Depletion of tristetraprolin in breast cancer cells increases interleukin-16 expression and promotes tumor infiltration with monocytes/macrophages

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The RNA-binding protein tristetraprolin (TTP) destabilizes target messenger RNAs (mRNAs) containing AU-rich elements within their 3′ untranslated region. To date, it controls the expression of multiple inflammatory and tumor-associated transcripts. Moreover, a loss of TTP in tumors predicts disease-associated survival. Although tumor intrinsic functions of TTP have previously been studied, the impact of TTP on the interaction of tumors with their microenvironment remains elusive. As immune cell infiltration into tumors is a critical determinant for tumor progression, this study aimed at determining the influence of tumor cell TTP on the interaction between tumor and immune cells, specifically monocytes (MO)/macrophages (MΦ). Knockdown (k/d) of TTP in T47D breast cancer cells enhanced tumor growth both in vitro and in vivo and increased infiltration of MΦ into 3D tumor spheroids in vitro and of MΦ into tumor xenografts in vivo. Enhanced migration of MO toward supernatants of TTP-deficient tumor spheroids was determined as the underlying principle. Interestingly, we noticed interleukin-16 (IL-16) mRNA stabilization when TTP was depleted. In line, IL-16 protein levels were elevated in TTP-deficient spheroids and their supernatants as well as in TTP k/d tumor xenografts and critically contributed to the enhanced chemotactic behavior. In summary, we show that the loss of TTP in tumors not only affects tumor cell proliferation and survival but also enhances infiltration of MO/MΦ into the tumors, which is typically associated with poor prognosis. Moreover, we identified IL-16 as a critical TTP-regulated chemotactic factor that contributes to MO/MΦ migration.

Introduction

The RNA-binding protein tristetraprolin (TTP) binds to messenger RNAs (mRNAs) containing AU-rich elements within their 3′ untranslated region and facilitates rapid degradation of the respective target mRNAs (1). TTP plays a crucial role in the post-transcriptional regulation of immune responses as it promotes destabilization of several inflammatory mediators [e.g. tumor necrosis factor-α (TNF-α), granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin (IL)-6] (2–6). Moreover, TTP binds to mRNAs encoding factors associated with cancer [e.g. cyclin D1, c-Myc], angiogenesis (e.g. vascular endothelial growth factor, IL-8) and tissue invasion [e.g. uPA, PLAU, matrix metalloproteinase 1], thereby limiting their expression and consequently exerting tumor-suppressive functions (7–10). Recently, TTP expression analysis in a variety of human tumors and cancer cell lines revealed a progressive downregulation of TTP during tumorigenesis and metastasis (11–13). Decreased TTP expression was even put forward as a negative prognostic marker for tumor-associated survival (14).

It is increasingly acknowledged that tumorigenesis is strongly influenced by the interaction of neoplastic cells with the stroma including surrounding and infiltrating cells. Leukocytes, especially tumor-associated macrophages (TAM), have been recognized as a major component of tumors (15,16). Several studies investigating human breast tumor tissues and using various transgenic mouse models demonstrated a close link between TAM infiltration and tumor progression by showing a correlation between high macrophage (MΦ) numbers and poor prognosis (17–20). Recruitment of blood monocytes (MO), precursors of TAMs, is an important step that is orchestrated to a substantial degree by chemokines and chemotactic cytokines. Specifically, chemokine and chemotactic cytokines recruit leukocytes, and, thus, constitute a key determinant of tumorigenesis (21,22). IL-16 is a pleiotropic cytokine with a fundamental role in the pathophysiology of inflammatory diseases. It can be synthesized by both immune and non-immune cells, including T cells, mast cells, dendritic cells, fibroblasts and epithelial cells, and serves as a chemoattractant for CD4+ T lymphocytes, MO, MΦ and eosinophils (23–27). IL-16 has also been implicated in tumor development and progression as it was found to be overexpressed in different tumor types. Mechanisms by which IL-16 influences cancer development and progression are still under investigation (28–30).

In this study, we determined whether and how the loss of TTP in breast cancer cells affects MO recruitment and established a fundamental role of IL-16.

Materials and methods

Materials

Medium and supplements were purchased from PAA (Linz, Austria). Fetal calf serum was from Biochrom (Berlin, Germany). Nitrocellulose membranes, the enhanced chemiluminescence detection system and horseradish peroxidase-labeled secondary antibodies were supplied by GE Biosciences (Freiburg, Germany) and BRDyces 800CW secondary antibodies came from Li-COR Biosciences GmbH (Bad Homburg, Germany). Rabbit antiserum against TTP was kindly provided by Prof. Dr P.Kovarik (Vienna, Austria). Fluorescein isothiocyanate-conjugated cleaved caspase 3 antibody was from BD Biosciences (Heidelberg, Germany), caspase 3 antibody was from Cell Signaling Technology (Frankfurt, Germany), APC-conjugated CD14 antibody came from BD Biosciences and APC-conjugated CD16 antibody was from ImmunoTools (Friesoythe, Germany). IL-16 neutralizing antibody was purchased from Abcam (Cambridge, UK) and rabbit IgG control antibody was from R&D Systems (Wiesbaden, Germany). CD68 antibody came from DAKO (Hamburg, Germany) and F4/80 was purchased from AbD Serotec (Düsseldorf, Germany). Tubulin antibody was supplied from Sigma–Aldrich (Schnelldorf, Germany) and histone H3 antibody was from Upstate (Billerica, MA).

Cell culture

HEK293T and T47D cells (LGCC Standards GmbH, Wesel, Germany) were cultured in Dulbecco’s modified Eagle’s medium (HEK293T) or RPMI 1640 (T47D) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal calf serum and 2 mM l-glutamine. Cells were kept at 37°C in a humidified atmosphere with 5% CO₂.

Lentivirus-mediated shRNA expression

The bacterial glycerol stocks containing TTP short hairpin (shRNA) transfer vectors were delivered by Sigma–Aldrich. Lentivirus production using HEK293T cells and subsequent transduction of T47D cells were carried out following the protocols supplied by the manufacturer. Lentivirus expressing a non-target shRNA (sh ctr) served as negative control.

mRNA expression analysis

RNA from T47D tumor spheroids was extracted using peqGold RNAPure (Peqlab Biotechnologie, Erlangen, Germany). Total RNA was transcribed with iScript complementary DNA synthesis kit (Bio-Rad Laboratories, Munich, Germany). Quantitative real-time PCR was performed using Absolute Blue qPCR SYBR green fluorescein mix (Thermo Scientific, Karlsruhe, Germany).

Abbreviations: IL, interleukin; k/d, knockdown; MΦ, macrophage; MO, monocyte; mRNA, messenger RNA; IL-16, recombinant IL-16; TAMs, tumor-associated macrophages; TNF-α, tumor necrosis factor-α; TTP, tristetraprolin; sh ctr, non-target shRNA.
The following primers were used for quantitative real-time PCR: human actin, 5'-TGA CGG GGT CAC CCA CAC TGTC GCC CAT CTA-3' and 5'-CTA GAA GCA ACC TTT CTT CAC GTG GAT GIA GGG-3'; for human TTP, 5'-CAA GTA GCC AAA GCC GTT GCC AAA-3' and 5'-ATA CAA GGG AAG CAG ACC CAA-3' and for human IL-16, 5'-CGC AAA GCC GTT GCC AAA-3' and 5'-TGG GTG TTC AGA CCG ATT CTT CAA-3'. Real-time PCR results were quantified using the Bio-Rad CFX Manager (version 1.6; Bio-Rad Laboratories), with actin expression as internal control.

Western analysis
Cell extracts were prepared in lysis buffer [6.65 M urea, 10% glycerol, 1% sodium dodecyl sulfate, 10 mM Tris–HCl (pH 6.8) and protease inhibitor cocktail (pH 7.4)] by sonication. The protein content was determined by the Lowry method. About 90 µg of protein were resolved on 15% sodium dodecyl sulfate–polyacrylamide gels and blotted onto nitrocellulose membranes. Proteins were detected using specific antibodies and appropriate secondary antibodies. For visualization, enhanced chemiluminescence and the Odyssey infrared imaging system (Li-COR Biosciences GmbH) were used.

Peripheral blood MO isolation
MO were isolated from human buffy coats (DRK-Blutspendest Baden-Württemberg-Hessen, Institut für Transfusionsmedizin und Immunohematologie, Frankfurt, Germany) using Ficoll-Hypaque gradients (PAAR Laboratories, Colbe, Germany). Total MO or CD14+ MO were separated by magnetic cell sorting using microbeads for human pan MO or CD14, respectively, by the autoMACSTM separator (Milenyi Biotec, Bergisch Gladbach, Germany).

Fluorescence-activated cell sorting of MO subpopulations
For separation of MO subpopulations, 2 x 106 freshly isolated total blood MO were stained with APC-conjugated CD16 and APC-H7-conjugated CD14 antibodies. After washing, cells were filtered through a 70 µm sterile nylon mesh (BD Biosciences) before high-quality sorting with a FACSaria (BD Biosciences). Forward and side scatter, as indicators of size and granularity, as well as the specific fluorescence signals were recorded and sort gates were defined in the respective dot plot diagrams.

Co-culture of spheroids and MO
Multicellular spheroids were generated according to the liquid overlay technique using agarose-coated 96-well plates and single cell suspensions of 5 x 103 T47D cells per well. For co-culture experiments, 1 x 104 purified CD14+ cells were added to each spheroid. Medium was changed after 3 days of co-culture. After 5 days of cultivation, the co-cultures were employed for flow cytometric analysis and immunohistochemistry. For co-culture experiments with MO subpopulations, 5 x 105 cells per well were used.

Cell migration assays
For Boyden chamber assays, 5 x 105 starved purified CD14+ cells were added to serum-free RPMI 1640 medium in transwell inserts (5 µm pores; Costar, Corning, NY) and allowed to migrate toward spheroid supernatants in the transwell inserts. After 3 days of migration, the monolayer culture, we grew sh ctr and TTP k/d cells as 3D tumor spheroids (Figure 1D). This corroborates previous reports that besides proliferation and invasiveness (10,14). As we aimed at deciphering the influence of tumor cell TTP on the interaction between the tumor and immune cells, we established a stable knockdown (k/d) of TTP in the non-invasive, epithelial breast cancer cell line T47D. Efficient k/d by two independent shRNA clones (cl. 1 and cl. 2) targeting TTP were confirmed at mRNA and protein level (Figure 1A and B). TTP is involved in the regulation of cell proliferation. Therefore, we examined the growth of control cells (sh ctr) and TTP k/d cells. In line with previous reports, depletion of TTP significantly accelerated the cell growth in both TTP k/d clones (Figure 1C) (31). As both TTP k/d clones exhibited comparable characteristics, TTP k/d cl. 1 was selected for further experiments. To investigate the impact of a loss of TTP in a more physiologically relevant setting as compared with the monolayer culture, we grew sh ctr and TTP k/d cells as 3D tumor spheroids. Ten days after seeding the respective T47D cells, spheroids were stained with trypan blue to assess viability of the 3D structures. Interestingly, TTP k/d spheroids consistently showed less trypan blue staining compared with sh ctr spheroids, indicating that TTP k/d spheroids appeared to maintain a higher viability when grown as tumor spheroids (Figure 1D). This corroborates previous reports that besides proliferation, TTP also regulates cell death mechanisms (32,33). To further support the observation that reduced TTP expression promotes cell survival, we evaluated cleavage of caspase 3 as an indicator for apoptosis by western analysis. Indeed, lysates of TTP k/d spheroids displayed less cleaved (active) caspase 3 compared with sh ctr spheroids (Figure 1E). Quantitative analysis revealed a reduction of cleaved caspase 3 in TTP-depleted tumor spheroids to 0.75 ± 0.04-fold of sh ctr spheroids. Additional flow cytometric analyses corroborated our results showing that 19.2 ± 1.5% of the cells contained cleaved caspase 3 in sh ctr spheroids, whereas TTP k/d spheroids displayed cleaved caspase 3 in only 14.6 ± 1.0% of the cells (Figure 1F). Taken together, our data indicate that a loss of TTP in T47D cells enhances proliferation and prevents apoptosis in human 3D tumor spheroids.

Depletion of TTP in tumor spheroids increases infiltration with MO
It is widely accepted that the behavior of tumorogenic cells is strongly affected by microenvironmental conditions, including tumor-infiltrating immune cells. MO are especially involved in the...
Fig. 1. TTP depletion increases proliferation and viability of T47D cells. (A) Quantitative PCR of total TTP mRNA expression (expr.) in T47D cells stably transduced with different shRNA clones (cl. 1 and cl. 2) targeting TTP. A sh ctr clone was used as control. TTP mRNA levels were normalized to actin mRNA levels. (B) TTP and histone H3 protein expression in sh ctr and TTP k/d (cl. 1 and cl. 2) T47D cells was analyzed by western analysis. (C) Sh ctr or TTP k/d cells (cl. 1 and cl. 2) were grown for indicated time periods. Proliferation was determined relative to the cell numbers at day 0. (D) Ten-day-old sh ctr and TTP k/d spheroids were stained with trypan blue. Scale bars = 200 μm. (E) Pro-caspase 3 and cleaved (active) caspase 3 expression in sh ctr and TTP k/d spheroids was determined using western analysis and densitometrically evaluated using ImageJ software. The ratio of cleaved caspase 3 (cl. caspase 3) to tubulin (tub.) was used for quantification. Sh ctr was set to 1. (F) Quantification of active caspase 3 in 10-day-old spheroids was performed by intracellular flow cytometry using a fluorescein isothiocyanate-labeled antibody against cleaved caspase 3. Data represent means ± SEM (n ≥ 3; *P < 0.05, **P < 0.01, ***P < 0.001). Blots are representative for at least three individual experiments.

Development and spread of breast tumors (16,34). MΦ in tumors are derived mainly from blood MO. Therefore, we next determined how TTP in tumor cells affects their interaction with MO. Ten-day-old tumor spheroids were co-cultured with MO isolated from peripheral blood mononuclear cells for 5 days. Differentiating MO/MΦ can be identified by the specific expression of a number of proteins including CD14, CD11b and CD68. As we observed more prominent expression of CD14 in MO/MΦ isolated from human peripheral blood as compared with CD11b, we determined infiltration via flow cytometry by detecting CD14+ cells (Figure 2A). Interestingly, the rate of MO infiltration was 1.7 ± 0.3-fold higher in TTP k/d spheroids than in sh ctr spheroids (Figure 2B). Blood MO are heterogeneous and can be subdivided into at least three distinct subsets defined by diverging expression of CD14 and CD16 (35,36). Because different migratory and functional states are attributed to these subsets, we next analyzed the infiltration of purified classical (CD14++ CD16−), non-classical (CD14+ CD16++) and intermediate (CD14++ CD16+) MO subsets into tumor spheroids (flow cytometric separation, see Supplementary Figure 1, available at Carcinogenesis Online). Indeed, all three subsets showed a higher infiltration into TTP-deficient tumor spheroids (classical MO: sh ctr: 15.8 ± 2.1% and TTP k/d: 24 ± 3.7%; non-classical MO: sh ctr: 21 ± 0.3% and TTP k/d: 48 ± 1.9%; intermediate MO: sh ctr: 4.6 ± 0.8% and TTP k/d: 7.1 ± 1.9%) (Figure 2C). However, classical MO showed prominently higher basal infiltration rates into tumor spheroids. To further assess whether particular areas within the respective tumor spheroids were infiltrated by mature MO/MΦ, we stained sections of the co-cultures for the intracellular marker CD68, which allows for sensitive detection by immunohistochemistry. Remarkably, the distribution of infiltrated MO/MΦ differed in sh ctr and TTP k/d spheroids. Although MO/MΦ in TTP k/d tumor spheroids resided primarily in the outer spheroid areas, MO/MΦ were more widely distributed in sh ctr tumor spheroids (Figure 2D). Consistent with our findings of increased tumor spheroid infiltration with CD14+ cells, quantitative analysis of CD68+ cells in sections of MΟ-/tumor spheroid co-cultures revealed significantly elevated numbers of MO/MΦ in TTP k/d tumor spheroids (1001 ± 56 CD68+ cells/mm²) compared with sh ctr samples (528 ± 35 CD68+ cells/mm²) (Figure 2E). These results indicate that tumor spheroids are more efficiently infiltrated by MO/MΦ when they lack TTP.

TTP depletion in tumors accelerates their growth and enhances infiltration with MΦ in vivo

Next, we aimed to validate our experimental system in an in vivo setup. To this end, we injected female nude mice subcutaneously with either 5 x 10⁶ T47D sh ctr or TTP k/d cells in 50% Matrigel™ into the dorsal flanks (both cell lines in alternate flanks of one individual animal) and monitored the tumor growth for 35 days. TTP k/d xenografts displayed an elevated growth rate compared with tumors derived from sh ctr cell implants (Figure 3A). Analysis of the weight of the xenografts revealed a significantly increased tumor mass for the TTP k/d tumors at the end of the study (Figure 3B). To determine if lower TTP levels in tumor cells affected myeloid cell recruitment as seen in vitro, we examined the infiltration of CD11b+ cells into the tumor xenografts. In contrast to the human system, CD11b, but not CD14, is prominently expressed in murine cells of myeloid origin. Therefore, we used this marker to assess infiltration in our in vivo studies. Tumors were digested and CD11b+ cells were quantified using flow cytometry. To avoid misinterpretation due to different tumor masses of sh ctr and TTP k/d tumors, the numbers of infiltrated CD11b+ cells were normalized to the tumor weight. In line with the tumor spheroid model, infiltration of CD11b+ cells was increased 1.8 ± 0.4-fold in TTP k/d tumors as compared with sh ctr tumors (Figure 3C). To gain further evidence regarding the nature of the infiltrating cells, we stained tumor xenograft sections by immunohistochemistry for the murine MΦ marker F4/80. Consistent with our observation of increased infiltration of CD11b+...
Loss of TTP in tumors enhances MO/MΦ infiltration

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cells, elevated levels of F4/80+ cells appeared in TTP k/d tumors (Figure 3D).

TTP deficiency alters cytokine expression in TTP k/d tumor spheroids

To determine if enhanced infiltration of MO/MΦ was a result of secretion of chemoattractants by tumor cells, we analyzed migration of MO toward tumor spheroid supernatants in a Boyden chamber approach. The supernatants of TTP k/d spheroids significantly induced the migration of MO as compared with sh ctr supernatants.
IL-16 is upregulated upon TTP deficiency

As IL-16 displayed the most prominent increase in TTP k/d spheroid supernatants (2.7-fold) as compared with sh ctr supernatants, we further analyzed the role of IL-16 in our system. IL-16 is known to act as a chemotacticant not only for CD4+ T lymphocytes but also for MO and MΦ (23,24). To explore the contribution of IL-16 to migration in our experimental setup, we validated the expression of IL-16 in sh ctr and TTP k/d tumor spheroids. Interestingly, IL-16 mRNA expression was significantly elevated in TTP k/d spheroids (2.5 ± 0.2-fold) as compared with sh ctr spheroid media (Table I). Indeed, the stability of IL-16 mRNA significantly increased because TTP is an mRNA destabilizing protein, we analyzed the stability of IL-16 mRNA. Interestingly, although well-known chemotactic and angiogenic factors such as growth-regulated oncogene, epidermal growth factor, macrophage migration inhibitory factor, monocyte chemotactic protein-1, and angioiucin were downregulated in TTP k/d spheroid media, IL-16, interferon-inducible protein-10, transforming growth factor-β 2 and osteopontin levels were increased (Table I). These data indicate that TTP k/d spheroids secrete a distinct set of chemokines and cytokines, which, although it lacks various prominent chemotactic factors, provoked increased MO migration toward and infiltration into tumor spheroids.

Neutralization of IL-16 affects chemotaxis of MO

As the IL-16 levels quantified in both sh ctr and TTP k/d spheroid supernatants were rather low, we questioned their impact on MO chemotaxis. To this end, human rIL-16 was used to induce migration of MO in a Boyden chamber assay. Importantly, chemotaxis of MO was significantly increased at concentrations of rIL-16 as low as 20 pg/ml (3.9 ± 0.9-fold) (close to the concentration in TTP k/d spheroid supernatants) and further increased when 200 pg/ml rIL-16 were used (23.3 ± 4.7-fold) (Figure 5F). Furthermore, the addition of rIL-16 to sh ctr spheroid supernatants at comparable concentrations to those found in TTP k/d supernatants increased MO migration (1.3 ± 0.06-fold) to similar values as seen with TTP k/d supernatants (Figure 5G). To verify that IL-16 within the complex tumor spheroid supernatants contributes to the migratory phenotype of co-cultured MO, we depleted supernatants of IL-16 using a neutralizing antibody against IL-16. Indeed, neutralization of IL-16 significantly attenuated migration of MO towards supernatants of TTP k/d spheroids (Figure 5H). In line with lower IL-16 levels found in sh ctr supernatants, IL-16 depletion only minimally affected the migratory response induced by these supernatants.

In summary, elevated IL-16 levels, as observed in supernatants of TTP k/d spheroids, enhanced the migration of MO toward and being the underlying mechanism for enhanced MO/MΦ numbers in TTP k/d spheroids (Figure 4A). In order to identify factors secreted by the tumor cells that contribute to enhanced migration of MO toward TTP k/d spheroid supernatants, we evaluated the abundance of major immune cell chemokines/cytokines in supernatants of sh ctr and TTP k/d spheroids using a human cytokine antibody array (Figure 4B). Densitometric analysis revealed that the levels of the established TTP targets TNF-α, IL-1β, interferon γ and IL-3 were higher in TTP k/d spheroid supernatants compared with sh ctr spheroid media (Figure 4A). In order to identify factors secreted by TTP k/d spheroids secrete a distinct set of chemokines and cytokines, which, as IL-16 displayed the most prominent increase in TTP k/d spheroid supernatants (2.7-fold) as compared with sh ctr supernatants, we further analyzed the role of IL-16 in our system. IL-16 is known to act as a chemotacticant not only for CD4+ T lymphocytes but also for MO and MΦ (23,24). To explore the contribution of IL-16 to migration in our experimental setup, we validated the expression of IL-16 in sh ctr and TTP k/d tumor spheroids. Interestingly, IL-16 mRNA expression was significantly elevated in TTP k/d spheroids (2.5 ± 0.2-fold) as compared with sh ctr spheroid media (Figure 5A). Because TTP is an mRNA destabilizing protein, we analyzed the stability of IL-16 mRNA. Indeed, the stability of IL-16 mRNA significantly increased when TTP was depleted. The half-life of IL-16 mRNA was markedly prolonged in TTP k/d tumor spheroids (t1/2 > 4h) compared with sh ctr samples (t1/2 = 2.7 ± 0.7h) (Figure 5B). Furthermore, as the data in Figure 4B revealed only relative expression changes, we quantified absolute amounts of IL-16 in spheroid supernatants by enzyme-linked immunosorbent assay. As shown in Figure 5C, TTP k/d spheroid supernatants contained significantly higher IL-16 levels (16.4 ± 3.9 pg/ml) compared with the amounts detected in sh ctr media (6.1 ± 1.2 pg/ml). Western analysis of tumor spheroid lysates for IL-16 supported the finding of higher IL-16 expression in TTP-deficient cells (Figure 5D). Similarly, IL-16 appeared to be elevated in TTP k/d tumor xenografts as compared with ctr tumors (Figure 5E). Taken together, the loss of TTP in tumor cells enhanced IL-16 expression in vitro and in vivo.

Table I. Cytokine antibody array results with a selective panel of cytokines showing differential expression in sh ctr versus TTP k/d spheroid supernatants

<table>
<thead>
<tr>
<th>Location on membrane</th>
<th>Cytokine</th>
<th>Fold change in TTP k/d (rel. to sh ctr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upregulated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C 7</td>
<td>IL-16</td>
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</tr>
<tr>
<td>E 2</td>
<td>IL-3β</td>
<td>1.6586</td>
</tr>
<tr>
<td>D 3</td>
<td>IFNγ</td>
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</tr>
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<td>D 7</td>
<td>IP-10</td>
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<tr>
<td>F 8</td>
<td>TGF-β 2</td>
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</tr>
<tr>
<td>B 8</td>
<td>Osteopontin</td>
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<tr>
<td>G 4</td>
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</tr>
<tr>
<td>J 1</td>
<td>Gro</td>
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</tr>
<tr>
<td>I 4</td>
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<td>H 7</td>
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<tr>
<td>K 4</td>
<td>Angioiucin</td>
<td>0.4658</td>
</tr>
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</table>

Changes in relative expression between sh ctr and TTP k/d spheroid supernatants are expressed as fold change. Cytokines displaying a minimal change of 30% are presented. Cytokines below the minimal detection threshold are not shown. EGF, epidermal growth factor; Gro, growth-regulated oncogene; IFNγ, interferon γ; IP-10, interferon-inducible protein-10; MCP-1, monocyte chemotactic protein-1; MIF, macrophage migration inhibitory factor; TGF-β 2, transforming growth factor-β 2.

For a complete list of cytokines, see Supplementary Table I, available at Carcinogenesis Online.

*Described TTP target (1,37–39).
Loss of TTP in tumors enhances MO/MΦ infiltration

consequently infiltration into tumor spheroids. Importantly, even seemingly low levels of IL-16 sufficed to induce such responses. Enhanced infiltration of MΦ into and elevated IL-16 expression within TTP k/d tumors is an indicator that IL-16 might be important for the recruitment of MO in vivo as well.

Discussion

In this study, we show that the loss of TTP increases proliferation and prevents apoptosis of T47D breast cancer cells, consequently enhancing growth of tumor xenografts. We further provide evidence that TTP-deficient tumors are infiltrated with MO and MΦ to a higher degree in vitro and in vivo, and identify IL-16 as a critical chemoattractant contributing to MO recruitment into TTP-deficient tumor spheroids.

Our study demonstrates that depletion of TTP in T47D cells promotes proliferation and increases the viability in a 3D tumor spheroid system. Specifically, we observed reduced caspase 3 activity in TTP-depleted cells, which is in line with previous reports showing that overexpression of TTP sensitizes tumor cells to apoptosis induction (14,33,40). These observations are substantiated by our in vivo xenograft experiments, which demonstrated an inverse correlation between TTP expression and tumor growth. Our findings support recent reports indicating a tumor-suppressive function of TTP, which is also implicated by reports demonstrating a loss of TTP in various tumorigenic tissues as compared with their non-neoplastic origins (11–14).

The notion that TTP deficiency increases infiltration of MO, precursors of tumor-associated MΦ, into tumor spheroids and elevates the number of MΦ within tumors has previously been correlated with poor prognosis (17–19). Thus, the lack of TTP in tumor cells might contribute to tumorigenesis not only by affecting growth and survival of the tumor cells but also by enhancing recruitment of MO into the tumors. Increased infiltration with MO was a consequence of chemoattractant-induced migration. Specifically, we observed that TTP deficiency alters the secretome of a distinct set of mediators. Of note, well-characterized TTP targets such as TNF-α, IL-1β, interferon γ or IL-3 (4,37–40) were elevated. Despite the promigratory characteristics of the supernatants, we found decreased levels of various prominent chemotactic and proangiogenic factors like growth-regulated oncogene, monocyte chemotactic protein-1, macrophage migration inhibitory factor, epidermal growth factor and angiogenin when TTP was depleted. In contrast, a number of factors associated with chemotraction, growth, survival

Fig. 5. IL-16 is upregulated in TTP-deficient tumor spheroids and causes chemotraction of MO. (A) IL-16 mRNA expression in 10-day-old sh ctr and TTP k/d spheroids was assessed by quantitative PCR and normalized to actin mRNA. (B) IL-16 mRNA stability in 10-day-old sh ctr and TTP k/d spheroids was determined by blocking de novo transcription with actinomycin D (5 µg/ml) (act D). Remaining IL-16 mRNA was normalized to actin mRNA and is given relative to the respective mRNA expression before the addition of act D. The half-life (t1/2) ± SEM of IL-16 mRNA was calculated by regression analyses. (C) IL-16 protein (pg/ml) was determined in the supernatants of 10-day-old sh ctr and TTP k/d spheroids using an IL-16-specific enzyme-linked immunosorbent assay. (D) IL-16 and tubulin protein expression in sh ctr and TTP k/d tumor spheroids was analyzed by western analysis and densitometrically evaluated using ImageJ software. The ratio of IL-16 to tubulin was used for quantification. Sh ctr was set to 1. Blots are representative for three independent experiments. (E) Paraffin sections of sh ctr and TTP k/d tumor xenografts were stained for human IL-16 (brown). Nuclei were stained with Mayer’s hemalum (blue). Representative images are shown. Scale bars = 100 µm. (F) Chemotaxis of MO was determined using a Boyden chamber approach. Migration toward human rIL-16 (20 and 200 pg/ml) was normalized to fetal calf serum-free cell culture medium. (G) Chemotaxis of MO toward sh ctr supernatant supplemented with 20 pg/ml rIL-16 was normalized to sh ctr supernatant alone. (H) Chemotaxis of MO toward sh ctr and TTP k/d spheroid supernatants was determined in the presence of IL-16 neutralizing antibody (3 µg/ml) and compared with an isotypic IgG control antibody (3 µg/ml). Migration is given relative to sh ctr supernatants (IgG). In all experiments, the number of migrated cells was determined after 90 min. Data represent means ± SEM (n ≥ 3; ***P < 0.001).
and metastasis including IL-16, interferon-inducible protein-10, osteopontin and transforming growth factor-β 2 (24,41–44) were elevated in supernatants of TTP-deficient spheroids. Interestingly, although the IL-16 levels appeared rather low, they sufficed to induce migration of MO/MΦ. Besides higher MO/MΦ infiltration into TTP-deficient tumor spheroids, MO/MΦ distribution within the MO-tumor spheroid co-cultures was distinct. Differences in the depth of infiltration could result from larger areas of cell death in sh ctr spheroids accompanied by low oxygen tension that might provide microenvironmental conditions that facilitate invasion of deeper spheroid layers. In contrast, the small necrotic core accompanied by an attenuated hypoxic environment, might not suffice to recruit MO/MΦ to these areas in TTP k/d spheroids, thus, MO/MΦ reside preferentially in the outer layers. However, irrespective of tumor cell TTP expression, primarily classical MO (CD14+CD16+) infiltrated into tumor spheroids. Despite different expression patterns of receptors for chemotactic signals of distinct MO subtypes (45,46), we found similarly elevated infiltration of classical, non-classical and intermediate MO subpopulations into TTP k/d spheroids. Of note, all subsets were shown previously to express comparable levels of CD4, which is the receptor for IL-16, thus supporting the notion that IL-16 could emerge as a novel important chemotactic mediator in the tumor microenvironment (47). These findings are corroborated by previous reports that characterized IL-16 as a multi-functional cytokine with chemotactic properties for CD4 expressing MO, MΦ, T cells and eosinophils (24). Moreover, depletion of IL-16 efficiently blocked the enhanced chemotactic behavior of supernatants of TTP-deficient spheroids. Additionally, elevated IL-16 expression in TTP k/d xenografts, paralleled by enhanced MΦ infiltration, suggests that IL-16 might play a role in the immune cell recruitment in vivo as well. Although the role of IL-16 in tumorigenesis is poorly understood, high IL-16 levels were observed in tumor patients (28–30,48–49). Our study now provides evidence for a MO/MΦ attracting function of IL-16, which sheds further light on the still elusive role of IL-16 in tumorigenesis. Elevated IL-16 protein levels in spheroid supernatants were closely associated with enhanced IL-16 mRNA expression in TTP-deficient tumor spheroids. Moreover, increased IL-16 mRNA levels correlated with stabilization of the IL-16 mRNA in TTP-depleted cells. As TTP is an mRNA destabilizing RNA-binding protein (RBP) and IL-16 mRNA contains six AU-rich elements within its 3’ untranslated region as potential binding sites for TTP, this observation might be taken as an indication for a direct regulation of IL-16 mRNA by TTP. Yet, alternative indirect mechanisms of regulation cannot be excluded. For instance, TNF-α and IL-1β, both of which were elevated in TTP-deficient spheroid supernatants, were previously reported to induce IL-16 transcription (25). Thus, autocrine signals might also contribute to the enhanced IL-16 expression in TTP-depleted spheroids. Conversely, IL-16 was shown to induce the expression of TNF-α in MO (50). Thus, it might be speculated that tumor cell-derived IL-16 affects MO responses. Although there is ample evidence for the TTP-dependent regulation of IL-16, further studies are required to unequivocally determine the direct and/or indirect impact of TIP on IL-16 expression.

In summary, we provide evidence that TTP deficiency in breast cancer cells promotes recruitment of MO/MΦ into tumors. Furthermore, we identify IL-16 as a novel TTP-regulated cytokine contributing to the enhanced MO attraction. These findings will be of interest for the development of molecular targets of therapeutic interventions.

Supplementary material

Supplementary Figures 1 and 2 and Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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References

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50. Mathy, N.L. et al. (2000) Interleukin-16 stimulates the expression and production of pro-inflammatory cytokines by human monocytes. Immunology, 100, 63–69.

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