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ORIGINAL ARTICLE

TGF-β modulates the functionality of tumor-infiltrating CD8$^+$ T cells through effects on TCR signaling and Spred1 expression

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Abstract This study demonstrates that CD8$^+$ T cells in the tumor microenvironment display reduced functionality and hyporesponsiveness. TGF-β contributed markedly to the tumor-infiltrating CD8$^+$ T cells' (TILs) reduced functionality, which could be reversed using a small molecule TGF-β inhibitor. Upon T-cell receptor (TCR) activation, the activation of ITK and ERK kinases were reduced in CD8$^+$ TILs, as compared to splenic CD8$^+$ T cells: TGF-β inhibitor could reverse this phenomenon. This study demonstrates for the first time the association of the Spred-1 gene, an inhibitor of the Ras/MAPK pathway, with CD8$^+$ TILs and TGF-β activity. Spred-1 was upregulated in CD8$^+$ TILs and TGF-β enhanced the expression of Spred-1 in effector/memory CD8$^+$ T cells and not in rested/memory CD8$^+$ T cells. Based on these findings, this study supports the hypothesis that TGF-β mediates an inhibitory mechanism on CD8$^+$ TILs involving TCR-signaling blockade and the upregulation of Spred-1, thus implicating Spred-1 as a potential new target for future anti-tumor immune studies.

Keywords T cells · Tolerance/suppression/anergy · Tumor immunity · Signal transduction

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Introduction

T cells specific for tumor antigens have been identified both within the tumor itself and in the peripheral blood of cancer-bearing individuals. A number of studies on tumor-associated CD4$^+$ T cells have revealed a T-cell–specific hyporesponsiveness related to the T-cell receptor (TCR) signaling pathway [1–7]. Moreover, other studies have similarly demonstrated impairment of functionality in CD8$^+$ T cells in tumors [8–12]. A number of biologically active agents synthesized by either tumor or stromal cells, such as TGF-β, exert suppressive effects on the immune system [13].

Transforming growth factor (TGF-β) is a pleiotropic immunosuppressive cytokine that inhibits T-cell activation, proliferation and differentiation [14]. It has been shown that TGF-β is produced by many different tumors and it has been suggested that its immunosuppressive effects may contribute to the failure of tumor-associated T cells to control tumor progression [15, 16]. These phenomena might explain how tumors escape immune recognition. Elevated TGF-β expression in the tumor microenvironment modulates a complex web of intercellular interactions that promotes metastasis and progression [17]. TGF-β not only exerts its influence on the hyporesponsiveness of CD4$^+$ and

$CD8^+$ T cells in the tumor microenvironment, but also regulates the development and function of other leukocytes, including regulatory T cells, dendritic cells, NK and B cells [14]. TGF-β can induce the transformation of normal effector cells into regulatory T cells by inducing the expression of Foxp3 through the inactivation of ERK [18, 19]. TGF-β may also actively subvert the $CD8^+$ T cells into directly promoting tumor growth by producing IL-17, an apoptosis suppressor of tumor cell lines in vitro [20]. Moreover, membrane-associated TGF-β has been demonstrated to be involved in the blockade of the TCR signaling pathway of memory T cells in the tumor microenvironment and non-malignant inflammatory microenvironment [21]. In vitro studies have shown that the TGF-β signaling pathway intersects with and modulates the TCR signaling pathway through the inactivation of TCR signaling components like the inactivation of IL2-inducible T-cell kinase (ITK) and inhibition of ERK phosphorylation in activated $CD4^+$ T cells from non-tumor-bearing mice [13, 22]. However, the role of TGF-β in the molecular mechanism that affects the TCR signaling hyporesponsiveness of T cells in the tumor microenvironment is still not well established.

A recent study has characterized Spred-1 (Sprouty-related Ena/VASP homology 1-domain-containing protein-1) as a novel protein involved in the inhibition of the Ras/Raf-1/ERK pathway impairing the growth-factor-mediated activation of ERK1/2 kinases. It has been shown that Spred-1 specifically potentiates the interaction between activated Ras and Raf creating a Spred-Ras–Raf complex. This complex prevents Raf activation by Raf kinase, thus suppressing the activation of the downstream pathway [23]. The negative effect of Spred-1 on the Ras-MAP kinase pathway was investigated first in muscle cell lines and brain tumor cells and then in a variety of other cells like human hepatocellular carcinomas as well as hematopoietic cells [23–25]. In this study we have defined, for the first time, the overexpression of the Ras/MAPK inhibitor Spred-1 in tumor-associated $CD8^+$ T cells and we have correlated Spred-1 expression with TGF-β activity. The data reported here thus provide evidence that TGF-β plays an important role in impairing the functionality of tumor-infiltrating $CD8^+$ T cells (TILs), which then lose their ability to respond to TCR stimulation. Moreover, Spred-1 can play an important role in the action of TGF-β to induce anergy in tumor-infiltrating $CD8^+$ T cells.

Materials and methods

Tumor model

All animal studies were approved by the National Institutes of Health Animal Care and Use Committee. Female C57BL/6 mice were injected s.c. on the abdomen with 3×10^5 MC38, a murine colon adenocarcinoma cell line. Mice were sacrificed at varying specific intervals following tumor cells injection.

Mice, peptide and reagents

TCR/Tg F5 mice that are transgenic for an influenza nucleoprotein (NP) peptide ($NP_{366–374}$), designated NP68-specific, and C57BL/6 were obtained from Taconic Farms. The NP-68 peptide (^{366}ASNENMDAM^{374}) was synthesized by the American Peptide Company. Recombinant TGF-β1 was purchased from PeproTech Inc. A small molecule inhibitor of the type I TGF-β receptor kinase (SB505124) was obtained under a Materials Transfer Agreement from Glaxo Smith Kline.

Proliferation assay

To perform the proliferation assay of $CD8^+$ T cells from tumor infiltrate and from spleens of control and tumor-bearing mice, mice were sacrificed 3 weeks after tumor implant. Tumors were removed and digested (as described in Ref. [26]). Cells were stained for CD8 and, using a FACS-Vantage flow cytometer (BD Biosciences), $CD8^+$ cells were purified. Splenic $CD8^+$ T cells preparation and proliferation assays were set up as described in Ref. [7].

Flow cytometry

Cells from spleen and tumor were stained with Ab against CD8 (53–6.7), purchased from BD Biosciences. For BrdU studies, in vitro labeling of the cells was performed adding BrdU to the cell culture at a final concentration of 10 μM for 6 h. Following cell-surface marker staining, cells were stained for BrdU incorporation using the BrdU Flow Kit from BD Biosciences and anti-BrdU (B44).

For CFSE assay, cells were labeled with CFSE: cells were washed twice with PBS, incubated with 1 μM CFSE in PBS for 10 min at 37°C and washed twice with PBS.

For intracellular staining of pITK (pY551) and pERK1/2 (pT202/pY204), specific antibodies were purchased from BD Biosciences. Single-cell suspensions from the spleen and tumor infiltrate were prepared and incubated for 24 h in vitro. Cells were treated with Fc block for 15 min on ice. For the activation of pITK, cells were incubated with 10 μg/ml anti-CD3 (BD Biosciences) for 20 min on ice. The single-cell suspensions were then incubated in a 37°C waterbath for 5 min before adding 60 μg/ml goat anti-hamster cross linker (Jackson Immunoresearch) for 1 min. For activation of pERK 1/2, cells were incubated in a 37°C waterbath with PMA/Ionomycin (15 ng/ml/850 nM) for 15 min. After activation, cells were fixed, permeabilized

and stained for pITK or pERK 1/2 for 1 h at room temperature in the dark. The samples were then analyzed on a FACSCalibur flow cytometer (BD Biosciences). Data were analyzed using CellQuest software (BD Biosciences) and gates were placed on $CD8^+$ cells.

Detection of intracellular cytokines

For intracellular detection of cytokine production, cells were incubated at 37°C in a CO_2 incubator with 1 μg/ml GolgiPlug (BD Biosciences) for at least 15 h. After incubation, cells were harvested and stained for $CD8^+$ surface marker for 15 min on ice, washed with PBS-FCS and fixed with Cytofix/Cytoperm (BD Biosciences) for 30 min at room temperature. After fixation, cells were washed with Perm/Wash buffer (BD Biosciences), stained with anti-TNF-α (MP6-XT22), anti-INF-γ (XMG1.2) and anti-IL-2 (JES6-5H4) (all Abs from BD Biosciences) for 15 min on ice, washed with Perm/Wash Buffer and analyzed on a FACSCalibur apparatus.

Generation of effector/memory and rested/memory $CD8^+$ T cells in culture

Spleens from F5 transgenic mice were removed and pressed through a 70-μm filter to obtain a total splenocytes cell suspension. Effector/memory $CD8^+$ T cells were generated incubating in culture the total splenocytes with 10^{-4} μg/ml NP-68 peptide for 3 days. A CD8 positive selection was then performed using anti-CD8 microbeads from Miltenyi Biotec, according to the manufacturer's protocol.

Rested/memory $CD8^+$ T cells were generated by culturing the total splenocytes with 10^{-4} μg/ml NP-68 peptide for 3 days. After 3 days, the live cells were collected using centrifugation with Ficoll gradient, washed and rested for 14 days in six-well plates with 140 ng/ml of murine IL-15 in medium. The cells were washed and given IL-15 every 3 days. A CD8 positive selection was then performed as described above.

Expression analysis by real-time PCR

RNA was isolated from cells using a NucleoSpin RNA II Kit (Macherey-Nagel). cDNA was prepared using 0.5 μg of RNA with the high capacity RNA-to-cDNA kit (Applied Biosystems). The real-time PCRs were performed using TaqMan Gene Expression Master Mix (Applied Biosystems) and TaqMan Gene Expression Assays for mouse Spred-1 (FAM) and mouse GAPD (VIC) as housekeeping gene (Applied Biosystems). The reaction was performed using the 7300 Real-Time PCR System apparatus (Applied Biosystems). Cycling conditions were 52°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. The results were analyzed using the ΔΔCt formula.

Statistics

Data are presented as the mean ± SD. The significance of the difference between groups was evaluated with Student's *t* test; $P < 0.05$ was considered significant.

Results

Tumor-infiltrating $CD8^+$ T cells are functionally impaired

In order to investigate the functionality of the tumor-infiltrating lymphocytes (TILs), MC38 cells were implanted subcutaneously and 3 weeks later TILs were compared to $CD8^+$ T cells from spleens of either control mice or tumor-bearing mice. This tumor produced high levels of TGF-β, as shown in Fig. S1.

To address the functionality of $CD8^+$ T cells, the proliferation of splenic $CD8^+$ T cells from control mice and from tumor-bearing mice was compared to TILs 24 h post-TCR activation. While splenic $CD8^+$ T cells from control and tumor-bearing mice showed a similar level of proliferation, tumor-infiltrating $CD8^+$ T cells showed a significantly reduced proliferation as compared to the controls (Fig. 1a, $P < 0.005$).

Moreover, tumor-infiltrating $CD8^+$ T cells demonstrated a reduced ability to produce cytokines such as TNF-α, IL-2 and IFN-γ, compared to splenic $CD8^+$ T cells isolated from tumor-bearing spleen after 24 h of TCR activation in vitro (Fig. 1b). These data collectively demonstrated that in an MC38 tumor model TILs displayed a hyporesponsive (anergic) status compared to splenic $CD8^+$ T cells of either control or tumor-bearing mice.

Role of TGF-β in the induction of anergy of $CD8^+$ T cells in vitro

To investigate the direct effect of TGF-β on $CD8^+$ T cells, normal unfractionated splenocytes were incubated with or without TGF-β for 24 h. $CD8^+$ T cells were purified, CFSE labeled and activated in the presence of anti-CD3 and irradiated APCs for an additional 24 h. As depicted in Fig. 2a, $CD8^+$ T cells that were treated with TGF-β lagged in cell cycle progression both at 24 h (left panels) and strongly at 72 h post-activation (right panels). INF-γ production was also measured by intracellular staining 24 h post-TCR activation in $CD8^+$ T cells from splenocytes cultured with or without TGF-β. Treatment with TGF-β led to a reduction in the number of $CD8^+$ T cells that produced INF-γ (from 10.9 to 3.5%, Fig. 2b). These data

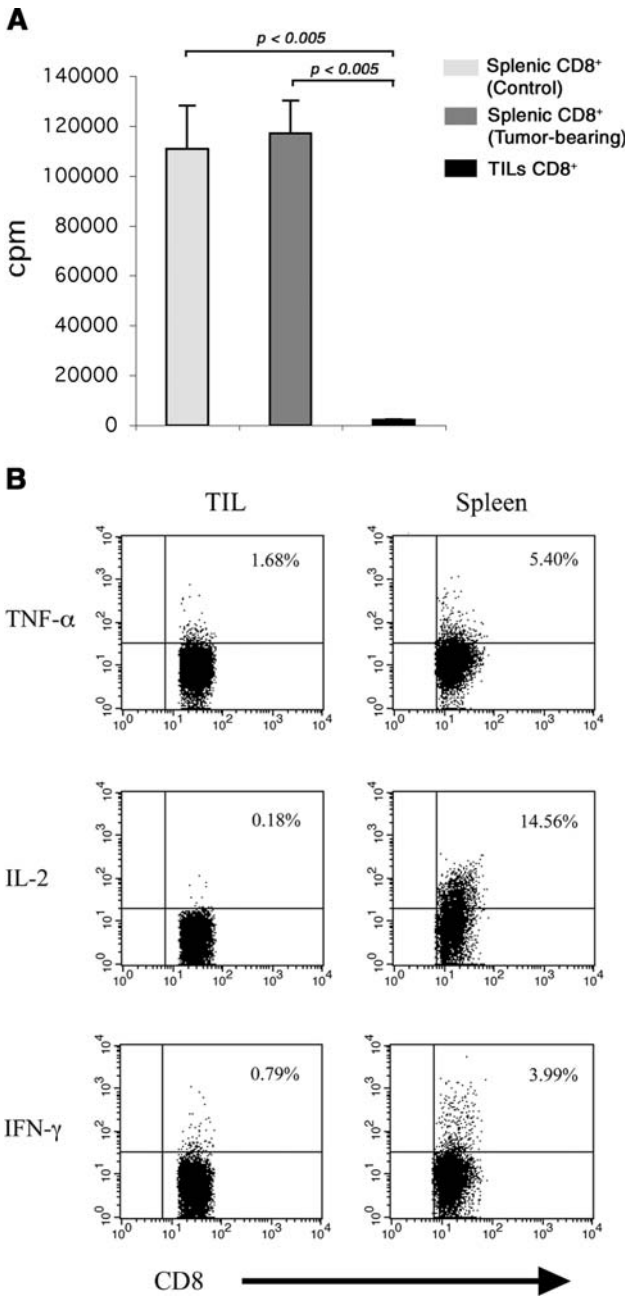

Fig. 1 Functionality of CD8$^+$ T cells from spleens and tumor infiltrate of tumor-bearing mice. **a** Proliferation assay of tumor-infiltrating CD8$^+$ T cells compared to normal and tumor-bearing splenic CD8$^+$ T cells. CD8$^+$ T cells were stimulated with anti-CD3 (1 μg/ml) and irradiated APCs at a ratio of CD8$^+$/APC 1:2. Proliferation was measured by [^{3}H]thymidine incorporation. Data are representative of $n = 6$ experiments. **b** Cytokine production of tumor-infiltrating CD8$^+$ T cells or CD8$^+$ T cells from the spleen of tumor-bearing mice. CD8$^+$ T cells were stimulated with anti-CD3 and irradiated APCs at a ratio of CD8$^+$/APC 1:2. TNF-β, IL-2 and IFN-γ productions were measured by intracellular staining. Data are representative of $n = 3$ experiments

suggest that TGF-β treatment in vitro indeed affected CD8$^+$ T cells and reduced the responsiveness of CD8$^+$ T cells to TCR stimulation.

Inhibition of TGF-β partially restored tumor-infiltrating CD8$^+$ functionality ex vivo

To further investigate the effect of TGF-β in establishing the anergic state of CD8$^+$ T cells in the tumor microenvironment, a small molecule TGF-β inhibitor (specific inhibitor of the TGF-β receptor I activity) was used on ex vivo MC38 total tumor digestion. Three-week s.c. tumors were removed and digested to a single cell suspension. The total tumor digest, in which the TILs were also present, was incubated for 24 h in vitro with 5 μM TGF-β inhibitor SB505124 or with its solvent DMSO as control. The functionality of the tumor-infiltrating CD8$^+$ T cells, in the presence or absence of SB505124, was measured by BrdU incorporation and intracellular IFN-γ production after TCR activation by the addition of anti-CD3 in the total cell suspension (containing APCs). As depicted in Fig. 3, the BrdU incorporation by the TILs treated with TGF-β inhibitor SB505124 was increased compared with the same cells incubated with DMSO (Fig. 3a). The incubation of the total tumor digestion for 24 h with TGF-β inhibitor SB505124 also led to an increase in INF-γ production by the TILs as shown by intracellular staining (Fig. 3b). These results demonstrated that the inhibition of TGF-β activity by a small molecule inhibitor for only 24 h in vitro could partially reinstate the functionality of the tumor-infiltrating CD8$^+$ T cells. Based on these observations, it can be concluded that TGF-β is an active player in the induction of anergy in CD8$^+$ T cells inside the tumor.

Tumor-infiltrating CD8$^+$ T cells downregulate gene expression of molecules involved in TCR signaling and T-cell proliferation

The results of the microarray analyses conducted on the individual gene level demonstrated that the mRNAs of many molecules involved in proximal and distal TCR signaling pathway were downregulated in the tumor-infiltrating CD8$^+$ T cells as compared with CD8$^+$ T cells from the spleen of tumor-bearing mice (Table S1). The altered mRNA expression of CD3 and TCR in microarray results was further confirmed on a protein level by FACS analysis of cell-surface staining (Table S2). To assess the level of ITK phosphorylation following TCR activation, intracellular staining of phospho-ITK was performed. MC38 tumor digest and unfractionated splenocytes from tumor-bearing mice were incubated for 24 h in vitro with DMSO or TGF-β inhibitor SB505124 and then activated through the TCR with anti-CD3 and crosslinker. As depicted in Fig. 4, phospho-ITK was detected by intracellular staining using FACS analysis. CD8$^+$ T cells from the tumor suspension did not show any increase in phospho-ITK after TCR stimulation (middle panel, Fig. 4) as

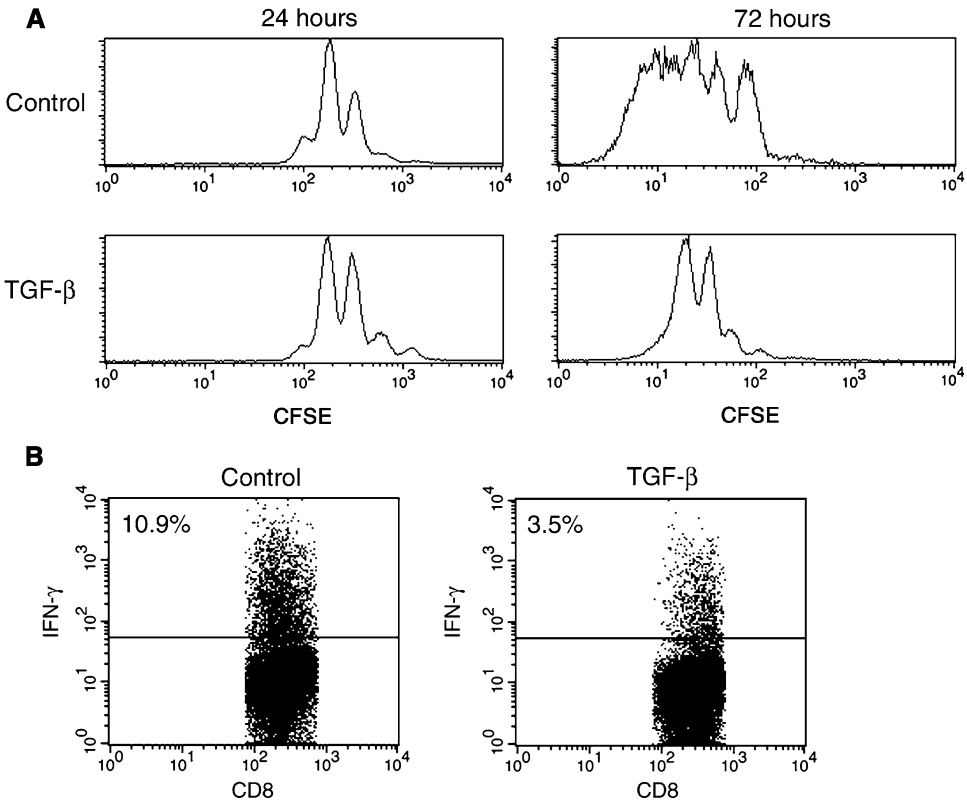

Fig. 2 Effect of in vitro exposure to TGF-β on CD8$^+$ T cells functionality. **a** Unfractionated splenocytes from healthy C57BL/6 mice were incubated with 2 ng/ml of TGF-β for 24 h. Then, CD8$^+$ T cells were purified, labeled with CFSE and incubated with anti-CD3 (1 μg/ml) together with irradiated APCs at a ratio of CD8$^+$/APC 1:2. CFSE profile was analyzed after 24 and 72 h. Data are representative of $n = 3$ experiments. **b** Unfractionated splenocytes from healthy C57BL/6 mice were incubated with 2 ng/ml of TGF-β for 24 h. Then CD8$^+$ T cells were purified and incubated with anti-CD3 (1 μg/ml) together with irradiated APCs at a ratio of CD8$^+$/APC 1:2. IFN-γ production by CD8$^+$ T cells was measured by intracellular staining after 24 h. Data are representative of $n = 3$ experiments

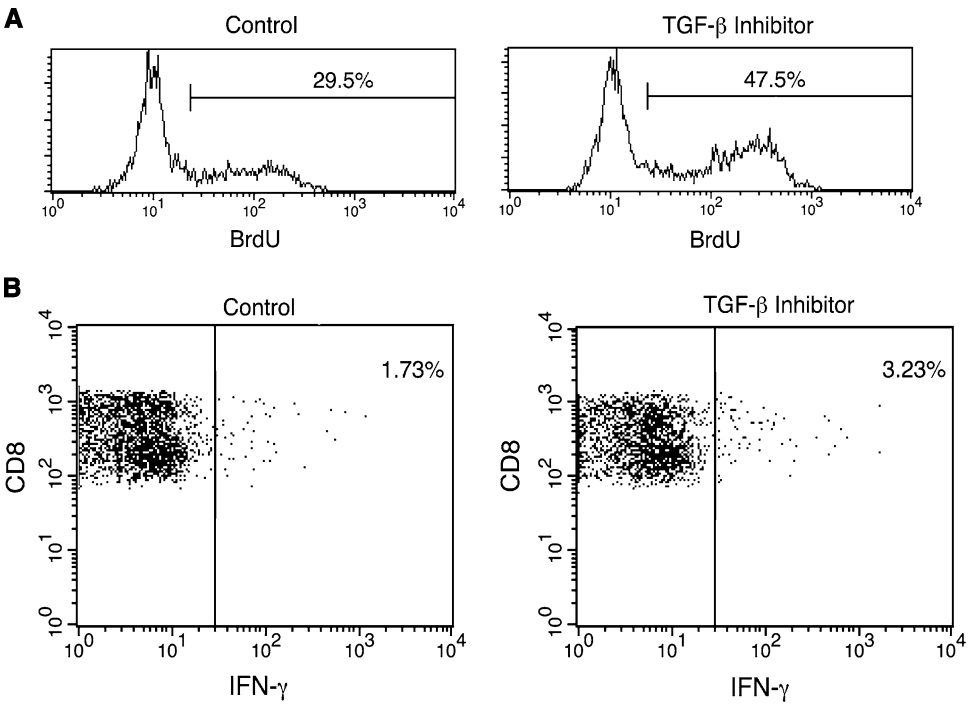

Fig. 3 Effect of TGF-β inhibitor SB505124 on functionality of tumor-infiltrating CD8$^+$ T cells. MC38 tumor were grown for 3 weeks, removed from C57BL/6 mice and digested to a single cell suspension. Total digest cells were incubated in vitro with TGF-β inhibitor SB505124 (5 μM [27]) or DMSO (control). Twenty-four hours later anti-CD3 (1 μg/ml) was added to the culture and incubated for another 24 h together with BrdU. **a** Cells were stained for BrdU incorporation and CD8 cell surface marker. For FACS analysis gates were placed around CD8$^+$ cells. Values indicate the percentage of BrdU$^+$ cells on total CD8$^+$ cells. Data are representative of $n = 3$ experiments. **b** IFN-γ production was measured by intracellular staining on gated CD8$^+$ cells. Values indicate the percentage of IFN-γ^+ cells. Data are representative of $n = 3$ experiments

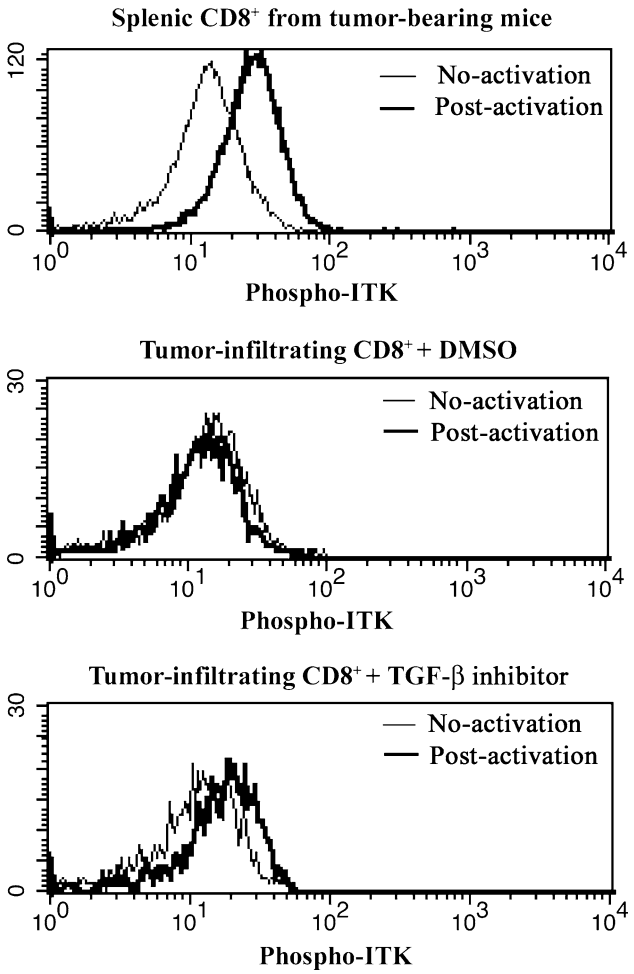

Fig. 4 ITK phosphorylation, following TCR activation, is impaired in tumor-infiltrating CD8+ T cells in a TGF-β-dependent manner. MC38 tumors were grown for 3 weeks and then removed from C57BL/6 mice as well as the spleen. A single cell suspension of tumor cells, as well as unfractionated splenocytes from tumor-bearing mice, were incubated in vitro for 24 h with DMSO or with TGFβ inhibitor (10 μM) and then activated with anti-CD3/crosslinker. Intracellular staining for phospho-ITK was performed as well as CD8 surface staining. For FACS analysis, gates were placed around CD8+ cells. Data are representative of $n = 3$ experiments

contrasted to the increase seen in splenic CD8+ T cells from tumor-bearing mice (top panel, Fig. 4). The presence of the TGF-β inhibitor SB505124 in the culture was able to restore the phosphorylation of ITK after TCR stimulation (bottom panel, Fig. 4). These results parallel with the microarray analysis demonstrating that the downregulation of TCR pathway components, in tumor-infiltrating CD8+ T cells, corresponds to impairment in phosphorylation of ITK, a proximal event in the TCR signaling cascade. Moreover, TGF-β activity seems to play a role in the impaired phosphorylation of ITK in CD8+ TILs.

Spred-1, an inhibitor of the activation of ERK/MAPK kinase, is upregulated in tumor-infiltrating CD8+ T cells in a TGF-β–dependent manner

At the individual gene level, microarray analysis demonstrated that Spred-1 expression is highly upregulated in the TILs as compared with normal CD8+ T cells from tumor-bearing mice (70-fold, Table S1). Additionally, real-time PCR confirmed that the expression of Spred-1 was markedly upregulated ($P < 0.005$) in these cells as depicted in Fig. 5a.

Since tumor-infiltrating CD8+ T cells showed altered proliferation and cytokine production compared with splenic CD8+ T cells from tumor-bearing mice, we focused our attention on a single gene, Spred-1, which has been shown to inhibit the activation of the ERK/MAPK pathway. To investigate whether TGF-β plays a role in Spred-1 upregulation in CD8+ T cells in the tumor microenvironment, MC38 tumors were digested to a single cell suspension. In order to generate a large number of CD8+ T cells that have been exposed to tumor cells, additional normal purified splenic CD8+ T cells were cultured together with the tumor digest (to allow the purification of a high number of CD8+ T cells after treatment for real-time PCR assay). TGF-β inhibitor SB505124 was added to the mixed cell culture 24 h before the addition of anti-CD3, which triggers the TCR activation. After 24 h of activation, the CD8+ T cells were purified from the mixed culture and real-time PCR was performed. The inhibition of the endogenous TGF-β activity by the inhibitor SB505124 led to a significant reduction ($P < 0.005$) of Spred-1 expression in CD8+ T cells in a dose-dependent manner compared to the DMSO control (Fig. 5b). These data demonstrated that TGF-β could directly upregulate Spred-1 expression in CD8+ T cells.

To further investigate the effect of TGF-β on Spred-1 expression in CD8+ T cells, three different populations of CD8+ T cells were treated: naive, effector/memory, and rested/memory CD8+ T cells. These cells were incubated with TGF-β in vitro for 24 h and the expression of Spred-1 was examined by real-time PCR. As depicted in Fig. 5c, Spred-1 was slightly upregulated ($P < 0.05$) in the presence of TGF-β in the naive CD8+ T cells as compared to the same cells incubated with media without TGF-β (Fig. 5c, left panel). TGF-β treatment led to enhanced upregulation ($P < 0.005$) of Spred-1 in the effector/memory CD8+ T cells (Fig. 5c, middle panel), whereas the expression of Spred-1 did not change in the rested/memory CD8+ T cells post–TGF-β treatment (Fig. 5c, right panel). Therefore, our in vitro data demonstrated that TGF-β could directly upregulate Spred-1 primarily in effector/memory CD8+ T cells.

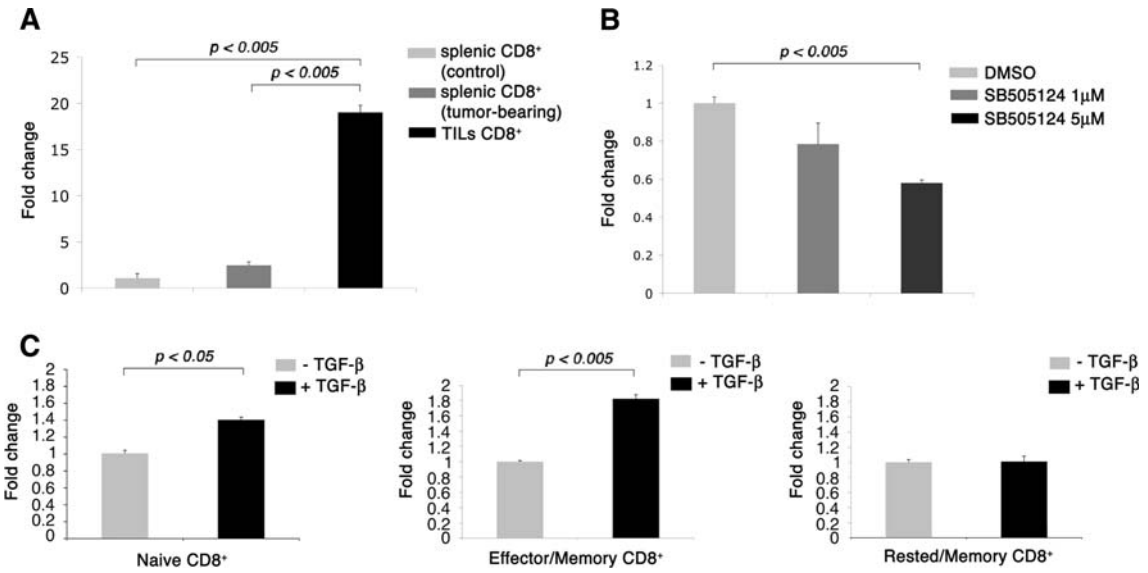

Fig. 5 Spred-1 gene expression in CD8$^+$ T cells is dependent on TGF-β activity. **a** Spred-1 gene expression was measured by real-time PCR on cDNA generated from splenic CD8$^+$ T cells of healthy (control) and tumor-bearing mice and from tumor-infiltrating CD8$^+$ T cells. The fold change of expression is reported in relation to expression of Spred-1 in splenic CD8$^+$ T cells of healthy mice. Data are representative of $n = 4$ experiments. **b** MC38 tumor were grown for 3 weeks, removed from the C57BL/6 mice and digested to a single cell suspension. Total digest cells were incubated in vitro with normal splenic CD8$^+$ T cells at a ratio of 10:1 in the presence of two different concentrations of TGF-β inhibitor SB505124. Twenty-four hours later anti-CD3 was added to the cell culture and, after an additional 24 h, CD8$^+$ T cells were purified for real-time PCR. The fold change of Spred-1 expression is reported in relation to the expression in CD8$^+$ T cells treated with DMSO. Data are representative of $n = 3$ experiments. **c** Naïve, effector/memory and rested/memory CD8$^+$ T cells were generated from the spleen of TCR/Tg-F5 mice. They were incubated in vitro for 24 h with TGF-β and real-time PCR was performed. The fold change of Spred-1 expression is reported in relation to its expression in CD8$^+$ T cells incubated without TGF-β. Data are representative of $n = 3$ experiments

Phosphorylation of ERK kinases, following activation, is impaired in tumor-infiltrating CD8$^+$ T cells in a TGF-β-dependent manner

Since it has been previously shown that Spred-1 can inhibit the activation of ERK kinase pathway in neuronal and muscle cell lines [23], we investigated the consequences of the upregulation of Spred-1 on ERK kinase phosphorylation following TCR activation in tumor-infiltrating CD8$^+$ T cells. To address the role of Spred-1 in the activation of ERK in tumor-infiltrating CD8$^+$ T cells, MC38 tumor digest and unfractionated splenocytes from the tumor-bearing mice were incubated in vitro for 24 h with DMSO or TGF-β inhibitor SB505124 and then activated with PMA/Ionomycin, which mimics full activation by TCR signaling. Following this, intracellular staining for phospho-ERK was performed using FACS analysis. ERK phosphorylation in CD8$^+$ T cells from the tumor infiltrate was markedly reduced as compared to CD8$^+$ T cells from the spleen (Fig. 6, top and middle pannel). However, the treatment of tumor single cell suspension with TGF-β inhibitor could restore the phosphorylation of ERK in CD8$^+$ TILs after PMA/Ionomycin activation (Fig. 6, bottom panel).

These results demonstrated that CD8$^+$ TILs present a low level of ERK phosphorylation following TCR activation and this response is linked to the TGF-β activity. This outcome might explain the impaired proliferation responses of the TILs upon TCR activation.

Discussion

In this study we have established that CD8$^+$ T cells present in the tumor microenvironment displayed impaired functionality and hyporesponsiveness to TCR stimulation in the presence of TGF-β. We have shown that the inhibition of TGF-β activity, using a specific small molecule inhibitor SB505124 [27], can reverse the anergic state of ex vivo tumor-infiltrating CD8$^+$ T cells. This result suggests that TGF-β is one of the cytokines that leads to impaired functionality of CD8$^+$ T cells in the tumor microenvironment.

To explore possible molecular mechanisms involved in the altered functionality of the TILs, we examined changes at the gene level in these cells. Microarray analysis of the TILs revealed that many genes involved in TCR signaling pathway were altered in tumor-associated CD8$^+$ T cells in accordance with previous reports [1, 28]. In our study we have reported that TCR chains and signaling molecules required for TCR signal transduction, such as TCR beta chain, CD3 zeta chain, ZAP70 and ITK, are downregulated in tumor-infiltrating CD8$^+$ T cells. Another cell-surface molecule overexpressed on the CD8$^+$ TILs' surface

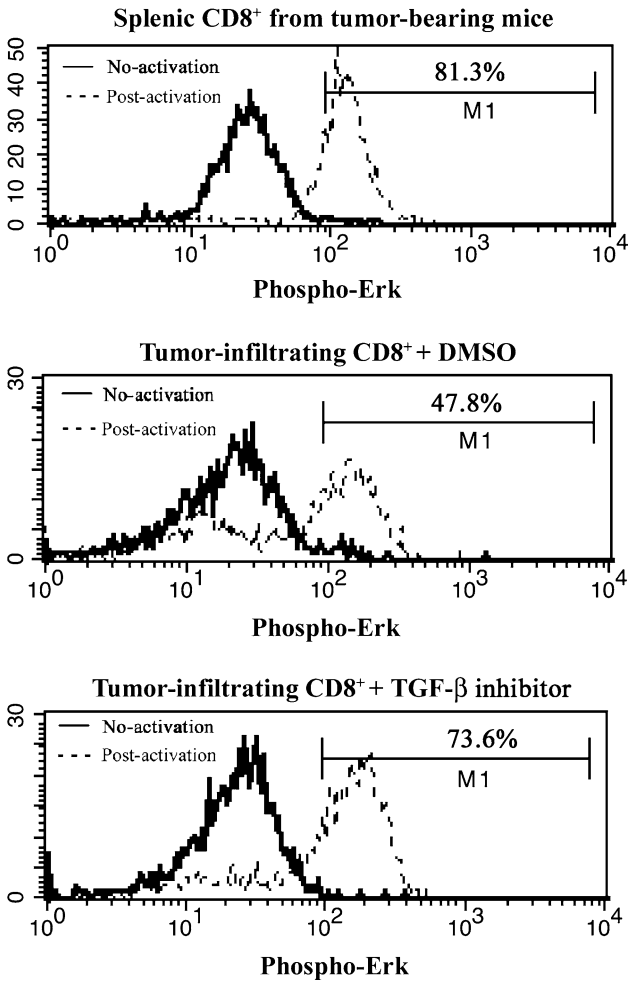

Fig. 6 ERK1/2 kinase phosphorylation is reduced in tumor-infiltrating $CD8^+$ T cells following TCR activation and restored by TGF-β inhibitor. MC38 tumors were grown for 3 weeks and then removed from C57BL/6 mice as well as the spleen. A single cell suspension of tumor cells, as well as unfractionated splenocytes from tumor-bearing mice, were incubated in vitro for 24 h with DMSO or with TGF-β inhibitor (10 μM) and then activated with PMA/Ionomycin. Intracellular staining for phospho-ERK was performed as well as CD8 surface staining. For FACS analysis, gates were placed around $CD8^+$ cells. Values indicate the percentage of phospho-ERK^+ cells out of $CD8^+$ cells. Data are representative of three separate experiments

compared to the splenic $CD8^+$ T cells is LAG-3: it has already been shown, using two murine systems, that LAG-3 is involved in the maintenance of the tolerance to self and tumor antigens via direct effects on $CD8^+$ T cells [29].

Previously, it has been demonstrated using confocal microscopy that TGF-β impaired the nuclear translocation of NF-κB, following the TCR activation, in memory T cells isolated from human lung tumor and from nonmalignant chronic inflammatory tissues [21]. Another study demonstrated that TGF-β inhibits phosphorylation and activation of ITK in normal $CD4^+$ T cells resulting in the lack of $CD4^+$ T cell differentiation into Th1/Th2 lineages [22]. In our study we demonstrated for the first time, by flow cytometry at a single cell level, that ITK phosphorylation following TCR stimulation was completely inhibited in tumor-associated $CD8^+$ T cells as compared to splenic $CD8^+$ T cells from the same tumor-bearing mice. These results suggest that the hyporesponsiveness of the $CD8^+$ TILs to TCR stimulation could be due to the downregulation and/or inactivation of TCR signaling components such as ITK. The hyporesponsiveness of the $CD8^+$ TILs could also be due to TGF-β activity, since the inhibition of TGF-β can reverse the reduced functionality of ITK.

An important event that could contribute to the induction of the hyporesponsiveness of the $CD8^+$ TILs is the presence in the tumor microenvironment of $CD4^+$ regulatory T cells (T_{REGs}). This hypothesis is supported by the already described intratumoral accumulation of $CD4^+CD25^+$ T_{REG} cells [7] and by the involvement of TGF-β in the induction of Foxp3 expression in $CD4^+CD25^-$ T cells during T cell activation [19].

Recently the concept has been emerging that $CD8^+$ T_{REGs}, as a subset of $CD8^+$ T cells, may either inherently possess or be induced under specific activation conditions to exert suppressor activities [30–33]. The compartmentalization of peripheral $CD8^+$ T_{REGs} in environments rich in IL-10 and TGF-β, such as tumor microenvironments of tumor-bearing mice, has also been reported [34]. Moreover the $CD8^+$ TILs, when stimulated with anti-CD3, produced high amounts of IFN-γ, a molecule that plays a crucial role in either the induction or the expression of $CD8^+$ T_{REGs} activity [35]. One can thus speculate that $CD8^+$ T_{REGs} may be present in the $CD8^+$ TILs population contributing to the inhibition of $CD8^+$ T cells activity in the tumor microenvironment.

We have also shown here for the first time that Spred-1 expression can be related to impaired functionality of tumor-infiltrating $CD8^+$ T cells. Spred-1 is a described inhibitor of growth factor-induced ERK kinases [23]. A previous study has shown the involvement of Spred-1 in tumor progression, in particular in human hepatocellular carcinoma where the downregulation of Spred-1 expression is one of the causes of the acquisition of malignant features by hepatic cells [25]. Spred-1 also plays an important role in lymphatic vessel development during embryogenesis and negatively regulates hematopoiesis suppressing stem-cell-factor (SFC)-induced and IL-3-induced ERK activation [24, 36]. Despite the fact that Spred-1 has been associated with the suppression of IL-5-dependent proliferation of $CD4^+$ T cells in allergic asthma, there is no study addressing the role of Spred-1 in $CD8^+$ T lymphocytes or in tumor-associated T cells [37]. ERK1/2 kinases activation is a crucial event in the signaling cascade of several growth factors in T cells; it is also a downstream event in the TCR signaling pathway leading to the activation and proliferation of T cells [38, 39].

We demonstrate here that Spred-1 is highly upregulated in the tumor-infiltrating $CD8^+$ T cells. Our results illustrate that this upregulation of Spred-1 is accompanied by more than 60% reduction of ERK activity in the TILs' post-TCR activation. This ERK inhibition could be the consequence of the upstream impairment of TCR signaling components as well as the overexpression of Spred-1. Since ITK can function upstream to ERK inactivation, TGF-β could suppress ERK by two different mechanism, i.e., inhibition of ITK and/or induction of Spred-1.

Moreover, the inhibition of TGF-β using a small molecule inhibitor SB505124 in an ex vivo tumor microenvironment led to a decreased expression level of Spred-1 in $CD8^+$ T cells, as well as a restored ERK activity post-TCR activation, suggesting a correlation between the TGF-β pathway and Spred-1/ERK kinases. This correlation was also supported by our in vitro studies in which Spred-1 expression was increased only in effector/memory $CD8^+$ T cells after TGF-β treatment.

The differential effect of TGF-β on the $CD8^+$ T cells' differentiation status and activation history has been recently investigated in different models [40, 41]. In tumor antigen-specific human memory $CD8^+$ T cells TGF-β attenuates the acquisition and expression of effector function [40]; in an in vitro mouse model, TGF-β suppresses the activation of naïve $CD8^+$ T cells but promotes their survival and function once they are antigen-experienced [41].

In conclusion, we suggest that TGF-β plays a pivotal role in T-cell anergy in the tumor microenvironment. High levels of TGF-β in the tumor can result in downregulation of upstream TCR signaling components, accompanied by upregulation of Spred-1. This outcome leads to the inhibition of the ERK pathway, thereby contributing to the impairment of proliferation/cytokine production of tumor-infiltrating $CD8^+$ T cells.

The results shown here may have important implications for the development of novel anti-tumor immune strategies based on the inhibition of TGF-β by a small molecule inhibitor directed against the TGF-β receptor; this strategy has the potential to be an alternative to the use of monoclonal antibody against TGF-β. This strategy is based on the inhibition of Spred-1 function that blocks the ERK1/2 activation, which is an important event controlling several growth factor and cytokines cascades.

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