with 3 × 106 CFSE-labeled splenocytes from RAG-1−/− OTI mice, together or not with 3 × 105 TPIN-SCs. After 5 additional days, mice were killed, and CFSE−CD3+CD8+ cells in the spleens and tumors were quantified by FACS. Data are representative of at least three independent experiments. Data are reported as average ± SD. The Student’s t test; *, P < 0.05; **, P < 0.01; ***, P < 0.0001.

Pyruvate metabolism (Supplementary Table S2), which is a peculiarity of resting T cells (25), and failed to upregulate the amino acid transporter CD98 and the transferrin receptor CD71 (Supplementary Fig. S4B), suggesting an impaired signaling for CSC and metastasis initiating cells (26), and can be immunosuppressive in vitro (27), yet each of these functions had been independently reported.

Expression of TNC in TPIN-SC and not in TNE-SC was confirmed by both Western blot analysis (Fig. 3B), and immunofluorescence (Fig. 3C). Moreover, when soluble TNC, whose sequence is conserved between humans and mice, was added to the cultures of human (Fig. 3D) or mouse T cells (Fig. 3E), it mirrored the effects of TPIN-SC on proliferation, expression of CD62L, and cytokine production. Thus, TNC was a strong candidate for mechanistic TPIN-SC-mediated immunosuppression.

Whereas in the prostate of WT mice at any age (Fig. 4A), expression of TNC was absent (Fig. 4B); in mPIN lesions from

Figure 2. TPIN-SC dampen antigen-specific T-cell activation both in vitro and in vivo. A and B, OTI (CD4+) white bars) and OTII (CD8+ black bars) cells were primed in vitro with OVA257-264 and OVA323-339 peptides, respectively, alone (Nihil) or in the presence of irradiated TPIN-SCs (ratio, 1:10), and analyzed after 3 (OTI) or 5 (OTII) days for proliferation (left), expression of activation markers (middle), IFN production (right; A), or apoptosis (B). C, C57BL/6J mice bearing s.c. B16-OVA melanoma (at least 4 × 4 mm diameter) were inoculated intra-spleen with 3 × 106 CFSE-labeled splenocytes from RAG-1−/− OTI mice, together or not with 3 × 105 TPIN-SCs. After 5 additional days, mice were killed, and CFSE−CD3+CD8+ cells in the spleens and tumors were quantified by FACS. Data are representative of at least three independent experiments. Data are reported as average ± SD. The Student’s t test; *, P < 0.05; **, P < 0.01; ***, P < 0.0001.

Figure 1. TPIN-SC arrest in vitro T-cell activation. A, left, representative dot plots of in vitro proliferation of CD4+ (top) and CD8+ (bottom) measured by FACS as CFSE dilution after 5 or 3 days, respectively, of stimulation with anti-CD3/CD28 beads alone (Nihil) or in the presence of irradiated TPIN-SC or TNE-SC (1:10 ratio). Each panel of the figure is representative of at least three independent experiments. Data are reported as average ± SD. ANOVA followed by the Tukey test and the Student’s t test; *, P < 0.05; **, P < 0.01; ***, P < 0.0001.

TNC is overexpressed in TPIN-SC and in both human and TRAMP prostate cancer lesions and is involved in TPIN-SC-mediated immunosuppression

We compared the transcriptome of TPIN-SC with that of TNE-SC (12), founding 37 differentially expressed genes (fold change > 2; P < 0.001), among which we identified the ECM protein TNC, upregulated in TPIN-SC (Fig. 3A) as a potential immunosuppressive molecule. TNC provides physical and signaling support for CSC and metastasis initiating cells (26), and can be immunosuppressive in vitro (27), yet each of these functions had been independently reported.

Expression of TNC in TPIN-SC and not in TNE-SC was confirmed by both Western blot analysis (Fig. 3B), and immunofluorescence (Fig. 3C). Moreover, when soluble TNC, whose sequence is conserved between humans and mice, was added to the cultures of human (Fig. 3D) or mouse T cells (Fig. 3E), it mirrored the effects of TPIN-SC on proliferation, expression of CD62L, and cytokine production. Thus, TNC was a strong candidate for mechanistic TPIN-SC-mediated immunosuppression.

Whereas in the prostate of WT mice at any age (Fig. 4A), expression of TNC was absent (Fig. 4B); in mPIN lesions from

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TRAMP mice (Fig. 4A), TNC showed a patchy distribution mostly within the stroma surrounding affected acini, and in a few transformed epithelial cells (Fig. 4B). A faint cytoplasmic expression of TNC was present in TRAMP adenocarcinoma cells (Fig. 4B), and absent in neuroendocrine tumors (Fig. 4B). In good correlation with the TRAMP model, TNC was expressed in human PIN (Fig. 4B), and not in human adenocarcinoma (Fig. 4B). TNC was also expressed in metastatic LN, confirmed by cytokeratin staining (Fig. 4C), of both TRAMP (Fig. 4C) and prostate cancer patients (Fig. 4C), but not in metastatic LN from TRAMP mice affected by neuroendocrine tumors (Fig. 4C, middle).

TPIN-SC were infected with lentiviral vectors encoding either a TNC-specific or a scrambled short hairpin RNA (shRNA-TNC and shRNA-ctr, respectively). Both Western blot analysis (Fig. 3B) and immunofluorescence (Fig. 3C) confirmed the specific silencing of TNC. Reduction of TNC expression in TPIN-SC did not influence their in vitro growth, because TPIN-SC infected with either shRNA-TNC or shRNA-ctr showed similar growth rate (data not shown). However, specific silencing of TNC substantially dampened the effects of TPIN-SC on T-cell proliferation and modulation of activation markers (Fig. 5A), and fully rescued the capacity of T cells to produce IFNγ upon activation (Fig. 5A). This was confirmed by the increase of ZAP70 and ERK2 phosphorylation levels (Fig. 5B and Supplementary Fig. S5A and S5B). Thus, TNC is directly involved in TPIN-SC-mediated immunosuppression. Interestingly, silencing of TNC did not abrogate the inhibition of T-cell priming. A, gene expression analysis of CSC with Affymetrix Mouse Gene 1.0 ST Array. Heatmap reports the global gene expression data in TPIN-SCs versus TNE-SCs. Differentially expressed genes with a P < 0.001 are red (upregulated) or green-colored (downregulated). B, Western blot analysis for TNC expression in CSC reported in A. C, immunofluorescence analysis for TNC expression in TPIN-SC, TNE-SC, or TPIN-SC infected with lentiviral vectors encoding for anti-TNC (TPIN-SC shRNA-TNC) or unspecific (TPIN-SC shRNA-ctr) shRNA (red, TNC; blue, DAPI). D, proliferation (i.e., CFSE dilution measured at FAC5) of PBMC at day 6 of stimulation with ConA alone (Nihil) or in the presence of soluble TNC (0.5 μg/mL). E, CFSE dilution (left), expression of CD62L (middle), and IFNγ production (right), as measured by flow cytometry, in CD4+ (white columns) and CD8+ splenocytes (black columns) at days 5 or 3, respectively, of stimulation with anti-CD3 and anti-CD28 beads alone (Nihil) or in the presence of TNC (0.5 μg/mL). Data are representative of at least three independent experiments. Data are reported as average ± SD. The Student t test: *, P < 0.05; **, P < 0.01; ***, P < 0.0001.
Figure 4. TNC expression in mouse and human prostates and metastatic LN. A–D, hematoxylin and eosin staining (A), immunohistochemistry for TNC (B and D), or pan-cytokeratin (C) on the indicated samples. Slides are representative of at least three different cases.
Figure 5.
Silencing of TNC modulates the inhibitory activity of TPIN-SC. A, CFSE dilution (left), expression of CD62L (middle), or IFNγ production (right) by CD4⁺ (white columns) and CD8⁺ splenocytes (black columns) at days 5 or 3, respectively, of stimulation with anti-CD3/CD28 beads alone (Nihil) or in the presence of irradiated TPIN-SC shRNA-TNC or TPIN-SC shRNA-ctr (1:10 ratio). B, phosphorylation of Zap70 (pZAP70), ERK (pERK), and STAT5 (pSTAT5) in cells described in A as measured by FACS. C, FACS analysis of spleen and tumor cells from B16-OVA-bearing mice treated as described in Fig. 3C, with the exception that CFSE-labeled splenocytes were inoculated together or not with 3 × 10⁶ TPIN-SC shRNA-TNC. D, frequency of tumor formation in C56BL/6 (WT; white bars) or RAG-1⁻/⁻ OTI male mice (OT1; black bars; n = 5 for each group) challenged s.c with 2 × 10⁶ TPIN-SC shRNA-TNC or TPIN⁺-SC shRNA-ctr mixed 1:1 with Matrigel. Mice with no apparent tumor were sacrificed 100 days after challenge. Each panel of the figure is representative of at least two independent experiments performed with at least 3 mice per group. Data are reported as average ± SD. ANOVA followed by the Tukey test and Student t test: *, P < 0.05; **, P < 0.01; ***, P < 0.0001.
of TPIN-SC of STAT5 phosphorylation (Fig. 5B and Supplementary Fig. S5C), suggesting that TNC does not affect IL2 signaling pathway, and that additional mechanisms cooperate in the immunosuppressive activity of TPIN-SC on T cells.

TNC silencing abrogated the inhibitory activity of TPIN-SC on T cells also in vivo. Indeed, the frequency of CFSE-labeled OTI cells was similar in the spleens and tumors of mice bearing B16-OVA melanoma, irrespective of the fact that they were infused 5 days earlier with naïve CFSE-labeled OTI cells either admixed or not to TPIN-SC-shRNA-TNC cells (Fig. 5C).

TPIN-SC infected with either shRNA-TNC or shRNA-ctr were injected subcutaneously within Matrigel plugs into fully immunocompetent C57BL/6 mice or RAG-1-/- mice, which lack B and T cells but OVA-specific CD8+ T cells. In agreement with our previous findings (13), TPIN-SC-shRNA-ctr cells generated tumors in 100% of RAG-1-/- OTI mice, and 60% of C57BL/6 mice (Fig. 5D), supporting a role for adaptive immunity in surveillance against TPIN-SC-derived tumors. Because no tumor grew in C57BL/6 mice challenged with TPIN-SC-shRNA-TNC cells (Fig. 5D), we conclude that TNC produced by TPIN-SCs negatively affected adaptive immune surveillance. TNC also has a function on tumor biology, because only 60% of RAG-1-/- OTI mice developed tumor upon challenge with TPIN-SC-shRNA-TNC cells (Fig. 5D).

TNC produced by CSC interacts with α5β1 integrin and inhibits T-cell proliferation by blocking stress fiber formation

TPIN-SC required a cell-to-cell contact to arrest T-cell activation (Fig. 1A). To further investigate the role of TNC in this cell-to-cell interaction, contacts between CFSE-labeled prostate CSC and CMTMR-labeled CD8 T cells were recorded by multichannel time-lapse fluorescent live cell imaging during the first 4 hours of coculture (Fig. 6A). Stimulated T cells were engaged in more prolonged contacts with TPIN-SC than with TNE-SC, and, more importantly, silencing of TNC in TPIN-SC reduced...
the duration of contacts to the level of TNE-SC (Fig. 6B and C). Thus, TNC appeared to be directly involved in CSC–T cell contacts, eventually leading of T-cell inhibition.

Intracellular signaling triggered by TCR stimulation is intimately related to the reorganization of the actin/myosin and microtubule cytoskeleton through small GTPases, including Rho, Rac, and Cdc42 (28). Inhibition of Rho in T cells suppresses T-cell proliferation (29), and TNC inhibits Rho activation (30). Thus, we hypothesized that TNC arrests T-cell proliferation by inhibiting cytoskeleton reorganization. In support of this hypothesis, SILAC analysis on T cells inhibited by TPIN-SC showed downregulation of actin gamma 1 (Actg1) and myosin heavy chain 10 (Myh10), two molecules involved in actin stress fibers assembly and focal adhesion formation (Supplementary Table S1). SILAC analysis revealed that other molecules involved in Rho and Rac pathways were differentially expressed in T cells inhibited by TPIN-SCs. Indeed, in TPIN-SCs conditioned T cells, we found upregulation of the Rho GDP dissociation inhibitors 1 and 2 (Arhgdia and Arhgdib), which prevent Rho activation by blocking the release of GDP, an essential step for GTP loading and activation (Supplementary Table S1; ref. 31). Cofflin, a downstream effector of both RhoA and Rac, which inhibits actin polymerization (32) was also upregulated in TPIN-SC–conditioned T cells (Supplementary Table S1). Conversely, the Rho GTPase-activating protein 1 (Arhgap1) was downregulated (Supplementary Table S1). Also Crk, which can activate small GTPases including Rac (33), and the Rac downstream effectors Vinculin and Gelsolin were downregulated in TPIN-SCs-conditioned T cells (Supplementary Table S1). Thus, to corroborate the hypothesis that TNC inhibits cytoskeleton reorganization, polymers of F-actin were quantitated in T cells by staining with FITC-conjugated phalloidin. As expected, T cells increased actin polymerization after PMA stimulation and this process was inhibited by pretreatment with cytocalasin D (Fig. 6D and data not shown), which selectively blocks the ATP-dependent actin polymerization process. Actin polymerization was inhibited also when T cells were activated in the presence of TPIN-SCs conditioned T cells. This led us to selectively block the ATP-dependent actin polymerization process. Actin polymerization was inhibited also when T cells were activated in the presence of TPIN-SC or TNC and, most importantly, silencing of TNC in TRAMP mice gave rise to TNC in mice (Fig. 7C). As confirmation of their origin, TLN-SC expressed the prostate antigens STEAP (37) and PSCA (Fig. 7D; ref. 38). We found higher TNC in TRAMP PDNL than in NDNL of TRAMP mice or in PDNL of WT mice (Fig. 7E). More importantly, TLN-SC expressed TNC (Fig. 7F) and recapitulated the immunosuppressive effects of TPIN-SC on mouse T cells (Fig. 7G).

The existence of an immunosuppressive environment in PDNL of 12-week-old TRAMP mice was confirmed by the finding that CD8+ T cells from TRAMP PDNL proliferated less than T cells from PDNL of age-matched WT mice (Fig. 7H). Importantly, when TRAMP mice were treated with AMD3100 starting at 4 weeks of age, CD8+ T-cell proliferation in their PDNL at 12 weeks returned comparable with WT mice either treated or not with the drug (Fig. 7H).

Thus, CSCs early disseminate to PDNL, where they use TNC to avoid immune surveillance, and a precocious treatment with a CXCR4 inhibitor can positively affect local CSC-mediated immunosuppression.

**Discussion**

We have found that CSC, already at the stage of mPIN, deploy to PDNL that at pathologic examination appear nonmetastatic. Thus, prostate cancer appears to adhere to the model of parallel metastatic spread (1). In support of this model, 13.3% of patients with pT3 prostate carcinoma treated by radical prostatectomy and bilateral LN dissection, and classified as node negative, harbored instead occult LN metastases, which were an independent predictor of recurrence and death in a multivariable analysis (39).
We also found that CSC either from mPIN or PDLN of mice affected by mPIN likely overcome immune surveillance by inhibiting T-cell proliferation and effector functions. Several experimental evidences let us conclude that TNC is one major mechanism by which CSCs inhibit T-cell response in TRAMP mice. Indeed, prostate CSC overexpressed TNC and TNC silencing abrogated CSC-mediated immunosuppression. Furthermore, TNC in vitro recapitulated the immunosuppressive activity of CSC.

Whereas TNC does not correlate with prostate cancer Gleason score (40), data reported herein in ref. 40 indicate that in normal prostate and low-grade PIN, TNC is weakly expressed in the ECM and acinar basement membrane, its expression increases in high-grade PIN, returns to basal levels in prostate adenocarcinoma, and is again overexpressed in metastases. TNC also is overexpressed in melanoma spheres enriched in CSC, supporting their growth, metastatic potential, and resistance to chemotherapy (41). Accordingly, while TNC was dispensable for TPIN-SC growth in vitro, it had a role in supporting their tumor formation in vivo. Thus, TNC has a dual role for prostate CSC in vivo; on the one hand, it sustains tumor growth, and on the other hand, it inhibits T cell–mediated immune surveillance. TNC is therefore a very interesting target of cancer therapy, and anti-TNC antibodies are already in the clinic (42). It is also tempting to speculate that overexpression of TNC in high-grade PIN and low Gleason score patients, because of the role of TNC in tissue remodeling.

Figure 7.
PAC-SCs expressing TNC are present in PDLN. A, representative dot plots (left) and quantification (n=5/group; right) of Lin− (CD45−CD31−) CD44+ Sca-1+ CD166+ cells in PDLN from 16- or 6-week-old TRAMP and WT mice. B, percentage of success in establishing CSC lines from PDLN or NDLNs from 12-week-old TRAMP (n=4/group) and WT mice (n=2). C, FACS analysis of TLN-SC, TPIN-SC, or ex vivo prostate cells from 12-week-old TRAMP mice (red, specific staining; black, isotype control; gray, specific staining after IFNγ stimulation). D, STEAP (red) and PSCA (green) immunofluorescence on TLN-SCs (blue, DAPI). E, relative expression ± SD of TNC in PDLN or NDLN of TRAMP and WT mice as assessed by real-time PCR. Values were normalized to the positive control (i.e., TRAMP prostate). F, TNC immunofluorescence in TLN-SC (red, TNC; blue, DAPI). G, proliferation, expression of CD62L, and cytokine production (average ± SD) of CD4+ (white columns) and CD8+ (black columns) as measured by CFSE dilution at day 5 or 3, respectively, of stimulation with anti-CD3/CD28 beads alone (Nihil) or in the presence of irradiated TLN-SCs (1:10 ratio). H, CFSE dilution of CD8+ cells from PDLN of 12-week-old TRAMP and WT mice, which were treated with AMD3100 or vehicle (PBS), at day 3 of stimulation with anti-CD3/CD28 beads. Each panel is representative of at least two independent experiments performed with at least 3 mice per group if not differently indicated. ANOVA followed by the Tukey test and the Student t-test: **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
and metastasis (26), might identify a subgroup of patients with increased chances of early LN invasion. This hypothesis should be investigated in a large cohort of patients.

CSCs use TNC to block T-cell activation during priming/restimulation, but are ineffective on fully activated T cells, as it has been reported for TNC (27). Hence, CSC do not appear to directly interfere with TCR-MHC interactions, nor TNC interferes with LFA1-ICAM1 interaction (27). It is well known that TNC can bind several molecules among integrins (15). In particular, the fragment FnIII 1–5 of TNC inhibits α5β1-dependent T-cell adhesion to fibronectin (43), while the fragment FnIII A1A2 inhibits T-cell proliferation through a yet unknown mechanism (44). Although we did not investigate which TNC fragment is involved in the CSC-mediated immunosuppressive activity, our in vitro findings strongly support a mechanism by which TNC interacts with α5β1 integrins on T-cell surface, thus likely inhibiting Rho activation as previously reported in fibroblasts (30), and blocking actin stress fibers assembly and focal adhesion formation. The signaling cascade triggered by TCR engagement activates Rho, Rac, and Cdc42 GTPases (31). In particular, phosphorylated Zap70 can activate a pathway leading to the formation of a complex of proteins, including VAV1, which facilitates the exchange from GDP to GTP and thus the activation of Rho, Rac, and Cdc42 (32). As TNC inhibits TCR signaling and Zap70 phosphorylation in particular, this could be a complementary mechanism by which TNC inhibit Rho, Rac, and Cdc42 GTPases.

Both CSCs and TNC significantly reduced phosphorylation of Zap70 and ERK2 and induced substantial metabolic alterations in T cells, as those described in T-cell anergy (24). The state of T-cell hyporesponsiveness induced by TPIN-SCs does not correspond to the physiologic state of activation-induced nonresponsiveness (45), because T cells lost not only proliferative competence, but also IFNγ production and cytotoxic activity; neither can it be considered as adaptive tolerance, because removal of CSC/TNC allowed full T-cell recovery. Thus, the effect mediated by CSC on T cells is characteristic more of cell-mediated immunosuppression (24).

Sensing of TNC did not completely abrogate the immune-suppressive activity of CSC and did not rescue STAT5 phosphorylation, suggesting that other molecules are involved in CSC-mediated immunosuppression. Indeed, microarray-based whole transcript showed that both TPIN-SC and TLN-SC produce several other immunomodulatory factors, currently investigated by us.

Peripheral tolerance to tumor-associated antigens in prostate cancer may be induced and maintained by several additional mechanisms. Indeed, CSC and/or other more differentiated tumor cells may generate immunosuppressive networks within the primary lesion, and anergic Tag-specific CD8+ T cells are found in the prostate of TRAMP mice (14). Both regulatory T cells and myeloid-derived suppressor cells accumulate in the prostate of cancer patients and TRAMP mice, although their targeting revealed insufficient for breaking T-cell tolerance and sustaining antitumor immunity (46, 47). Moreover, in TRAMP and in human prostate cancer lesions, a population of tolerogenic DCs induces CD8+ T cells to acquire suppressive functions through indoleamine-2,3-dioxynitri, arginase, TGFβ, and PDL-1 (48).

Our results merge the evidence of early tumor cell dissemination with tumor-induced immune escape, with relevant clinical implications. In our cohort, TRAMP mice never developed LN metastasis from prostate adenocarcinoma before week 17 (data not shown), suggesting that CSC that have colonized PD LN remain quiescent for several weeks. Immune surveillance appears to be involved in limiting the metastatic outgrowth, as cytostatic CD8+ T cells have been shown to maintain DTC dormancy in visceral organs (49). Thus, strategies that either block the interaction between TNC and α5β1, such as antibodies (42) or isoGDR peptides, or the CXCR4–CXCL12 axis (50), as the one described herein, or both TNC and CXCR4 should unsheath early-disseminated CSC and avoid full-blown metastasis development, and might be proposed either alone or in combination to subjects with high risk to develop prostate cancer or to patients at high risk for disease recurrence after radical prostatectomy. Although in our cohort of TRAMP mice, the earliest metastasis from adenocarcinoma was found in the kidney at 16 weeks of age, metastasis to soft tissues in this model is rather infrequent and stochastic. Hence, efficacy of the proposed treatments in preventing metastasis occurrence to soft tissues might better be investigated in other models.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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