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Mesenchymal Stem Cells Protect Breast Cancer Cells through Regulatory T Cells: Role of Mesenchymal Stem Cell-Derived TGF-β

Shyam A. Patel,* Justin R. Meyer,* Steven J. Greco,* Kelly E. Corcoran,* Margarette Bryan,* and Pranela Rameshwar*

Mesenchymal stem cells (MSCs) have been shown to support breast cancer growth. Because MSCs also increase the frequency of regulatory T cells ($T_{reg}$), this study tested the hypothesis that human MSCs, via Tregs, protect breast cancer cells (BCCs) from immune clearance. MSCs suppressed the proliferation of PBMCs when the latter were exposed to gamma-irradiated BCCs. Similarly, MSCs showed significant inhibition of PBMC migration toward BCCs and a corresponding decrease in CXCL12. MSCs also inhibited NK cell and CTL functions, which correlated with reduced numbers of CD8$^+$ and CD56$^+$ cells compared with parallel cultures without MSCs. The reduced NK and CTL activities correlated with a decrease in intracellular and secreted granzyme B. To explain these immunosuppressive findings, we compared $T_{reg}$ levels after coculture with MSCs and found an ~2-fold increase in $T_{reg}$, with associated decreases in antitumor Th1 cytokines and increases in Th2 cytokines.

MSC-derived TGF-β1 was largely responsible for the increase in $T_{reg}$ based on knockdown studies. In the presence of $T_{reg}$ depletion, PBMC proliferation and effector functions were partially restored. Together, these studies show an MSC-mediated increase in $T_{reg}$ in cocultures of PBMCs and BCCs. The results could be explained, in part, by the increase in Th2-type cytokines and MSC-generated TGF-β1. These findings demonstrate immune protection by MSCs to BCCs. The reduction in immune cell proliferation and recruitment mediated by MSCs has implications for treatment of breast cancer with chemotherapy.


Breast cancer cells (BCCs) have been reported to adapt a quiescent phenotype in regions close to the endostium in the bone marrow (BM) stromal compartment (1–3). Although changes in cytokine production and gap junctional intercellular communications have been implicated in BCC quiescence, the mechanisms of immune evasion remain to be elucidated. Recent studies suggested a role for CXCR4 and its ligand, stromal cell-derived factor 1α (or CXCL12), in the entry of BCCs into BM, as well as in attaining BCC quiescence in the endosteal region of BM (3). These findings are linked to the constitutive expression of CXCL12 in BM stroma (3). CXCL12 has a role in chemoattraction, immune responses, and the biology of stem cells (4–6).

It has long been held that a major mechanism of tumor cell destruction by the immune system involves NK cells and MHC class I (MHC I)-restricted CTLs (7). Tumor cells that downregulate MHC I are protected from CTL-mediated killing but are susceptible to NK-mediated killing (8). Tumor destruction by CTLs can occur via cross-linking of death receptors of the TNFR superfamily or activation of the cytotoxic granule secretion system involving perforin and granzyme (7). In the latter mechanism, perforin mediates the release of the serine protease granzyme B from endosomes, and granzyme B triggers apoptosis in the target cell (9).

The granzyme/perforin system functions alongside other complex immunological effectors and is regulated, in part, by regulatory T cells ($T_{reg}$), which have been implicated in modulating granzyme expression (10). At least two types of $T_{reg}$ have been shown to use granzyme-mediated killing: natural $T_{reg}$ can use granzyme A to induce cell apoptosis, and inducible $T_{reg}$ (Tr1/Th3 cells) can use granzyme B (10). However, $T_{reg}$ are mostly immunosuppressive, as they have been shown to suppress effector T cell functions and autoimmunity by mechanisms involving cell–cell contact and subsequent delivery of inhibitory signals (11, 12). A link between $T_{reg}$ and granzyme B is evident in that depletion of $T_{reg}$ enhances granzyme B expression in virally infected mice (11).

Recent attention in the arena of stem cell biology has focused on the immunomodulatory effects of mesenchymal stem cells (MSCs), which are found around vascular areas of the BM (13). MSCs exhibit diverse effects, with applications for graft-versus-host disease and autoimmune-mediated disorders (14, 15). The interaction between MSCs and CTLs could be complex, and studies have shown resistance of MSCs to T cell effector mechanisms and MSC-mediated inhibition of effector cell function (16, 17).

Studies have demonstrated that MSCs cause the dose-dependent inhibition of mitogen-activated T cell proliferation as a critical mechanism in immunosuppression (17). However, other mechanisms of immunosuppression by MSCs remain largely unknown, and these were addressed in this study. This article demonstrates immune evasion of BCCs by MSCs through induction, and perhaps expansion, of $T_{reg}$, as well as polarization toward Th2-type cytokines.
CD4⁺ T cells. These findings may have broad implications for breast cancer quiescence in the BM and resurgence after years of disease-free survival (3). To demonstrate MSC effects on immune cell function, we used two cell lines with mutually exclusive targeting by immune effectors: P815 murine lymphoblast-like mastocytoma cells are targeted by CTLs, and K562 myelogenous leukemic cells are targeted by NK cells (18, 19). Because immune effector cells that target CTL-sensitive P815 cells fail to target K562 cells, this strategy allows for the determination of whether MSCs hinder CTL-mediated and/or NK-mediated immune responses (18). In summary, this study demonstrates the immunomodulatory effects of MSCs in the context of breast cancer metastasis to various organs, but particularly to the BM, which is also a dominant site of MSCs (13). These studies have implications for BCC dormancy and immune evasion.

Materials and Methods

Reagents

DMEM and α-MEM were purchased from Life Technologies (Grand Island, NY), FCS was purchased from Hyclone Laboratories (Logan, UT), and Ficol-Hypaque and RPMI 1640 were purchased from Sigma-Aldrich (St. Louis, MO). [3H]thymidine (DuPont-New England Nuclear) and [32P] were purchased from PerkinElmer (Wellesley, MA). CellTracker Green 5-chloromethyl-fluorescein diacetate (CMFDA), Platinum SYBR Green qPCR SuperMix-UDG Kit, and SuperScript III reverse transcriptase were purchased from Invitrogen (Carlsbad, CA). Cytokine arrays were purchased from Ray BioTech (Norcross, GA). Human Granzyme B ELISA Kit was purchased from Abcam (Cambridge, MA). Western Lightning Plus chemiluminescence reagent was generously provided by the Metabolism Branch of the National Cancer Institute.

Abs and cytokines

Human TGF-β1 and rabbit neutralizing Ab to TGF-β1 and Human rCXCCL12 were purchased from R&D Systems (Minneapolis, MN). Anti-human CD8-FITC and anti-human CD56-allophycocyanin mAbs were purchased from BD Biosciences (San Jose, CA). Rabbit polyclonal anti-human CD8-FITC and anti-human CD56-allophycocyanin mAbs were purchased from eBioscience (San Diego, CA). Mouse anti–granzyme B was purchased from Abcam. Human Regulatory T Cell Staining Kit was purchased from BD Biosciences (San Jose, CA). CellTracker Green 5-chloromethyl-fluorescein diacetate (CMFDA), Platinum SYBR Green qPCR SuperMix-UDG Kit, and SuperScript III reverse transcriptase were purchased from Invitrogen (Carlsbad, CA). Cytokine arrays were purchased from Ray BioTech (Norcross, GA). Human Granzyme B ELISA Kit was purchased from Abcam (Cambridge, MA). Western Lightning Plus chemiluminescence reagent was generously provided by the Metabolism Branch of the National Cancer Institute.

Cell lines

Highly aggressive MDA-MB-231 breast adenocarcinoma, low-invasive MCF-7 breast adenocarcinoma, T47D breast adenocarcinoma, P815 murine mastocytoma, and K562 myelogenous leukemia were purchased from American Type Culture Collection and cultured as per manufacturer’s instructions. All cells were cultured at 37°C in 5% CO₂.

Human subjects

The use of human subjects was approved by the Institutional Review Board of University of Medicine and Dentistry of New Jersey, Newark Campus. After obtaining informed consent, peripheral blood was obtained from healthy volunteers ranging in age from 18–35 y. The mononuclear fractions (PBMCs) were isolated by Ficol-Hypaque density gradient separation. BM aspirates were obtained from healthy volunteers with informed consent and then used to expand MSCs.

MSC culture

MSCs were cultured from BM aspirates, as described (20). Briefly, unfractonated aspirates were diluted in DMEM. Aspirates were transferred to vacuum gas plasma-treated plates (BD Falcon, Franklin Lakes, NJ). After 3 d of incubation, RBCs and granulocytes were removed by Ficol-Hypaque density gradient centrifugation, and the mononuclear fraction was replaced. At weekly intervals, 50% of the media was replaced with fresh media. The adherent cells were serially passaged five times after the growth attained ~80% confluence. After four cell passages, the adherent cells were symmetric, CD14⁺, CD29⁺, CD44⁺, CD34⁺, CD45⁺, CD105⁺, prolyl-4-hydroxylase⁺.

Proliferation assays

PBMC proliferation was determined by [3H]Thd incorporation. PBMCs (10⁵/ml) were diluted in RPMI 1640 with 10% FCS (R10 media), and 1 ml was added to 12-well tissue culture plates (BD Falcon). BCCs were gamma-irradiated (20 Gy) using a cesium source. MSCs were similarly gamma-irradiated. The cancer cells and MSCs were added to the PBMCs in 200-μl volumes to achieve ratios of 50:1 PBMCs to T47D or MSCs. In parallel cultures, TGF-β1 neutralizing Ab was added at 5 μg/ml. After 48 h, cells were transferred to 96-well plates, and each well was pulsed with 1 μCi/ml [3H]Thd. After 16 h, the cells were collected and placed on glass fiber filters in a vacuum chamber. The filters were thoroughly washed and dried with 70% ethanol and then with absolute ethanol. Additional drying was done by leaving the filters in a fume hood overnight. The dried filters were analyzed for radioactive incorporation in a liquid scintillation counter (Beckman Coulter, Fullerton, CA). PBMC proliferation is presented as stimulation indices (SI), in which the dpm of experimental points was divided by the values of stimulated PBMCs alone.

Transmigration assay

Transmigration assay was performed as described (20). Briefly, Boyden chambers with 8-μm inserts (BD Falcon) were used to evaluate PBMC migration toward BCCs and/or MSCs. PBMCs were labeled with 3 μM CellTracker Green CMFDA and then added to the inner chamber. BCCs (with or without MSCs) were added to the outer chamber in serum-free DMEM. Parallel cultures were established with media containing human rCXCCL12 (6 ng/ml). The mean fluorescence intensity of each well was quantified, and the percentage of cell migrations was calculated on a standard curve of total PBMCs versus fluorescence intensities.

Establishment of effector cells

Effector cell cultures for cytotoxicity assays were established by coculturing PBMCs for 5 d with gamma-irradiated (20 Gy) T47D and MSCs. PBMCs (5 ml) were added at 10⁶/ml in T25 tissue flasks (BD Biosciences) and placed in a 37°C incubator in a standing position to allow for cellular interactions. T47D and MSCs (2×10⁵/ml each) were added in 500-μl volumes to attain a 50:1 ratio of PBMC/MSC and PBMC/T47D.

f⁵¹Cr-release assays

[51Cr] radionuclide assays for cytotoxicity were performed as described (21). Briefly, P815 and K562 represent target cells, and PBMCs represent effector cells. The establishment of effector cell cultures is described immediately above.

Targets (10⁵/ml) were labeled with [⁵¹Cr] (200 μCi/10⁵ cells) and then added to equal volumes (100 μl) of various effectors to attain different E:T ratios (100:1, 50:1, 25:1, and 12.5:1). Spontaneous [⁵¹Cr] release was determined by incubating targets alone, and total release was determined in parallel incubations with 1% Triton X-100. After 4 h of incubation of effectors and targets, cell-free supernatants were collected, and the amount of [⁵¹Cr] release was determined in a γ-counter. The percentages of lyses were calculated as follows: (experimental release (dpm) − spontaneous release (dpm)) × 100 /

Cytokine microarrays

Cytokine protein analyses were performed as described (22). Briefly, RayBio Cytokine Ab Arrays 3 and 5 membranes were incubated for 1 h in media, followed by consecutive incubations with biotin-conjugated cytokine Abs and HRP-streptavidin. The densities of spots were quantitated with UN-SCAN-IT (Silk Scientific, Orem, UT). The background signals with media alone were subtracted from experimental spots, and the unknowns were calculated after normalizing to internal positive controls, which were arbitrarily assigned values of 10.

ELISA

Quantiﬁcations of TNF-α and IL-12 and -10 were done in immunoasays with the Luminex 100 system (Luminex, Austin, TX) at Cytokine Core Laboratory, University of Maryland (Baltimore, MD). A standard curve was created to determine cytokine concentrations.

Anti-granzyme B and anti-granzyme B ELISA was performed on cell culture supernatants as per the manufacturer’s instructions. Briefly, supernatants were incubated on microtiter strips with biotinylated monoclonal anti-granzyme B. Streptavidin-peroxidase and substrate were added, and absorbance was measured at 450 nm. Quantitation of secreted granzyme B was determined based on a standard curve and calculated on a per-cell basis.
Western blot analysis
Western blots were performed as previously described (23). PBMC extracts were electrophoresed on 4–20% SDS-PAGE gels (Bio-Rad, Hercules, CA). Membranes were incubated overnight with rabbit polyclonal IgG granzyme B Ab (1:1000). This was followed by a 2-h incubation with polyclonal goat anti-rabbit IgG (1:1000). Detections were done by chemiluminescence, using Western Lightning chemiluminescence reagent. Membranes were stripped with Restore Western blot Stripping Buffer and then reprobed with mouse anti–β-actin mAb (1:2000). Bands were normalized to β-actin.

Bioassay for TGF-β1 quantitation
Quantitation of bioactive TGF-β1 was performed with a bioassay, as described (22). Briefly, CCL64 cells were seeded in 24-well culture plates, and culture supernatant was added after the adherence of cells. After 72 h, growth inhibition was determined by counting viable cells. TGF-β1 levels were determined in a standard curve established with known concentrations of the cytokine. Specificity was determined by repeating assays with neutralizing anti–TGF-β1 at 1.0 pM.

TGF-β1 and MHC I knockdown in MSCs
TGF-β1, β2-microglobulin, and HLA-A siRNA duplexes (Sigma-Aldrich) were used to knock down these respective genes in MSCs. Briefly, MSCs (10^6) were seeded in 75-cm² flasks; after 24 h, 100 nM siRNA was delivered via DharmaFECT Transfection reagent. siRNA duplex sequences were as follows: TGF-β siRNA: 5'-guu aua aua ecu ggc gau a-3' and 5'-uuu cgc cag gaa uug uug c-3'; β2-microglobulin siRNA: 5'-ggu aaa uaa aug uua aca u-3' and 5'-aug uua aca uua uaa c-3'; and HLA-A siRNA: 5'-gag uua caa cua ggc ugc a-3' and 5'-ugc age cuu gua acu c-3'. Knockdown of TGF-β1 was confirmed by bioassay using CCL64, semi-quantitative PCR of cDNA, and real-time PCR of cDNA. Knockdown of MHC I was confirmed by semiquantitative and real-time PCR of β2-microglobulin and HLA-A cDNA.

RT-PCR and quantitative real-time PCR
Whole-cell RNA extraction was performed using RNeasy Mini Kit from Qiagen (Valencia, CA). RNA isolates (2 μg) were immediately converted into cDNA via reverse transcription using 2'-deoxynucleoside 5'-triphosphates (0.2 mM), random hexamers (50 μM), and SuperScript III reverse transcriptase (200 U). Incubation conditions were 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min. Real-time quantitative PCR was performed on 200 ng cDNA using Platinum SYBR Green qPCR SuperMix-UDG Kit on the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). Samples were incubated at 50°C for 2 min and at 95°C for 2 min. Cycling conditions were 94°C for 15 s and 60°C for 45 s for 40 cycles. Primer sequences were as follows: TGF-β1 F: 5'-cag atc ttg cag ctc-3'; R: 5'-tcc tgt tga aag cag ctc-3'; β2-microglobulin F: 5'-aat gaa ttc aac ccc act-3'; R: 5'-tcc tca aac ctc gat gct-3'; and HLA-A F: 5'-aga tgg gac ctg ttg-3'; R: 5'-gtt ctt cct ctt cct cat-3'. Amplification of β-actin cDNA was used for normalization of target mRNA.

Flow cytometry
PBMCs were incubated with fluorochrome-conjugated Abs to CD3, CD4, CD8, and CD56 at 1:200 dilutions and then analyzed on FACSScan. The data were analyzed with CellQuest software (BD Biosciences). Labelings for T regs were done with a Human Regulatory T Cell Staining Kit, which includes FITC–anti-CD4, allophycocyanin–anti-CD25, and PE–anti-FoxP3. Analyses were performed using the FACSCalibur system (BD Biosciences). PE-rat IgG2a isotype was used as control for PE-FoxP3 emissions.

Statistical analysis
Data were analyzed using ANOVA and the Tukey-Kramer multiple comparisons test. A p value ≤0.05 was considered significant.

Results
Effects of MSCs on PBMC proliferation and migration toward BCCs
We and other investigators have reported on interactions between MSCs and BCCs via CXCR4 and membrane-bound CXCL12 (20, 24). We examined whether CXCR4 was involved in the attraction of BCCs toward MSCs when the cells were placed at a distance from each other. We began with three BCC lines: low-invasive MCF-7, low-invasive T47D, and highly aggressive MDA-MB-231. We first seeded MSCs and BCCs separately on slides. Upon adhesion, the media were mixed, and the cultures were observed at various times. Cultures showed cellular attraction between MSCs and the following BCC lines: MCF-7, T47D, and MDA-MB-231. The results also showed clustering of CXCR4 in BCCs at the projections of MSCs (data not shown).

MSCs have been shown to alter chemotraction of immune cells (25). Because immune responses to cancer cells require attraction and expansion of PBMCs, we determined whether MSCs could affect the proliferation and the migration of PBMCs toward BCCs. Cell proliferation was determined by [3H]Tdr uptake in assays in which PBMCs were cocultured with gamma-irradiated BCCs and/or MSCs. The results, presented as SI ± SD, indicate significant reductions for all three BCC lines in the presence of MSCs compared with cultures without MSCs (p ≤ 0.05) (Fig. 1A). Because CD4+ T cells are highly abundant within the lymphocyte subsets (60%) of PBMCs, we analyzed CD4+ cell levels by flow cytometry in culture with BCCs, in the presence or absence of MSCs. Analyses of similar events indicated a reduction in total CD4+ cells in the presence of MSCs. Representative graphs are shown in Fig. 1B.

The next set of studies determined whether MSCs could affect the migration of PBMCs toward T47D. The purpose of these studies was to recapitulate the presence of BCCs in BM, where they are expected to attract immune cells. Migration studies were performed in transwells to which labeled PBMCs were added to the upper/inner chambers, and T47D and/or MSCs were added in the lower/outier chambers (Fig. 2A, inset). The results showed a 6.3 ± 0.7-fold reduction in PBMC migration in the presence of MSCs compared with assays without MSCs (p ≤ 0.05) (Fig. 2A). No cell death was observed.

Because chemokine production is significant for the migration of immune cells, we analyzed the media for chemokine release using cytokine protein arrays (26). We observed significant decreases in CCL2, CCL5, inflammatory protein-10, and CXCL12 (Fig. 2B). Together with previous studies from our laboratory (3, 20), the results of chemokine analyses suggested that soluble CXCL12 could be critical to the interaction. Therefore, we performed these
studies in the presence of exogenous CXCL12-purified cytokine to determine whether the MSC-mediated decrease in PBMC migration could be restored (Fig. 2C). We observed partial, but significant ($p \leq 0.05$), restoration of transmigration to approximately half-maximal levels in the presence of exogenous CXCL12. In summary, this section describes the studies that showed that MSCs hinder PBMC proliferation and migration.

**NK and CTL responses in the presence or absence of MSCs**

MSCs have been reported to promote BCC growth (27, 28). However, MSCs exert immune suppression, such as inhibition of T cell proliferation, through the production of IL-10 (29). Immune responses to tumors predominantly involve effector CTLs and NK cells (25). Because MSCs inhibit the migration of PBMCs to T47D (Fig. 2A), we next determined whether the addition of PBMCs to T47D could initiate immune responses against the tumor cells. To address this question, we studied the NK and CTL responses in the presence or absence of MSCs.

In the presence of MSCs, there were significant reductions in CTL and NK responses (Fig. 3A, 3B, respectively). Significant differences ($p \leq 0.05$) were observed at E:T ratios of 100:1 and 50:1 for K562 target cells and at 25:1 for P815 target cells. CTL responses at a 50:1 ratio were decreased by 17.1 ± 1.8-fold. In the case of NK activity, the decrease with the 50:1 ratio was 4.2 ± 1.0-fold; $p \leq 0.05$. We next sought to determine the effects of MSCs after knockdown of MHC I, based on studies demonstrating the role of MHC I in NK-mediated lysis of MSCs (30). We selectively knocked down β2-microglobulin and HLA-A genes in MSCs and then established the cocultures and performed [51Cr]-release assays. Knockdown confirmation is shown by semiquantitative PCR (Fig. 3C). Cytotoxicity assays demonstrated a significant increase ($p \leq 0.05$) in CTL-mediated lysis in the presence of MHC I-knockdown MSCs at all E:T ratios (Fig. 3D). For NK-mediated lysis, increases in cytotoxicity were observed at E:T ratios of 100:1 and 50:1 (Fig. 3E). In summary, the results demonstrate that MSCs suppress CTLs and NK cells and that selective MHC I knockdown in MSCs partially reverses these effects.

**Effects of MSCs on granzyme B levels and NK and CTL phenotypes**

In general, NK cells and CTLs exert cytotoxicity via granzyme B/perforin system, which targets tumor cells and viruses via pore formation for apoptosis (7, 31). Because MSCs suppress the functions of NK cells and CTLs (Fig. 3A, 3B), we asked whether this could be explained by the inhibition of granzyme B release. To address this question, we performed Western blots and ELISA for granzyme B, using the conditioned media from the cocultures with PBMCs and T47D, with or without MSCs.

Representative Western blots for intracellular granzyme B from PBMCs are shown in Fig. 4A. As expected, there were intense bands when PBMCs were cultured with T47D. However, bands were reduced in the presence of MSCs. Normalized densitometry demonstrated a significant ($p \leq 0.05$) reduction (by 12.2 ± 0.9-fold) compared with PBMCs cultured with T47D. Regardless of the presence of MSCs, in the absence of T47D, there were comparable bands, although they were significantly low compared with cultures with T47D. We next performed granzyme B ELISA, which allowed for precise quantitation of granzyme B in supernatants of cultured cells (Fig. 4B). A titration of granzyme B per cell was calculated based on a standard curve. Results of two independent experiments showed a 4.7 ± 0.3-fold reduction in secreted granzyme B in the presence of MSCs compared with T47D with no MSCs.

We next asked whether the reduction in granzyme B could be explained by reduced numbers of CTLs and NK cells. This was addressed by flow cytometry for CD8 and CD56, as indicators of CTLs and NK cells, respectively. In the presence of MSCs, we observed a reduction in the peaks for CD8* and CD56* cells (Fig. 4C). We ruled out cell death as the reason for the reduced number...
of cytotoxic cells by performing daily trypan blue exclusion analyses; the results showed <5% cell death. In summary, MSCs cause a decrease in intracellular and secreted granzyme B from PBMCs and a significant reduction of phenotype consistent for CTL and NK cells. These observations are not due to cell death.

**MSC-mediated expansion of Tregs**

In vivo studies demonstrated that MSCs facilitate graft survival, and this could be explained, in part, by increases in Tregs (32). Therefore, we asked whether increased numbers of Tregs could explain reduced NK and CTL responses (Fig. 3). Effector cells were prepared as for Fig. 3, and the PBMC population was studied for Treg frequency via flow cytometry at days 3 and 5. Control cultures were done in parallel with PBMCs with or without T47D. Fig. 5 represents six independent experiments, each performed with cells from a different donor. In summary, the results showed increases in Tregs in the presence of MSCs over time. The increase at day 3 showed a trend toward increase (4.2% ± 0.1% without MSCs versus 6.1% ± 0.3% with MSCs) (Fig. 5, left panel) compared with significant (p < 0.05) increases at day 5 (4.1% ± 1.0% and 5.8% ± 0.5% without MSCs versus 9.9% ± 1.0% and 9.3% ± 0.9% with MSCs) (Fig. 5, middle and right panels).

**FIGURE 3.** NK cell and CTL effector responses. Effector cells (PBMCs) were cocultured with gamma-irradiated T47D and/or gamma-irradiated MSCs. Target cells P815 for CTL activity (A) and K562 for NK activity (B) were labeled with 200 μCi [51Cr]/10⁶ targets. Effectors and targets were added at varying ratios; after 4 h, supernatants were collected, and radioactive releases were determined. Data are shown as mean percentage lysis ± SD, compared with maximal release by incubation of targets with 1% Triton X-100 (n = 5). Each experiment was performed with cells from a different donor. Next, MHC I was selectively knocked down in MSCs by β2-microglobulin or HLA-A siRNA transfection. RT-PCR confirmed efficient knockdown of β2-microglobulin and HLA-A (C). [51Cr]-release assays for CTL effector activity (D) and NK effector activity (E) were performed in the presence of MHC I-knockdown MSCs. *p ≤ 0.05 versus all other experimental points.
Furthermore, for cultures containing MSCs, there was a significant increase in Tregs between day 3 (6.1% ± 0.3%) and day 5 (9.9% ± 1.0%).

**Cytokines in MSCs-mediated effects**

Cytokines are soluble glycoproteins that are key factors in determining T cell responses. To gain insights into their roles, we first screened the media of cocultures with cytokine arrays. The presence of MSCs in cocultures resulted in significant increases in IL-2 at day 3, which was decreased by day 5 (Table I). Similarly, the other Th1 cytokines, TNF-α and IFN-γ, were significantly decreased by day 5 in the presence of MSCs (Table I).

Because Th1-type cytokines were decreased, we next analyzed the Th2 cytokines and observed increases in IL-4, -5, -6, -10, and -13 at day 5 (Table I). It is known that IL-12 favors Th1 polarization (33, 34). Therefore, we examined IL-12 levels by ELISA and observed a significant decrease (Fig. 6), consistent with a predominance of Th2 cytokines at day 5.

We next focused on IL-10 and TGF-β1 because they are both linked to Treg expansion and/or functions (35, 36). The increase in IL-10 levels recorded by the arrays (Table I) was confirmed quantitatively by ELISA, and the results showed a significant increase in 5-d cultures (Fig. 6). Because TGF-β1 protein could be in its active and latent form, we performed a functional assay to

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**Table I.** Cytokine production in cocultures of PBMCs, T47D, and MSCs

<table>
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<tr>
<th>Cytokine</th>
<th>Day 3 Cultures</th>
<th>Day 5 Cultures</th>
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<th>MSCs</th>
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<tr>
<td><strong>Th1 subset</strong></td>
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<tr>
<td>IL-2</td>
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<td>8.1 ± 0.2</td>
<td>0.8 ± 0.04*</td>
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<td>1.0 ± 0.03</td>
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<tr>
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<td>IL-13</td>
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*Media from 3- and 5-d cocultures of PBMCs, gamma-irradiated T47D, and gamma-irradiated MSCs were studied for cytokine proteins using a cytokine array. The spots were normalized with the internal positive controls, which were arbitrarily assigned values of 10. The results represent the mean normalized densities ± SD and are categorized into those linked to Th1 and Th2 polarizations.

*p ≤ 0.05 versus cultures without MSCs.

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**Figure 5.** Flow cytometry for Treg population in PBMCs. Cocultures were established with PBMCs and gamma-irradiated T47D and/or gamma-irradiated MSC. After 3 and 5 d, cells were triple-labeled with FITC–anti-CD4, allophycocyanin–anti-CD25, and PE–anti-FoxP3. Rat IgG2a served as isotype control. The dot plots are shown for FoxP3 versus CD4 and CD25 (x-axis). Left panel, 3-d cocultures; middle and right panels, 5-d cocultures. The data are presented within each panel as mean percentage ± SD (n = 5). Each experiment was performed with cells from a different donor.

**Figure 6.** IL-10 and IL-12 levels in cocultures of PBMCs and gamma-irradiated T47D with or without gamma-irradiated MSCs. Media from cocultures were collected at day 5 and then evaluated for IL-10 and IL-12 using Luminex ELISA. The results were calculated from a standard curve and are presented as mean ± SD (n = 4). *p ≤ 0.05 versus T47D alone.
quantitate the bioactive form. The standard curve based on effects of purified TGF-β1 demonstrated 90% growth inhibition at 10–100 pM and 50% inhibition at 0.3–1.0 pM. The results show upregulation from 3.0 pM in 3-d cultures to 24.1 pM in 5-d cultures (Table II).

Role of MSC-derived TGF-β1 in Treg expansion

TGF-β1 has been linked to the expansion of Tregs (37). Therefore, we performed cause–effect studies to determine whether MSC-derived TGF-β1 could explain, at least in part, the increase in Tregs (Fig. 5). To address this question, we performed knockdown of TGF-β1 in MSCs before adding them to cocultures of PBMCs and gamma-irradiated T47D. Knockdown MSCs were studied for active TGF-β1 production. RT-PCR showed nearly undetectable TGF-β1 mRNA (Fig. 7A), and quantitative real-time PCR showed 46-fold knockdown (Fig. 7B). Production of TGF-β1 by MSCs was also assessed by bioassay, with mutant siRNA-transfected MSCs showing 4.8 ± 0.02 pM TGF-β1 and wild-type siRNA-transfected MSCs showing undetectable levels after overnight incubation (data not shown).

At days 3 and 5, the PBMC population was studied for Treg frequency. In the absence of TGF-β1 in MSCs, there were no significant ($p > 0.05$) differences in Tregs (6.2% ± 0.3% and 5.8% ± 0.5% for T47D alone versus 5.2% ± 0.5% and 6.8% ± 0.5% for T47D with MSCs) (Fig. 7C). These results contrast with studies using wild-type MSCs (Fig. 5) or mutant siRNA, which showed a significant increase (4.8% ± 0.4% versus 9.9% ± 1.0%) (Fig. 7C, left panel). Together, the results indicate a significant role for TGF-β1 production by MSCs in the increased number of Tregs.

Role of Tregs in functional responses

The Treg fraction within the PBMC population was depleted by the addition of CD25 mAb (5 μg/ml) or TGF-β neutralizing Ab (5 μg/ml) to culture media. Effective Treg depletion was confirmed by flow cytometry (data not shown). Effector cells, including BCCs, were maintained for 5 d in anti-CD25 prior to the addition of [51Cr]-labeled targets. CTL and NK cell responses were assessed by [51Cr]-release assays on a γ-counter, as for Fig. 3. We observed significant increases in CTL-mediated lysis (Fig. 8A) at all E:T ratios for cultures containing MSCs in the presence of anti-CD25. Levels of cytotoxicity approached those of PBMC and T47D cultures alone. For NK-mediated lysis (Fig. 8B), a significant increase was observed after Treg depletion at E:T ratios of 100:1 and 50:1. In summary, Treg depletion resulted in partial restoration of cytotoxicity in cultures containing MSCs, with minimal effects on cultures containing PBMCs and T47D alone. We next determined the role of MSCs after Treg depletion on PBMC proliferation (Fig. 8C). We noted significant increases in PBMC proliferation by [3H]Tdr uptake for PBMCs stimulated with MCF-7, T47D, and MDA-MB-231 in the presence of neutralizing TGF-β1 Ab. In summary, these results suggest that Tregs may play a role in the MSC-mediated effects on components of the immune system that normally serve to eliminate cancer cells (Fig. 9).

Discussion

In other studies, MSCs were shown to exert immune protection, and, in the case of breast cancer, MSCs can support cancer growth (38). These two properties of MSCs are consistent because immune protection would give the cancer cells a survival advantage. Indeed, this study showed MSCs conferring immune protection through the generation of FoxP3+ Tregs. This T cell subset is known for its anti-inflammatory and immunosuppressive roles, including the suppression of autoimmunity (39, 40).

The results showed a role for TGF-β1 in the increase in Tregs, with the source mostly from MSCs (Figs. 5 and 7). However, the maturation of Tregs is a complex process, because timeline studies show an initial increase in Th1 cytokines, such as IL-2 (Table I). A
PBMCs and T47D. Data are shown as mean percentage lysis performed as described for Fig. 3. All experimental points contained absence of anti–TGF-β was assessed using three BCC lines as alloantigens in the presence or p

Assays were performed as described for Fig. 3. All experimental points contained PBMCs and T47D. Data are shown as mean percentage lysis ± SD (n = 5). *p ≤ 0.05 versus MSC + α-CD25. C, PBMC proliferation in MSC culture was assessed using three BCC lines as alloantigens in the presence or absence of anti–TGF-β1. [3H]Tdr uptake was determined by a liquid scintillation counter, as for Fig. 1. Data are shown as mean SI ± SD (n = 3). *p ≤ 0.05 versus MSC cultures containing nonimmune IgG.

**FIGURE 8.** Treg depletion was achieved through the use of TGF-β1 neutralizing Ab or anti-CD25. [3H]Tdr-release assay was performed for assessment of CTL (A) or NK cell (B) effector function. Assays were performed as described for Fig. 3. All experimental points contained PBMCs and T47D. Data are shown as mean percentage lysis ± SD (n = 5). *p ≤ 0.05 versus MSC + α-CD25. C, PBMC proliferation in MSC culture was assessed using three BCC lines as alloantigens in the presence or absence of anti–TGF-β1. [3H]Tdr uptake was determined by a liquid scintillation counter, as for Fig. 1. Data are shown as mean SI ± SD (n = 3). *p ≤ 0.05 versus MSC cultures containing nonimmune IgG.

**FIGURE 9.** Differences in Treg and other T cell responses when breast cancer-challenged PBMCs are cocultured without (A) or with (B) MSCs. A, Shown are breast cancer cells (green) added to PBMCs. The addition of cancer cells resulted in immune activation and an increase in T cells, with a predominance of Th1 cytokines, and increases in CTL and NK activities. B, Shown are cultures with MSCs (spindle-shaped black cells). The cytokine profile has shifted to a reduced Th1 profile and an increased Th2 profile. Also shown are reductions in the activities of NK and CTL effector responses, with concomitant increases in Tregs, which could be explained, at least in part, by MSC-derived TGF-β1 (arrow).

MSCs favoring polarization away from a Th1 phenotype and toward a Th2 phenotype supports their immune-protective effect (Fig. 9). Amplified Th1 responses exert antitumor effects in cancers (45). The observed increase in levels of IL-10 (Fig. 6, Table I) favors the argument for aberrant activation of the Th2 response (46). Although we did not address the Th17 subset, its development may also be important for antitumor responses (45). The interaction between Tregs and Th17 in the development of breast cancer dormancy requires future studies.

A major question is why there is a delay for efficient immune suppression by Tregs. It is possible that not all subsets of the cancer cells are responsible for the observations. Ongoing studies are attempting to determine whether the immune responses are specific to BCC subsets. Regardless, our findings are consistent with other studies demonstrating that MSC promotion of graft tolerance in heart transplant could be explained by increases in Tregs (32).

We previously implicated the CXCR4–CXCL12 axis in the metastasis of BCCs (20). Our observations showed evidence of projections by MSCs toward BCCs (data not shown). These are intriguing observations that merit further study, especially because MSCs inhibit PBMC migration toward cancer cells (Fig. 2A). This may be explained by chemotactic functions, because several chemokines were decreased in the presence of MSCs, especially CCL2 and CXCL12, which are involved in PBMC trafficking (Fig. 2B). Our data are interesting in light of other reports demonstrating that BCCs stimulate CCL5 production from MSCs (47). Our observation of decreased CCL5 in culture with MSCs may indicate that the source of CCL5 is a cell type within the PBMC population or the BCCs themselves.

Previous studies demonstrated that BCC-derived CXCL12 regulates interaction with the BM stromal cells (3). Furthermore, our laboratory previously showed a role for CXCL12 in the integration...
of BCCs within the BM compartment and in the entry into the BM cavity (3, 20). We show a different role for CXCL12, i.e., its role in attracting PBMCs to the site of BCCs, even in the presence of MSCs (Fig. 2C). Because we did not observe total restoration of PBMC migration, it seems that other chemotactic factors are involved. Previous studies showed that contact between BCCs and MSCs occurs by double interactions between CXCR4 on both cell types and membrane-bound CXCL12 (20). Similar interactions might explain the reduction in CXCL12. This requires additional studies to determine whether a particular cancer cell subset might be involved and, if so, whether there are alterations in the level of CXCL12.

Because immune surveillance of cancer cells typically involves NK cells and CTLs (48), we explored these mechanisms in detailed studies. Other investigators reported on MSCs as inhibitors of IL-2–primed NK functions (43). In our studies, with breast cancer as a stimulator to activate effectors from PBMCs, we observed that MHC I on MSCs was important in the reduced functions of NK activity (Fig. 3D, 3E). This could be due to increased killing of MS by NK cells after the loss of killer Ig-like receptor interaction with MHC I. Our data are in concordance with previous findings (30, 43). We delved further into the NK and CTL mechanisms and demonstrated that the MSC-mediated decrease in target cell lysis was due to decreased effector function per cell, as indicated by the reduction in granzyme release (Fig. 4B) and the decrease in NK and CTL numbers (Fig. 4C). Treg depletion assays indicated its role in PBMC proliferation and effector cell function (Fig. 8).

We did not study whether MSCs could induce Tregs in activated T cells, whereby stimulation of the CD3 molecule could address this question. These studies were omitted because we observed a predominance of Th1 cells in the absence of MSCs (Table I). These studies confirmed that Tregs could be induced and/or expanded by MSCs. Although TGF-β1 production is expected by multiple immune cells, in this study, a critical source was MSCs (Fig. 7). This finding indicates that MSCs have an active role in the increase in Tregs when they are in contact with BCCs.

It is important to discuss our observation of decreased PBMC proliferation by MSCs in light of other studies showing increased tumor progression by MSCs (49, 50). MSCs could be producing other factors, such as vascular endothelial growth factor, when MSCs interact with cancer cells (46, 49). If this is the case, we should observe an increase in cancer cell proliferation. Although this study did not address the role of MSCs on BCC proliferation, these findings are important and have implications for treatment with chemotherapy. MSCs may behave differently toward immune cells versus BCCs, based on different chemokine receptor expression profiles or other characteristics. These are interesting prospects that warrant further investigation because MSCs interact with immune cells and BCCs when BCCs metastasize to the narrow cavity (20).

Our findings have implications for treatment with chemotherapy, which mostly targets cancer cells that are rapidly proliferating. An immune-competent individual would likely clear the few remaining cancer cells by immune surveillance or innate immunity (7). However, in the presence of MSCs, if a particular subset of cancer cells is protected, these could remain dormant and result in resurgence of cancer after years of disease-free survival. This is particularly significant for metastasis to the BM, an organ for which BCCs show preference (20). Upon entering BM, BCCs would encounter perivascular MSCs, which might hinder the immune functions that normally destroy cancer cells (Fig. 9). In cases in which immune therapies for cancer are indicated, the effects of MSCs will require serious consideration. The findings of this study might not be limited to breast cancer because these phenomena may include other cancers that metastasize to the BM, including prostate cancer and melanoma (51).

In summary, this report shows significant findings that might explain phenomena caused by MSCs during the treatment of breast cancer and might begin to unravel the reasons for the high incidence of recurrence of breast cancer from BM after years of event-free survival (52, 53).

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Disclosures

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