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Tumor-associated mesenchymal stem cells inhibit naïve T cell expansion by blocking cysteine export from dendritic cells

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Mesenchymal stem cells (MSCs) represent an important cellular constituent of the tumor microenvironment, which along with tumor cells themselves, serve to regulate protective immune responses in support of progressive disease. We report that tumor MSCs prevent the ability of dendritic cells (DC) to promote naïve $CD4^+$ and $CD8^+$ T cell expansion, interferon gamma secretion and cytotoxicity against tumor cells, which are critical to immune-mediated tumor eradication. Notably, tumor MSCs fail to prevent DC-mediated early T cell activation events or the ability of responder T cells to produce IL-2. The immunoregulatory activity of tumor MSCs is IL-10- and STAT3-dependent, with STAT3 repressing DC expression of cystathionase, a critical enzyme that converts methionine-to-cysteine. Under cysteine-deficient priming conditions, naïve T cells exhibit defective cellular metabolism and proliferation. Bioinformatics analyses as well as *in vitro* observations suggest that STAT3 may directly bind to a GAS-like motif within the cystathionase promoter (−269 to −261) leading to IL-10-STAT3 mediated repression of cystathionase gene transcription. Our collective results provide evidence for a novel mechanism of tumor MSC-mediated T cell inhibition within tumor microenvironment.

The significance of the tumor microenvironment (TME) and stroma in evolution of solid tumors is well-established.[1] Along with mature stromal elements that contribute to carcinogenesis, attention has now been shifted towards the progenitor cells including mesenchymal stem cells (MSCs),[2] adult stem cells exhibiting the properties of self-renewal and multi-lineage differentiative potential.[3] Within TME, MSCs directly interact with tumor cells, promoting tumor progression, metastasis, angiogenesis, epithelial mesenchymal transition (EMT) and autophagy.[4]

The immunoregulatory properties of normal MSCs have been implicated in immune evasion by progressor tumors.[5] Normal MSCs display suppressive effects on both innate and humoral immunity by inhibiting dendritic cell (DC) maturation,[6] natural killer/B-cell activation[7] and T cell proliferation,[8] while simultaneously supporting the regulatory T cell development.[9] Several MSC-associated mediators have been linked to MSC-mediated inhibition of T cell proliferation, including PGE_2, IDO, TGFβ1, HGF, iNOS and hemoxygenase-1.[5,10–12] However, the direct interaction between tumor resident

Key words: tumor microenvironment, mesenchymal stem/stromal cells, T cells, DC, cystathionase, cysteine/cystine, IL-10, STAT3

Abbreviations: APC: antigen presenting cell(s); BM: bone marrow; DC: dendritic cell(s); EMT: epithelial mesenchymal transition; GAS: gamma activated sequence; HGF: hepatocyte growth factor; IDO: indoleamine 2,3-dioxygenase; IFNγ: interferon γ; IL-10: interleulin-10; iNOS: inducible nitric oxide synthase; MDSC: myeloid derived suppressor cells; β-ME: beta marcaptoethanol; MHC: major histocompatibility complex; MSC: mesenchymal stem cells; PGE2: prostaglandin E2; STAT3: signal transducer and activator of transcription; TC-MSC: tumor conditioned MSC; TGFβ: transforming growth factor beta; TME: tumor microenvironment; VEGF: vascular endothelial growth factor

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What's new?

Mesenchymal stem cells (MSCs) within a tumor can suppress the immune response against that tumor. In this study, the authors discovered a pathway by which MSCs can block antigen-presenting cells (APCs) from stimulating cytotoxic T cells. This pathway inhibits an enzyme involved in the production of cysteine. Surprisingly, this cysteine deficiency doesn't suppress activation of the T cells, only their proliferation. These results suggest that targeting gene expression in MSCs may provide a useful therapeutic strategy to enhance the ability of T cells to eradicate tumors.

MSCs and naïve T cells, and the subsequent impact(s) on T cell functionality remain understudied.

Reportedly, T cell proliferation and cytotoxicity is significantly modulated by glutathione, an important component of redox signaling pathways.[13,14] Glutathione synthesis rate is limited by cysteine,[15] which can be available *via* two distinct pathways. Cells expressing the Xc-transporter (composed of the xCT-light chain and the 4F2-heavy chain) on their plasma membrane can import cystine from the extracellular milieu, with subsequent reduction to cysteine in the cell cytoplasm[15,16] or can be generated *in situ* by cystathionase catalyzed conversion of methionine to cysteine.[17] Because, naïve T cells fail to express either xCT chain of Xc- transporter or cystathionase,[18,19] they are completely dependent on DCs or other antigen presenting cells (APCs) for their source of metabolic cysteine. Cysteine generated in APCs is exported *via* plasma membrane ASC transporters[20,21] and could be directed in a polarized fashion to cognate T cells through immune synapses. Furthermore, thioredoxin secreting DCs and macrophages prevent conversion of cysteine to cystine within the oxidizing extracellular microenvironment, thereby "stabilizing" cysteine for uptake by cognate T cells.[22,23]

To our knowledge, the present study is the first to demonstrate that the tumor resident IL-10-secreting MSCs disrupt T cell proliferation and late-phase IFNγ secretion without affecting early activation and IL-2 production. This occurs *via* MSC regulatory mechanism inhibiting DC provision of cysteine to responder T cells. In particular, tumor-associated MSC (TC-MSC) secreted IL-10 represses cystathionase expression in DCs in a STAT3-dependent manner. Bioinformatic analysis along with ChIP and EMSA suggested possible direct interaction of STAT3 with cystathionase promoter leading to regulation of cystathionase gene transcription.

Material and Methods

Culture of C3H/10T1/2 cells

The cell line C3H/10T1/2 (ATCC#CCL-226) was purchased from the American Type Culture Collection (Manassas, VA), originally isolated from a line of C3H mouse embryo cells,[24] exhibit MSC-like properties and cultured at a sub-confluent density in DMEM (Invitrogen, Camarillo, CA) supplemented with 10% FBS, 233.6 mg/ml glutamine, 25 mM glucose and 80 U/ml penicillin/streptomycin.

Tumor conditioning of C3H/10T1/2 cells was accomplished by exposing MSCs to B16 melanoma confluent culture supernatants in hypoxia (37°C, 1–2% O_2 overnight in Hypoxia Chamber- Stem Cell Technologies, Vancouver, BC, Canada).

Mice

Wild-type (Wt) female C57BL/6 and Swiss mice (age, 4–6 weeks, body weight, 20–25 g on average) were procured from Animal Facilities of the National Institute of Nutrition (Hyderabad, India). IL-$10^{-/-}$ (KO) mice were obtained from Jackson Laboratories, Bar Horber, ME and subsequently reared/bred at NCCS, Pune, India. The care and treatment of animals conformed to guidelines established by the Institutional Animal Care and Ethics Committee.

Generation of wt and IL-10 deficient (IL-$10^{-/-}$) MSCs

Bone marrow cells from Wt and IL-$10^{-/-}$ mice on a C57BL/6 background were cultured in six-well plates (1.0×10^7 cells/well) in complete DMEM low glucose medium containing 15% (v/v) heat inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen, Camarillo, CA), at 37°C in 5% CO_2 for 14 days. Media was replenished in every 8–12 h for first 3 days and then, every 3 days interval for 14 days. After confluence, the primary culture was treated with 0.25% trypsin (0.5 ml) containing 0.02% EDTA for 2 min at RT (25°C). A purified MSC population (>90% $CD45^-CD34^-CD105^+$ $Vimentin^+$) was obtained after 3 weeks, as previously described.[25] Purity of primary and cultured MSCs was confirmed by CFU assay, followed by crystal violet staining.[25]

Generation of bone marrow-derived DCs

DCs were generated from bone marrow (BM) precursors, as described.[26] In brief, single-cell suspension was obtained after flushing bone marrow from the tibia and femurs of C57BL/6 mice. Erythrocytes were lysed by resuspending the cell pellet in a hypotonic buffer. The cells were cultured (2×10^6 cells/well) with complete RPMI 1640 medium containing 10% FBS, 10 ng/ml rm-GMCSF and 5 ng/ml rmIL-4. On Day 6 of culture, nonadherent cells obtained from these cultures were considered to be immature BmDC. For maturation, LPS was added separately for the final 2 days of culture.

On Day 8, cells were harvested and positively-selected with CD11c magnetic beads (Miltenyi Biotech, Bergisch Gladbach, Germany) and matured with LPS (1 μg/ml; Sigma-Aldrich) to culturefor 48 h at 37°C. Immature and LPS-

matured DCs were 85 to 90% $CD11c^+$ and expressed MHC II and CD86 as analyzed by flow cytometry (data not shown). Antigen-loading of Day 8 BmDCs (1×10^6 cells/ml) was accomplished by incubation with B16 melanoma antigen (5 μg/ml) overnight at 37°C. Semi-adherent cells were then collected and considered as melanoma antigen-pulsed BmDCs.

Antibodies

Antibodies against mCD45, mCD69, mIL-6, mIL-10 and Sp1 were purchased from BioLegend (San Diego, CA). Anti-CD4/CD8 magnetic particles, and anti-mKi-67, anti-mTGFβ, anti-mCD34, anti-mVimentin antibodies, pSTAT3, all neutralizing antibodies and rmIL-10 were purchased from BD Biosciences (San Jose, CA). Anti-mIFNγ and mCD105 antibodies were obtained from eBiosciences (San Diego, CA), while anti-mVEGF was purchased from Santa Cruz Biotechnology (Dallas, TX).

Isolation of T lymphocytes

$CD4^+$ and $CD8^+$ T cells were isolated from mice splenic cells by positive selection using BD IMag Anti-Mouse CD4 and CD8 Particles—DM (BD Biosciences, San Diego, CA). Flow cytometric analyses confirmed >95% purity. T cells were cultured either in complete RPMI medium (Invitrogen, Camarillo, CA) or in cystine/methionine free medium (CELLClone, Genetix, New Delhi, India) as indicated in text.

Rt-pcr

Cellular RNA was isolated using Trizol (Invitrogen, Camarillo, CA) and random hexamers used to generate corresponding cDNA (First Strand cDNA Synthesis Kit; Fermentas, Hanover, MD). Amplification was performed using 2X Bio Mix Red (Bioline, Tauntan, MA) with the following program: 94°C for 5 min; 35 cycles of 94°C for 30 sec, 54–57°C for 30 sec, and 72°C for 1 min; and 72°C for 5 min. PCR products were identified by image analysis software for gel documentation (Versadoc; BioRad Laboratories, Hercules, CA) after electrophoresis on 1.5% agarose gels and staining with ethidium bromide (Sigma-Aldrich). RT-PCR primers were designed and purchased from MWG-Biotech (Bangalore, India).

Flow cytometric staining

Flow cytometry for cell-surface phenotypes was performed after staining of cells (1×10^6) with fluorescently-labeled antibodies (specific and isotype-matched controls). After incubation for 30 min at 4°C in dark, labeled cells were washed twice with FACS buffer (0.1% BSA in PBS) before flow cytometric analysis. Similarly, intracellular molecules (*i.e.*, IFNγ) were stained with anti-mouse fluorescence-labeled antibodies using Cytofix/Cytoperm reagents per the manufacturers (BD Biosciences, San Diego, CA). For Ki67 staining, 70–80% chilled ethanol was added to fix the pelleted cells ($1–5 \times 10^7$ cells) with vortexing, followed by incubation at −20°C for 2 h. Fixed cells were then washed twice with staining buffer and centrifuged (10 min, 200 g), diluted to a concentration of 1×10^7 cells/ml for staining and corollary flow cytometry analyses.

Cells were then fixed with 1% paraformaldehyde in PBS, acquisition was performed using a FACSCalibur (Becton Dickinson, Mountainview, CA) along with suitable negative isotype controls. The percentage of positively-stained populations were determined using quadrant statistics established using Cell Quest (Becton Dickinson, Mountainview, CA) and FlowJo software (Tree Star, Ashland, OR).

Cytokine detection assay

Secreted cytokines in MSC/TC-MSC culture supernatants (IL-6, IL-10, VEGF, TGFβ) and in supernatants of DC-T cell-MSC cocultures (IFNγ/IL-2) were measured by ELISA (OptEIA kit, BD Pharmingen, San Diego, CA) and optical density was measured at 450 nm using microplate reader (BioTek Instruments, Vermont).

Treatment with cytokines and neutralizing antibodies

To study the cytokines' effect, cells were treated with recombinant murine cytokines (250–1,000 ng/ml/10^6 cells) for 24–48 h at 37°C in 5% CO_2. Neutralizing antibodies (0.5–5 μg) reactive against mIL-6, mIL-10, mTGFβ or mVEGF were added to cultures as indicated. After incubation, cells were washed thrice with FBS-free DMEM prior to experimental use.

Lymphocyte proliferation

Isolated $CD4^+$ and $CD8^+$ T cells (5×10^5/well) were cocultured with mitomycin C (80 μg/ml for 1 h) -treated DCs (2×10^5/well) in the presence or absence of MSC/TC-MSC-derived culture supernatants for 72 h. Assay wells containing no DCs and ConA treated T cells were used as negative and positive proliferation controls, respectively. After 72 h incubation at 37°C, 20 μl (0.5 mCi) $[^3H]$ thymidine was added to each well and plates were incubated for an additional 24 h. Cells were harvested with a cell harvester and analyzed for uptake of radioactivity on a β-scintillation counter (PerkinElmer, Waltham, MA).

Cytotoxicity assay

Cytotoxicity of $CD8^+$ T cells against mouse melanoma cells was determined by measuring lactate dehydrogenase (LDH) released by target cells using a commercially available kit (Roche Diagnostics, Mannheim, Germany).

Fluorescence imaging of tissue sections

Tumor and tissue samples were stained as previously reported.[27] MSCs were stained with PE-conjugated anti-mCD105 and anti-mVimentin or matching isotype controls. Imaging was performed using a Leica DM4000B fluorescence microscope (Wetzlar, Germany).

Si-RNA mediated STAT3 silencing

STAT3 specific Si-RNA (Santacruz Biotechnology, Dallas, TX) was added in 70% confluent BmDC culture to a final concentration of 100 nM. Si-RNA (50 μM/25 μl) and Lipofectamine (6 μl) were added to two Opti-MEM aliquots (250 μl each) and incubated for 5 min at RT. Then, the Si-RNA/Opti-MEM and the Lipofectamine/Opti-MEM (500 μl total volume) were mixed and allowed to incubate for 20 min at RT. Si-RNA-containing medium was then added to the DC culture. Finally, expression of stat3 was checked both in untreated and siRNA transfected DCs by RT-PCR.

STAT3 binding sites prediction in the promoter sequence of CTH

The promoter sequence of *CTH* gene (range, −499 to +100) was downloaded from the EPD database[28] and the PROMO 3.0 server[29] used to identify a required GAS (5′-TTCCGGGAA-3′) sequence or the probable binding site of STAT3 in this range (−499 to +100).

Docking of STAT3 with the promoter sequence of CTH gene containing the GAS motif

The three-dimensional duplex structure of *CTH* promoter sequence containing the GAS motif was formed by using the NAB program of Ambertools.[30] Phosphorylated STAT3 protein was then docked with the DNA structure using the HADDOCK server.[31]

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were conducted following the manufacturer's protocol (Millipore, Darmstadt, Germany). Briefly, DCs (1×10^6) were stimulated as described in Figure 6e. Paraformaldehyde fixed DCs were washed with chilled HBSS containing 1 mM PMSF and lysed in SDS lysis buffer. DNA was sheared by ultrasonication (Hielscher, NJ). Lysates were cleared by centrifugation and diluted in ChIP dilution buffer after preclearing using salmon sperm DNA/protein A-agarose and a sample of "input DNA" was collected. Protein-DNA complexes were immunoprecipitated with antibody (5 μg). Antibody-protein-DNA complexes were then captured using salmon sperm DNA/protein A-agarose. After washing beads protein/DNA complexes were eluted and DNA was extracted using phenol/chloroform to conduct PCR using promoter specific primers: GAS motif in cystathionase promoter (STAT3 binding region): sense 5′ GTGGGCACTGCTCTGTGCCA −3′, antisense 5′-TAAATGTGGTGGCCAACGAA-3′; GC motif in cystathionase promoter (SP1 binding site): sense5′-AGTTTTAGGTTCGGGCTGGT-3′ antisense 5′-TGGCACA-GAGCAGTGCCCAC-3′.

Co-immunoprecipitation and western blot

DCs were stimulated as described in Figure 6g. After 4 h of stimulation, the cells were lysed in RIPA buffer and cell lysates were precleared for 1 h with 40 ml of 50% (wt/vol) protein G-Sepharose beads and incubated overnight with pSTAT3 (Y705) antibody (BD Transduction Laboratories, San Jose) or control (IgG antibodies). The beads were added for 2 h and then washed extensively with lysis buffer; bound proteins were fractionated on 10% SDS-PAGE, transferred to a nitrocellulose membrane, and incubated with pSTAT3 or Sp1 antibodies. After washing, blots were incubated with HRP-conjugated secondary antibody for 2 h at RT. Bands were detected using Western lighting chemiluminescence detection kit (Pierce, Rockford, IL).

Electrophoretic mobility shift assay (EMSA)

Nuclear and cytosolic fractions were isolated from stimulated and nonstimulated DCs. EMSA was carried out in nuclear extract and oligonucleotide-containing consensus for GAS motif binding site in CTH. For super-shift assays, specific antibody, STAT3 was added for 20 min after the reaction with oligonucleotide was completed. A nonradioactive method (LightShift®) (Chemiluminescent EMSA Kit, Thermo Scientific, Waltham, MA) was used as per manufacturer's protocol.

Statistical analysis

All reported results represent the mean ± SD of data obtained in either six (for *in vivo* analysis) or three to six (*in vitro* assays) independent experiments. Statistical significance was established by unpaired *t test* using INSTAT 3 Software (Graphpad), with differences between groups attaining a p values ≤ 0.05 considered as significant.

Results

MSCs accumulate in tumors and tumor draining lymph nodes in significant extent

Because MSCs have the unique property of tropism to sites of tissue damage/injury, and to the hypoxic TME,[32,33] we screened progressively growing murine tumors (B16F10 melanoma in C57BL/6 mice, S180 sarcoma in Swiss mice; tumor volume, 100–200 mm^3) for the presence of $CD34^-CD45^-CD105^+$ $Vimentin^+$ MSCs *in vivo*. Flow cytometry and immunohistochemistry of growing tumors revealed major accumulation (approximately 35–45%) of MSCs in all tumors tested ($n = 6$ for each model) (Figs. 1b and 1c). Notably, we also observed significant accumulation (approximately 20–30%) of MSCs in tumor draining lymph nodes (TDLNs) when compared to tumor-uninvolved, contralateral lymph nodes (LNs) or LNs isolated from tumor-free mice (Figs. 1b and1c).

Tumor conditioning licenses MSCs to selectively inhibit T cell proliferation without hampering early activation

As the MSCs accumulate in TME and TDLN, sites of protective anti-tumor T cell effector function and priming, respectively, we next investigated the functional impact of TC-MSCs on T cells *in vitro*. To mimic the TME milieu as closely as possible *in vitro*, we exposed MSCs to B16 melanoma tumor supernatant under hypoxic conditions (1–2% O_2). Splenic $CD4^+$ and $CD8^+$ T cells isolated by MACS were co-cultured with melanoma antigen-pulsed BmDCs in presence

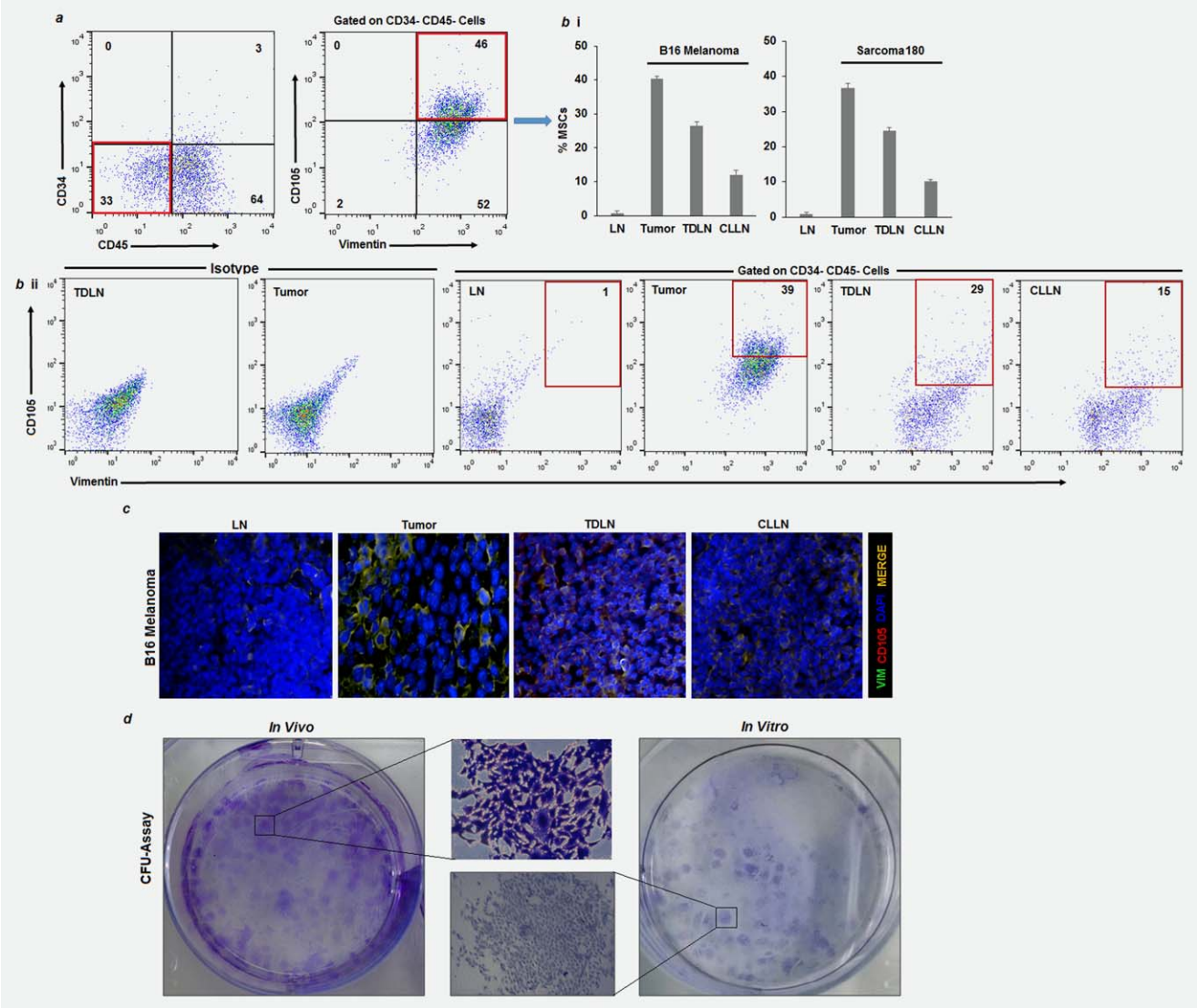

Figure 1. Accumulation of MSCs in tumor and TDLN. Tumor (Day 21 B16 melanoma or sarcoma 180), TDLN, CLLN and normal LN were removed from tumor-bearing mice (C57BL/6 and Swiss) or normal mice, respectively. (*a*) Tissues were processed into single cell suspensions and the percentage of $CD34^{-}CD45^{-}CD105^{+}$ $Vimentin^{+}$ MSCswas determined by flow cytometry as described in Materials and Methods; (*b*-i) bar diagram depicting aggregate data mean ± SD and (*b*-ii) representative dot plots presented. All data are reflective of six independent experiments performed for each tumor type. A representative dot plot of each case (B16 tumor) is presented. (*c*) Presence of MSCs in Day 21 B16 melanoma tumor sections and corresponding TDLN, CLLN and normal LN (C57BL/6 mice) was determined by immunofluorescence microscopy. Five-micrometer tissue sections were co-stained with anti-CD105 (red) and anti-Vimentin (green) Abs, with DAPI counterstain (blue) used to image cell nuclei. Representative four figures are presented. Original magnification ×200. (*d*) The proliferation and clonogenic property of MSCs isolated from B16 tumor and MSC cell line, cultured *in vitro* was measured by CFU assay (first passage). Representative data showed the colonies formed by MSCs in both *in vivo* and *in vitro* culture. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

or absence of TC-MSCs or MSCs (naive, untreated) for 24 or 72 hr and their activation was assessed by CD69 and CD25 expression. T cell expression of these markers remained unchanged in the presence of either MSCs or TC-MSCs (Fig. 2a). However, an assessment of T cell proliferation based on Ki67 expression revealed that TC-MSCs inhibited the proliferative potential of both $CD4^{+}$ and $CD8^{+}$ T cells from 60% to 20% approximately (Figs. 2b and2c). In line with the previous reports, normal MSCs also mediated some degree of inhibition of Ki67 expression by responder T cells, but to a lesser extent than TC-MSCs ($p < 0.01$). In conformational ^{3}H-thymidine incorporation assay, TC-MSCs significantly suppressed DC-induced T cell proliferation (Fig. 2b).

Given the ability of TC-MSCs to suppress the DC-induced T cell proliferation *in vitro*, we next evaluated the impact of TC-MSCs on responder T cell effector functions, such as cytokine production and anti-tumor cytotoxicity. Interestingly, TC-MSCs, but not control MSCs, show differential

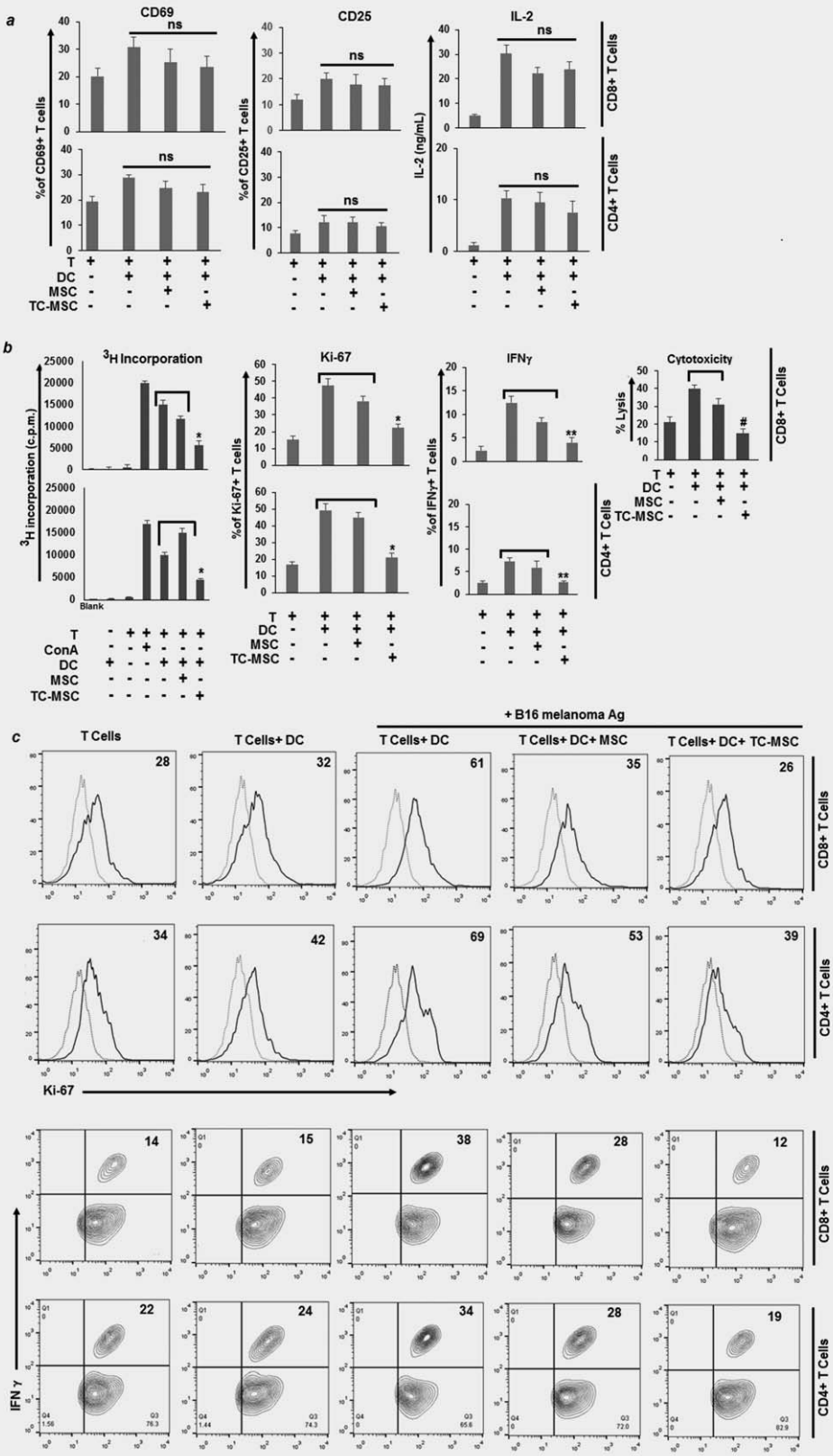

Figure 2.

regulatory effects on DC-induced IL-2 and IFNγ secretion from both subsets of T cells. Notably, IL-2 secretion at an early time point (24 h) was unaffected by TC-MSCs, but IFNγ secretion at a later time point (72 h) and cytotoxicity of $CD8^+$ T cells against B16 melanoma cells were significantly inhibited (Figs. 2a and 2b). These observations suggest that TC-MSCs selectively perturb the ability of cognate DC-T cell interaction leading to suboptimal T cell proliferation and type-1 effector functions (IFNγ production and cytotoxicity) without affecting the early T cell activation events or IL-2 production.

TC-MSCs inhibit T cell proliferation in a cell contact-independent manner

To investigate whether TC-MSC-mediated suppression of T cell function requires cell-cell contact, we established transwell co-cultures where MSCs (control or TC-MSCs) were placed in the lower well along with DC-T cells or they were placed in the upper well, with DC-T cells in the lower chamber (Fig. 3a). In either experimental setup, TC-MSCs inhibited T cell expression of Ki67 suggesting that TC-MSC regulates T cells in a contact-independent manner (Figs. 3b-i and 3*b*-ii). As before, TC-MSCs failed to have any significant effect on early T cell activation under either transwell protocol (Fig. 3b). In separate experiments, we added either normal or TC-MSC culture supernatant directly into DC-T cell co-cultures. We observed the concentration-dependent inhibitory effects of TC-MSC-derived supernatant on T cell proliferation (Fig. 3c). Our results suggest that tumor-conditioned MSCs secrete soluble factor(s) that are capable of inhibiting DC-induced T cell proliferative/effector functions.

TC-MSCs target cysteine export by DC to perturb cognate T cell proliferation and effector functions

Because the APC-derived cysteine differentially and independently regulates early T cell activation and proliferation,[34] we supplementedL-cysteine (20 μM/ml) and β-ME (0.05 nM/ml) into DC-T cell coculture containing either TC-MSCs or TC-MSC supernatant, in order to determine whether this manipulation could rescue deficient T cell proliferation and effector functions. We observed that L-cysteine prevented the inhibition of T cell Ki67 expression and thymidine incorporation by either TC-MSCs or their conditioned supernatants (Fig. 4a). These results suggest that TC-MSC secreted products might create an L-cysteine deficiency in the T cell induction environment that limits optimum T cell proliferation.

Since T cells are unable to synthesize cysteine from their intracellular stores of methionine given their lack of cystathionase and they cannot import extracellular cystine as a biosynthetic precursor (since their X^c transporter is not fully functional), T cells import cysteine from surroundings. This typically occurs *via* the elaboration of cysteine from APC through a plasma membrane ASC neutral amino acid transporter.[34] We next studied the influence of TC-MSCs on the expression of cysteine transporter pathway components (cystathionase, X^{c-} transporter (both xCT and 4F2) and ASC neutral amino acid transporter) in T cells, DCs and MSCs, using RT-PCR. In agreement with previous reports,[34,35] T cells lack expression of cystathionase and the xCT chain of the X^{c-} transporter, but they intrinsically express both the 4F2 heavy chain of X^c transporter and the ASC neutral amino acid transporter (Fig. 4b). Both MSCs and TC-MSCs show unaltered expression of cystathionase, Xc transporter as well as ASC transporter (Fig. 4b) indicating that TC-MSC mediated suppression of T cell proliferation is not due to the withdrawal of cysteine from surrounding media by MSCs/TC-MSCs as they themselves can synthesize cysteine. Surprisingly antigen-pulsed DCs (as used in our experimental set up to prime T cells) cultured for 12 hr in the presence of TC-MSC (but not naïve MSC) supernatant display significantly reduced expression of cystathionase (Fig. 4c). These collective data suggest that TC-MSCs may abrogate or decrease the generation of intracellular cysteine from methionine in DCs due to impaired cystathionase expression, which results in the inability of DCs to export cysteine into the local extracellular environment (for use by cognate T cells).

TC-MSC suppression of cystathionase expression in DCs is dependent on IL-10-STAT3 signaling

Because TC-MSC-mediated suppression of DC-induced T cell proliferation was found to be cell-cell contact-independent, but secretory factor(s)-dependent, we next investigated the nature of relevant MSC-shed regulatory molecules. TC-MSC-derived culture supernatant (24 h) was first evaluated for the presence of regulatory cytokines. ELISA results show that both IL-10 and IL-6 are highly-secreted in TC-MSC-derived supernatant, with

Figure 2. TC-MSCs suppress the proliferation of T lymphocytes but do not affect their early activation and IL-2 secretion. (*a*) $CD8^+$ and $CD4^+$ T cells and DCs were isolated from naïve C57BL/6 mice spleen and bone marrow, respectively, as described in the Materials and Methods. T cells and DCs (DC:T = 1:10) were cultured in the absence or presence of normal versus TC-MSCs (MSC:T = 1:5) for 24hor 72 hr, as indicated. Expression of CD69 and CD25 was analyzed on $CD8^+$ and $CD4^+$ T cells after 24 hr by flow cytometry, and IL-2 secretion as measured by ELISA from responder T cells. Bar diagrams depict the mean ± SD of aggregate data obtained in six independent experiments performed. ns, nonsignificant. (*b*) Measurement of T cell proliferation after 72 hr based on expression of Ki67 on $CD8^+$ and $CD4^+$ T cells and incorporation of [^{3}H] thymidine, with IFNγ expression on T cells analyzed by flow cytometry. Bar diagrams depict the mean ± SD of aggregate data obtained in six independent experiments performed. $^*p < 0.001$; $^{**}p < 0.05$ versus DC-induced T cells or DC-induced T cells in the presence of naïve MSC. Purified $CD8^+$ cells were cultured in presence or absence of normal as well as TC-MSC for 72 hr. Cytotoxicity of these differentially exposed $CD8^+$ T cells towards B16 melanoma cells was measured by LDH release assay. $^{\#}p < 0.01$ versus DC induced $CD8^+$ T cells or DC induced $CD8^+$ T cells in presence of naïve MSC. (*c*) The expression of Ki67 and intracellular IFNγ on $CD8^+$ and $CD4^+$ T cells is depicted in representative histogram and contour plots, respectively.

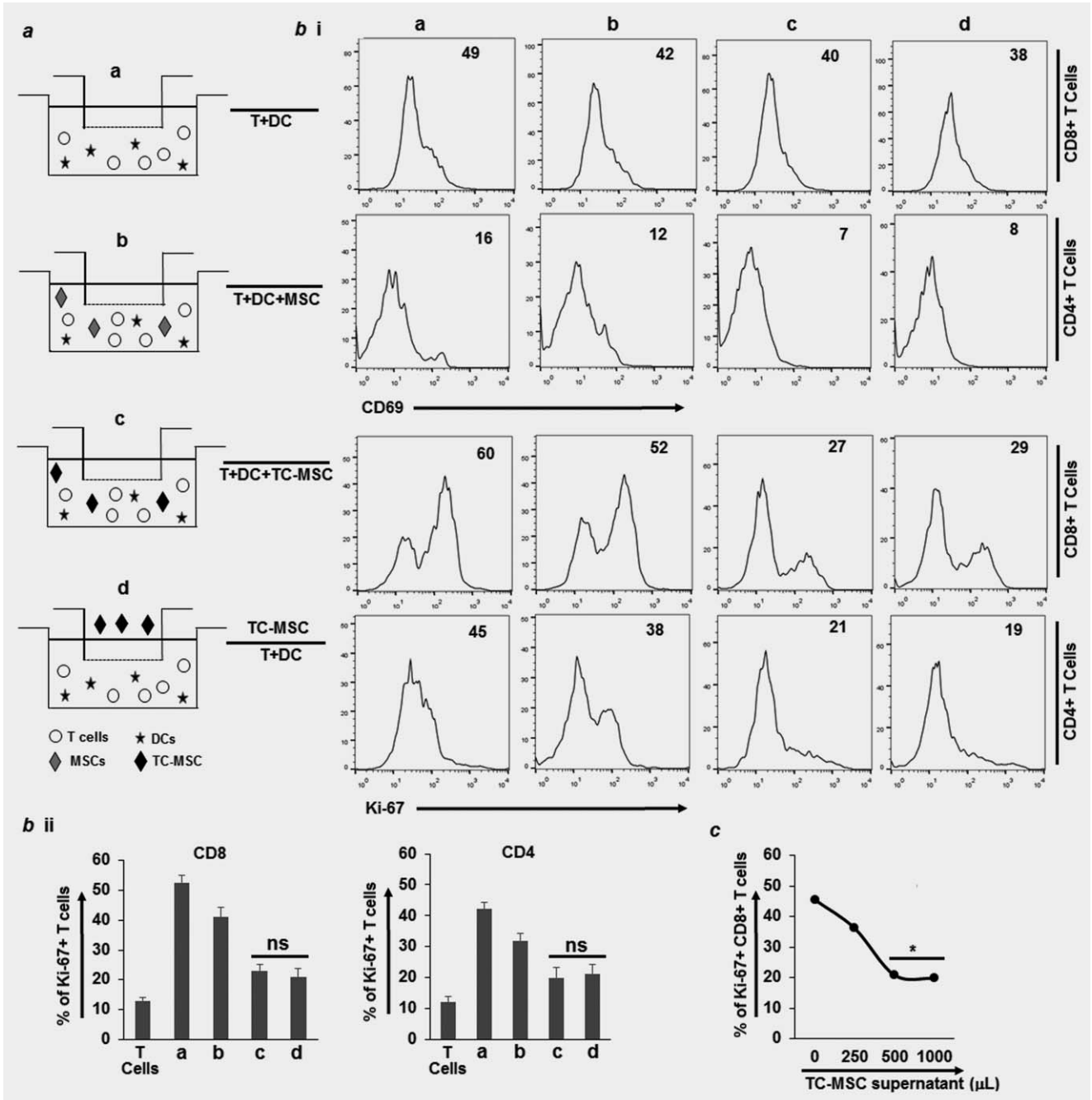

Figure 3. TC-MSC mediated suppression of DC-induced T cell proliferation is contact independent. (*a*) Diagrammatic representation of DC-T cell transwell co-cultures in which TC-MSCs were either placed in upper versus lower chambers, with DC and T cells always in the bottom chamber. (*b*) CD69 and Ki67 expression on $CD8^+$ and $CD4^+$ T cells in presence or absence of TC-MSCs were assessed by flow cytometry and (*b*-i) a representative histogram plot is depicted, with the % positive value reported in each plot. (*b*-ii) Aggregate data from 6 independent experiments is reported as the mean ± SD of $Ki67^+$ events amongst responder $CD8^+$ and $CD4^+$ T cells in transwell experiments. (*c*) The line diagram depicts proliferation of $CD8^+$ T cells based on Ki67 expression in presence of TC-supernatant at different concentrations, $*p < 0.01$ versus DC induced $CD8^+$ T cell (group) in the absence of TC-MSC supernatant.

somewhat lower levels of TGFβ and VEGF (Fig. 5a). To investigate the relevance of these cytokines in the regulatory action of TC-MSCs, DCs were exposed to TC-MSC supernatant along with corresponding neutralizing antibodies to each cytokine, and expression of cystathionase and the ASC transporter was assessed. IL-10 neutralization restored cystathionase expression to almost normal levels, while IL-6 neutralization yielded only partial restoration (Fig. 5b). Conversely, addition of rmIL-10 to normal MSC-supernatant led to complete abrogation of cystathionase expression in DCs, in an IL-10 dose-dependent manner (Fig. 5c). To further validate the involvement of IL-10, next we used IL-10 deficient MSCs. Culture supernatant

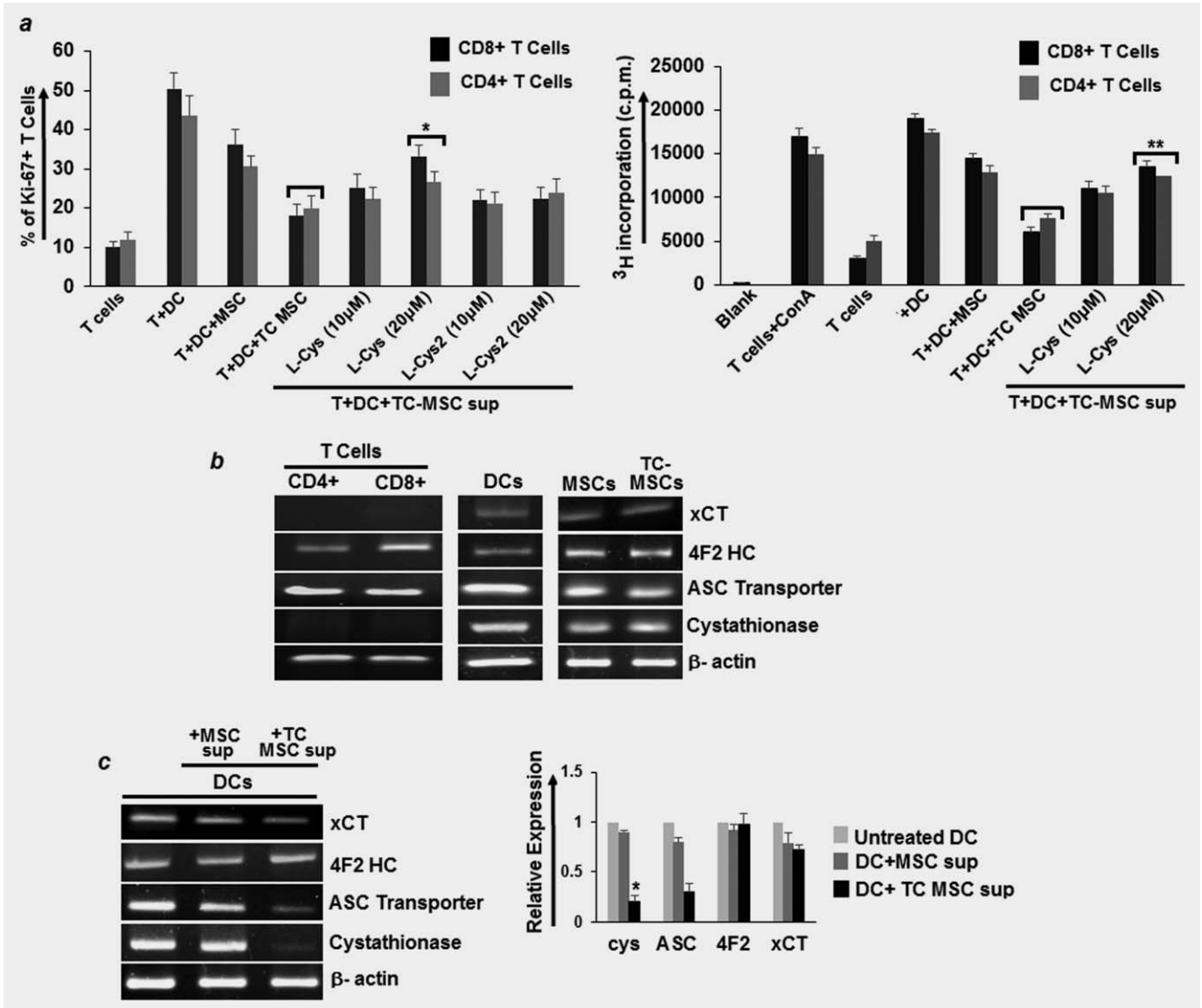

Figure 4. TC-MSC inhibits DC-induced T cell proliferation by blocking the ability of DC to provide cysteine to cognate T cells. (*a*) Purified $CD8^+$ and $CD4^+$ T cells were cultured with B16Mel Ag-pulsed DCs for 72 hr in the absence or presence of naive MSC and TC-MSC supernatant, with or without L-cysteine and β-ME. After 72 hrs the proliferation of T cells was assessed based on Ki67 expression or [^{3}H]-thymidine uptake as described in Materials and Methods. Data are reported as mean ± SD using bar diagram from six independent experiments performed. *$p < 0.01$, **$p < 0.001$ versus DC induced T cell proliferation in presence of TC-MSC supernatant (group). (*b*) $CD4^+$ T cells, $CD8^+$ T cells, DCs, MSCs and TC-MSCs were analyzed by RT-PCR for expression of xCT and the 4F2 heavy chain of the Xc transporter, the ASC transporter, cystathionase and β-actin as described in Materials and Methods. A representative figure from four independent experiments performed is presented. (*c*) The expression of xCT, 4F2, the ASC transporter, cystathionase, and β-actin in mRNA levels in DCs were analysed by RT-PCR in the absence or presence of naive MSC and TC-MSC supernatant, with representative data from five independent experiments depicted. Mean ±SD of relative expression of target gene normalized against β-actin is presented as a bar diagram. *$p < 0.001$ versus untreated DCs and DCs cultured in presence of naïve MSCs.

obtained from tumor-conditioned MSC-$IL10^{-/-}$ did not inhibit cystathionase expression in DCs, while supplementation of mrIL-10 into TC-MSC-$IL10^{-/-}$ culture supernatants led to inhibition of DC expression of cysthathionase (Fig. 5c right panel). Notably, the magnitude of IL-10 secreted by MSCs in the TME is comparable, or even slightly greater than that of other classical IL-10 secreting cells (Supporting Information Fig. S1).

Because STAT3 is essential in IL-10 signaling, we next assessed STAT3 induction by TC-MSC supernatant (Fig. 5d left panel), followed by examination of the role of STAT3 in TC-MSC modulation of DC expressed cystathionase. In particular, DCs were subjected to knockdown for STAT3 using specific siRNA and cultured with supernatant isolated from MSCs or TC-MSCs. Interestingly, in STAT3-silenced DC, TC-MSC supernatant was unable to

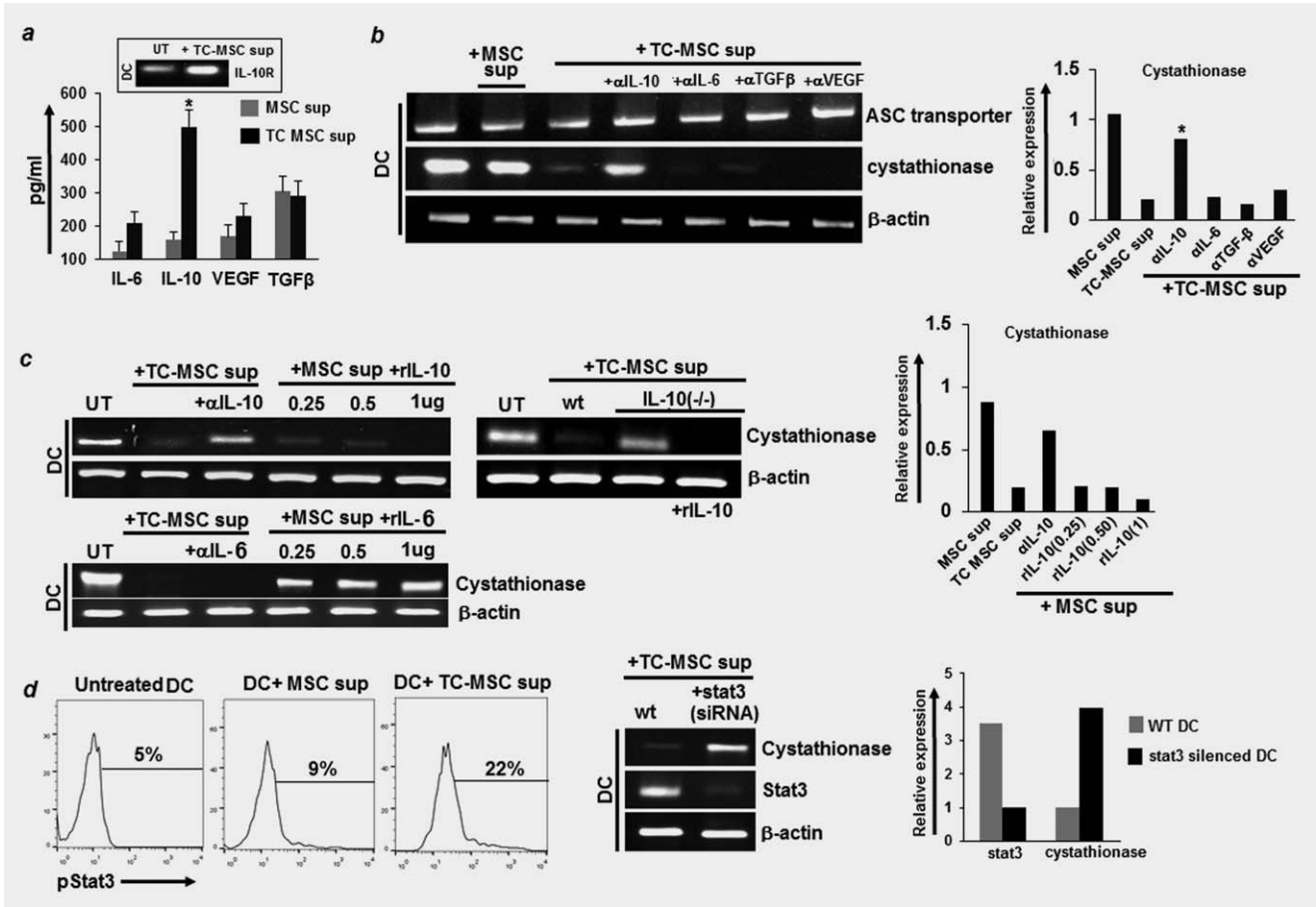

Figure 5. TC-MSC mediated inhibition of cystathionase expression in DCs is dependent on TC-MSC-derived IL-10 and STAT3 signaling in DC. (*a*) Cell-free supernatants harvested from naive MSCs versus TC-MSCs were assessed for their content of IL-6, IL-10, VEGF and TGFβ by ELISA (reported as mean ±SD values in pg/ml) using bar diagrams. Four independent experiments were performed. *$p < 0.001$ versus naïve MSC supernatant. IL-10R expression was analyzed on DC exposed to either naïve or TC-MSC supernatant and a representative figure is presented in the inset. (*b*) DCs were treated *in vitro* with TC-MSC supernatant, along with neutralizing antibodies (1 μg) against IL-6, IL-10, TGFβ or VEGF, and the expression of ASC transporter, cystathionase, and β-actin mRNA levels analyzed by RT-PCR. Representative figures are presented from 3 independent experiments performed, with data reported as the mean ±SD for relative expression of the indicated target gene normalized against β-actin transcript levels in bar diagrams. *$p < 0.001$ versus DCs cultured in presence of TC-MSC supertatant. (*c*) DCs were treated *in vitro* with either TC-MSC supernatant along with anti-IL-10 antibodies or with naïve MSC supernatant, supplemented with different concentrations of rmIL-10 and rmIL-6. In alternate experiments, DCs were exposed to supernatants harvested from TC-MSC generated either from wild-type C57BL/6 mice or from syngenic IL-$10^{-/-}$ mice +/- rmIL-10 (left panel). Expression of cystathionase and β-actin mRNAs level were analyzed by RT-PCR. Representative figures are presented from three independent experiments performed and mean ±SD, with the relative expression of target gene transcripts normalized against β-actin transcript levels as presented in bar diagrams. *$p < 0.001$ versus DCs cultured in presence of TC-MSC supernatant along with anti-IL-10 antibody. (*d*) DCs were exposed to naïve MSC versus TC-MSC culture supernatants and their expression levels of pStat3 evaluated by flow cytometry. Representative figures are presented from three different experiments (left panel). In another experiment, DCs were pre-treated *in vitro* with stat3 siRNA before being exposed to TC-MSC supernatant and the expression levels of cystathionase and β-actin mRNA determined by RT-PCR (middle panel). Representative figures are presented from three independent experiments performed (mean ±SD), with the relative expression of target gene transcripts normalized against β-actin mRNA as presented as bar diagram. **$p < 0.05$ versus untreated DCs cultured in the presence of TC-MSC supernatant.

inhibit DC expression of cystathionase (Fig. 5d middle panel). These data suggest that TC-MSC secreted IL-10 functions through STAT3 in DCs, leading to repression of cystathionase expression in these APCs. As a consequence, DC-derived cysteine export to T cells is blocked, leading to reduced T cell proliferation and late phase effector function(s).

TC-MSC mediated transcriptional inhibition of cystathionase might be regulated by direct binding of pSTAT3 with corresponding gene

Given the inability of IL-10 to repress cystathionase gene expression in STAT3-silenced DC, we next investigated the nucleotide sequence of the mouse cystathionase gene for the presence of candidate STAT3 binding sites. In the nucleus,

activated and tyrosine (407)-phosphorylated STAT3 binds to DNA-response elements (*i.e.*, interferon (gamma)-activated sequence; GAS) found in the promoter regions of target genes.[36] GAS is a 9-base-pair palindrome, having the consensus sequence, TTCCGGGAA. Interestingly, we found the sequence 5′- TTGCCGGAA −3′ in the region −269 to −261 which is very similar to the consensus GAS sequence (Fig. 6a). Using bioinformatics tools, we generated a putative model of the complex of phosphorylated STAT3 with the cystathionase target DNA sequence (−269 to −261) including ten

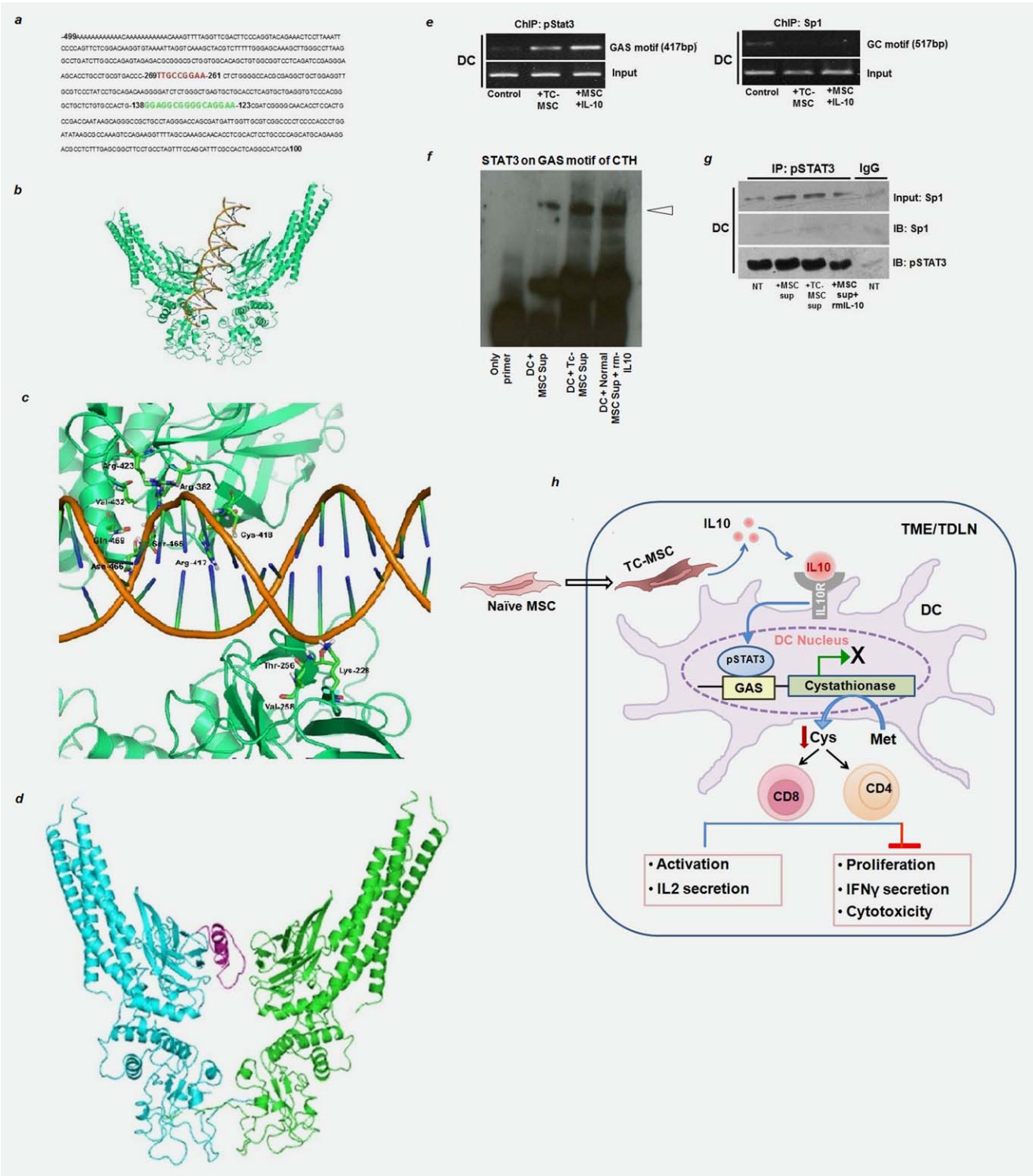

Figure 6.

extra constitutive flanking nucleotides. Overall, we determined that there are 12 STAT3 amino acid residues within 4 Å of DNA (Figs. 6b and 6c). Importantly, most of these interactions are very similar to those reported in the crystal structure of STAT3 with DNA (PDB code, 1BG1).

Furthermore, *p*STAT3 can form complexes with other binding partners such as Sp1 or NFκb that are competent to regulate the expression of target genes. A reinspection of the nucleotide sequence of the mouse cystathionase gene revealed the presence of a GC box (with the consensus sequence 5′-GGGCG(A/G)(G/A)−3′[37] at −138 to −122 of promoter sequence which is recognizable by Sp1 (Fig. 6a). To determine if the STAT3:Sp1 complex could bind DNA, we docked the two proteins. In the resulting heteroduplex (Fig. 6d), SP1is predicted to bind to STAT3 in a region, that is, very close to the DNA-binding site of STAT3, suggesting that SP1 may sterically block the ability of STAT3 to then bind to cystathionase promoter DNA. It is therefore more likely that *p*STAT3 and/or Sp1 may independently bind to the cystathionase gene promoter, leading to the regulated expression of this enzyme.

To validate the bioinformatic predictions, next we explore ChIP assays to examine the binding of *p*STAT3 to the cystathionase promoter. Unstimulated control DCs exhibited virtually no binding of *p*STAT3 to the cystathionase promoter. Pretreatment of DCs with TC-MSC supernatant or normal MSC supernatant along with rIL-10 significantly promoted the binding of *p*STAT3 to the cystathionase promoter (Fig. 6e). In particular, above mentioned treatment caused increase in *p*Stat3 binding at GAS motif present in cystathionase promoter corroborate with bioinformatics prediction. On the other hand, stimulating DCs with TC-MSC supernatant did not induce binding of Sp1 on GC motif present in cystathionase promoter. Furthermore, EMSA/Supershift assays confirmed STAT3 as a part of GAS motif (CTH):DNA ternary complex (Fig. 6f). However, co-immunoprecipitation of *p*STAT3 and Sp1 demonstrated that both TC-MSC and IL-10 does not induce binding between *p*STAT3 and Sp1 in DCs (Fig. 6g). Therefore, initial obtained data suggested that TC-MSC might induce the direct binding of *p*STAT3 to the GAS motif which in turn results in downregulation of cystathionase gene expression.

Discussion

MSCs have been most commonly applied clinically in adoptive cell therapy approaches based on their potent regenerative capacity.[38] Despite some controversy on the role of MSCs in tumor setting,[38] it is believed that these cells may support tumor progression by suppressing the host immune response.[39,40] Inadequate evidence on T cell inhibition by tumor conditioned MSCs prompted us to explore the role of TC-MSCs on DC/T cell function(s) *in vitro*. We report the following novel observations: first, TC-MSCs suppress proliferation and late phase effector functions (IFNγ secretion and tumor cell cytotoxicity) of T cells without affecting their early activation; second, TC-MSC mediated suppression of T cell proliferation is contact independent and mediated by TC-MSC-produced IL-10; third, this IL-10 represses cystathionase gene expression in DCs and thereby inhibits DC export of cysteine required by cognate T cells for their optimal proliferation; and finally, *p*STAT3 may directly bind with a GAS motif present within the cystathionase promoter of DC after IL-10 stimulation, leading to repression of cystathionase transcription.

In agreement with previous studies,[40,41] we documented significant accumulation of $CD45^{-}CD34^{-}CD105^{+}$ $Vimentin^{+}$ MSCs within murine progressor sarcomas and melanomas. Significantly higher numbers of MSCs were also detected within TDLN in comparison to control LNs. As TDLN and tumors are the primary sites of DC-mediated priming of naïve anti-tumor T cells,[42] we investigated the direct impact of TC-MSCs on DC-T cell crosstalk. To

Figure 6. pSTAT3 may serve as a repressor by directly binding to the cystathionase promoter. (*a*) Promoter sequence of *CTH* gene. The positions of GAS (shown in red) and GC box (shown in green) consensus sequences are −269 to −261 and −138 to −123, respectively. (*b*) The docked structure of STAT3 with a stretch containing GAS sequence in the promoter region of *CTH* gene. (*c*) Protein residues involved in interaction with DNA. (*d*)The docked structure representing the complex between STAT3 and Sp1. The two subunits of STAT3 are represented in cyan and green, and the Sp1 protein is represented in pink. (*e*)DCs (1×10^6 cells/ml) were either treated with TC-MSC supernatant or naïve MSC supernatant supplemented with rm-IL-10 or kept untreated. After 45 min of incubation, ChIP assays were conducted as described in Materials and Methods. Immunoprecipitations were performed using Abs specific to phosphorylated *p*STAT3or Sp1, and conventional RT-PCR was performed using primers specific to the GAS motif or GC motif present in cystathionase promoter. (*f*) EMSA/Supershift with the oligos of GAS motif and GC motif of CTH was performed on the nuclei isolated from unstimulated and differentially stimulated DCs. Arrowheads indicate the location of the shifted bands. (*g*) DCs were treated with naïve or TC-MSC supernatant and naïve MSC supernatant along with rm-IL-10 or kept untreated. After 4 hr, equal protein amounts from whole cell lysates were subjected to immunoprecipitation using an antibody against pSTAT3 or an anti-mouse IgG. The immunoprecipitated proteins were separated and subjected to immunoblot analysis using Sp1 antibody. No complexes were detected in lysates immunoprecipitated with normal mouse immunoglobulin G. The blots shown are representative of at least three independent experiments. (*h*) Proposed mechanism of TC-MSC mediated suppression of DC-induced T cell proliferation and IFNγ secretion. Naïve MSC are recruited into the TME where they are conditioned to secrete IL-10 which can bind to $IL\text{-}10R^{+}$ DCs, leading to STAT3 activation, the repression of DC-expressed cystathionase (responsible for the conversion of methionine-to cysteine) and cysteine deficiency in DC. Cysteine-deficient DC are incompetent to promote optimal priming of naïve $CD4^{+}$ and $CD8^{+}$ T cells based on reduced T cell proliferation and IFNγ secretion. Responder T cell expression of early activation markers and their ability to produce IL-2 remains unaffected. Bioinformatic analyses suggest that the IL-10-IL-10R interaction facilitates STAT3 phosphorylation, with *p*STAT3 translocation to the DC nucleus where it may bind to a "GAS-like motif" present within the cystathionase promoter. This results in the repression of cystathionase transcription. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

arguably best mimic the (immune)biology of tumor-MSCs *in vitro*, we used B16 melanoma tumor supernatant[43] and hypoxic (1–2% O_2) cultures to develop TC-MSCs. Surprisingly, TC-MSCs did not perturb early T cell activation associated with cognate DC stimulation, such as acquisition of CD25 or CD69 expression by T cells and their early (24 hr) IL-2 production. In marked contrast, TC-MSCs inhibited DC-induced T cell proliferation and late-phase (48 hr) IFNγ production by responder T cells. Consistent with current findings, Levering *et al*. reported that early activation and proliferation of T cell can occur independently, and that T cell proliferation is critically dependent on the availability of extracellular cystine/cysteine.[34] Indeed, activated T cells can synthesize proteins like CD25, CD69, IL-2 and the cystine/cysteine transporter in the absence of exogenous cystine/cysteine.[34] However, T cell proliferative responses to mitogenic or antigen-specific stimulation are critically dependent on increased GSH synthesis, in which cysteine is rate-limiting as an amino acid substrate.[44] Naïve T cells are metabolically dependent on DCs, as a source of cysteine to support their proliferation as they fail to intrinsically express either cystathionase (the enzyme convert intracellular methionine to cysteine) or the cystine transporter Xc, and cysteine is the least abundant amino acid in the extracellular space.[22,45] Conversely, addition of exogenous L-cysteine (along with 2-ME to maintain it in a reduced state in extracellular media), but not cystine, is able to prevent the suppressive effects of TC-MSCs on DC-induced T cell proliferation. In contrast to naïve T cells, MSCs and TC-MSCs, as well as DCs, constitutively express cystathionase, the xCT chain of the Xc transporter (in addition to the 4F2 heavy chain of the Xc transporter and the ASC transporter required for cysteine uptake), making them self-sufficient with regard to cysteine production.

Strikingly, DCs exposed to TC-MSC (but not control MSC) supernatant drastically reduce their transcription of cystathionase, leading to a block in intracellular cysteine generation and limiting the capacity of these APCs to provide exported cysteine to cognate T cells. Thus, at least one functional consequence of MSC trafficking into TDLN or TME is a hypo-stimulatory population of DC which is incapable of supporting optimal responder T cell proliferation and late-phase effector function(s). Interestingly, de Silly *et al*. showed that, reduced level of cystathionase in DC is required to maintain tolerant state, which is also associated with downregulation of TH-1 type factor Tbet, IL-12 and IFNγ.[46] We observed that this suppressive effect (reduction in cystathionase expression) of TC-MSCs on DCs is cell-cell contact-independent and mediated largely by TC-MSC-secreted IL-10. Notably, a number of previous reports have suggested that naïve MSCs are capable of mediating immunosuppression (including inhibition of T cell proliferation and IFNγ secretion) *via* their elaboration of IDO and iNOS.[9,11,47] Our preliminary studies have thus far failed to support a role for NO as a suppressor molecule in current TC-MSC model (data not shown), while the impact of IDO antagonists (1-MT) on TC-MSC inhibitory function is yet to be investigated.

Overall, a novel role for IL-10 is supported in repressing DC stimulatory potential *via* limiting their capacity to "feed" responder T cells' metabolic cysteine by suppressing DC expression of cystathionase. The observation is well-correlated with reports for elevated IL-10 levels in cancer patients with T cell dysfunction.[48] Notably, IL-10^+ TC-MSCs appear to constitute approximately 25–40% of total TME cells, which dwarfs the frequency of TME-associated IL-10-secreting hematopoetic cells (MDSCs; TAMs, Tregs) (Supporting Information Fig. S1). These results urges again for development of clinical-grade IL-10/IL-10R antagonists that are capable of interfering with the suppressive action of TC-MSCs, leading to improved anti-tumor TIL effector function(s) and clinical benefit in cancer patients.

Inhibitory effects of IL-10 are exerted through IL-10R expressed on the DC surface. As STAT3 is the obligate downstream signaling mediator of IL-10, it is perhaps not surprising, that we observed STAT3-knock down protected DC expression of cystathionase after exposure to TC-MSC supernatants or rmIL-10. Subsequent bioinformatics analyses suggested that STAT3 may bind directly with a GAS-like motif present within the −269 to −261 promoter region of the cystathionase gene. In agreement with previous reports,[49,50] we also identified a GC box at −138 to −122 within the promoter sequence of cystathionase, that is, recognized by SP1. They reported SP1 as a positive regulator that can directly bind to the cystathionase promoter in smooth muscle or cancer cells. Interestingly, bioinformatic approaches also predicted that STAT3 can form complex with Sp1, however, after docking these two proteins (to generate a STAT3-Sp1 heterodimer), the resulting structure was incompatible with coordinate binding to DNA (*i.e*., the cystathionase promoter). Hence, it is rather implausible that both the GAS and GC box sequences could be occupied simultaneously by such a heterodimeric species. STAT3 could also sterically prevent Sp1 binding to the cystathionase promoter by forming a protein-DNA complex that limits SP1-induced transcription of cystathionase. However, co-immunoprecipitation with *p*STAT3 and Sp1 excluded such possibility. Interestingly, ChIP assay and EMSA with DC-stimulated with either TC-MSC supernatant or rmIL-10 suggested possible direct binding between *p*STAT3 and GAS-motif of cystathionase promoter (Fig. 6). However, these initial results warrant corroboration in further mechanistic studies.

In conclusion, our data decipher a novel mechanism exploited by MSCs within progressive TME/TDLN and its impact (inhibition of DC expressed cystathionase and the export of cysteine to T cells) leading to reduced T cell expansion and protective (anti-tumor) effector functions to avoid host immune surveillance to facilitate tumor growth.

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