Heat Shock Protein 27 Differentiates Tolerogenic Macrophages That May Support Human Breast Cancer Progression

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Abstract

Tumor cells release several factors that can help the progression of the tumor by directly supporting tumor growth and/or suppressing host antitumor immunity. Here, we report that human primary breast tumor cells not only express elevated levels of heat shock protein 27 (Hsp27) at the intracellular level but also release extremely high levels of Hsp27 compared with the same patients’ serum Hsp27 levels, predicting an acutely increased concentration of soluble Hsp27 in the human breast tumor microenvironment (HBTM). We demonstrate that Hsp27 levels in the HBTM can be extremely elevated as evidenced by high soluble Hsp27 levels in patients’ tumor interstitial fluid. Because increasing numbers of tumor-associated macrophages (TAM) in the HBTM negatively correlate to patients’ clinical outcomes and we have previously reported the immunoregulatory activity of soluble Hsp27, here, we tested for any specific effects of soluble Hsp27 on human monocyte to macrophage differentiation. We demonstrate that soluble Hsp27 causes the differentiation of monocytes to macrophages with immuno-tolerizing phenotypes (HLA-DRlow, CD86low, PD-L1high, ILT2high, and ILT4high). We detected the presence of TAMs with similar phenotypes in breast cancer patients. Hsp27-differentiated macrophages induce severe unresponsiveness/anergy in T cells. Moreover, these macrophages lose tumoricidal activity but become extremely proangiogenic, inducing significant neovascularization, a process that is critically important for tumor growth. Thus, our data demonstrate a novel immune escape and tumor growth–supporting mechanism mediated by soluble Hsp27 that may be operative in human breast cancer. Cancer Res; 71(2); 318–27. ©2011 AACR.

Introduction

Heat shock protein (Hsp) 27, an important member of the small Hsp family, is ubiquitously expressed in various cell types and is involved in actin polymerization, which is the protection (chaperoning) of intracellular peptides during the stress and inhibition of mitochondrial apoptosis (1–3). Intracellular expression of Hsp27 is highly elevated in human breast tumor cells (4–6) and correlates to increased resistance to chemotherapeutic drug-induced apoptosis (7, 8). Interestingly, elevated serum Hsp27 levels are also reported in breast cancer patients (9, 10), suggesting the possible release of Hsp27 from breast cancer cells. In fact, a recent report has shown the secretion of Hsp27 from a human monocytic cell line (11). Therefore, we hypothesized that human breast tumor cells expressing elevated levels of intracellular Hsp27 may secrete high levels of Hsp27, increasing its local concentration in the human breast tumor microenvironment (HBTM). Tumor-released factors contribute to tumor progression by perturbing host antitumor immunity and/or supporting tumor growth. We have shown that rhHsp27 (recombinant human Hsp27) exhibited significant biological activity on human monocytes, causing the secretion of high levels of IL-10 and moderate levels of TNF-α (12). We have also reported that soluble Hsp27 could inhibit the differentiation of human monocytes to dendritic cells (DC; ref. 13). Thus, increased Hsp27 levels in the HBTM may cause a severe reduction in the differentiation of monocytes to DCs. An increase in the number of DCs in human breast tumors is positively correlated with survival (14). In contrast, the presence of increased numbers of tumor-associated macrophages (TAM) in the HBTM is negatively correlated with clinical outcome (15, 16). The exposure of peripheral monocytes...
infiltrating the tumor to high soluble Hsp27 levels in the HBTM may induce the release of several immunosuppressive and/or tumor growth–promoting soluble factors, such as IL-10 (17), and may also induce their differentiation to macrophages (MAC) that are supportive for tumor cells. In fact, the role of TAMs in supporting tumor growth is well established in mouse tumors (17–20). TAMs have reduced tumoricidal activities (21). They promote tumor growth by causing the suppression of antitumor immunity and/or inducing neovascularization (angiogenesis; ref. 22). We tested whether an in vitro culture of human peripheral monocytes in the presence of soluble Hsp27 differentiates them into MACs that have TAM-like properties.

Patients’ breast tumor cells not only express elevated levels of Hsp27 at the intracellular level but also release high levels of Hsp27. Finally, Hsp27 levels in patients’ tumor interstitial fluid are extremely high, indicating a highly elevated concentration of Hsp27 in the HBTM. We further demonstrate that MACs differentiated in the presence of soluble Hsp27 are highly immunosuppressive, inducing anergy in T cells. These Hsp27–differentiated MACs lose antitumor cytotoxicity but become extremely proangiogenic, indicating that they have properties similar to TAMs. Thus, we demonstrate a novel immune escape and tumor growth–supporting mechanism mediated by extracellular Hsp27, which may be operative in human breast cancer.

Materials and Methods

Reagents
rhHsp27 and phospho-Hsp27 (Ser82) antibody were purchased from Stressgen Laboratories/Enzo Life Science. Any endotoxin contamination in the rhHsp27 preparation was removed by incubation with polymyxin B–bound agarose beads (Sigma –Aldrich) for 1 hour at 4°C on a rocker. This process was repeated for 1 more cycle and then filtered (0.2 μm). Bead-treated rhHsp27 was further incubated with polymyxin B (20 U/mL) for 30 minutes before storing at −80°C. rhHsp27 (250 ng/mL) containing culture medium did not show any evidence of endotoxin contamination when examined with a Limulus amoebocyte lysate assay. Polymyxin B (20 U/mL) was added in all culture medium.

rhM-CSF and rhIL-2 were purchased from Peprotech. Monoclonal antibodies (MAb) against CD14 (clone MFP99), HLA-DR (clone L243/G46–6), CD86 (clone 2331/FUN-1), and CD206 (clone 19.2) were purchased from BD Biosciences. Those against PD-L1 (program death–ligand; clone MIH1), PD-L2 (clone MIH18), CD16 (clone CB16), and CD163 (clone GHI/61) were purchased from eBioscience. A MAb against CD326 (clone HEA-125) was obtained from Miltenyi Biotech. Abs against ILT2 (immunoglobulin-like transcript; clone 292305), ILT4 (bionylated goat anti-human antibody), VEGF (clone 23410), and Hsp27 (rabbit polyclonal) were purchased from R&D Systems. MAbs against CD3 (clone OKT3) and CD28 (clone CD28.2) used for T-cell stimulation were obtained from eBioscience and BD Biosciences, respectively. Hsp27 and PGE2 ELISA kits were purchased from Calbiochem and Amer sham/GE Health Care, respectively, whereas MCP-1 and IL-12 (p40 + p70) ELISA kits were obtained from Biosource International/Invitrogen. Luminex multiplex kits for IL-1β, IL-2, IL-6, IL-8, IL-10, IL-13, IFN-γ, TNF-α, and VEGF were purchased from LNCX/Millipore. iScript cDNA Synthesis kit, and SYBR green jumpstart PCR kit were purchased from Qiagen, Bio–Rad, and Sigma–Aldrich, respectively. Breast cancer cell lines (MCF-7, ZR-75-1, and SK-BR-3) were purchased from ATCC, and a normal human mammary epithelial cell line (HMEC) was obtained from Lonza. A HUVEC cell line was purchased from ScienCell Research Laboratories. HMEC and HUVEC were purchased within 6 months of performing experiments, whereas MCF-7, ZR-75-1, and SK-BR-3 were authenticated by the Johns Hopkins Genetic Resources Core Facility with STR analysis.

Serum and cell isolation and cell culture
Peripheral blood was drawn from untreated breast cancer patients and age-matched healthy women for the isolation of serum, monocytes, and T cells. Informed consent was obtained from every human subject prior to blood collection, and the study was approved by the Institutional Review Board at the University of Rochester. T cells were isolated as described (13) followed by monocyte separation using a negative isolation kit (Miltenyi). Isolated T cells were ~95% CD3+ and monocytes were ~90% CD14+ (<5% CD14+CD16+). T cells were kept frozen at −80°C in 90% FBS/10% DMSO until the day of their use in a coculture assay with MACs. T cells were thawed and equilibrated for 4–6 hours at 37°C before setting up the coculture assay. Monocytes were cultured for 3–4 days in the medium alone (adherence alone) or in the presence of rhHsp27 (250 ng/mL), M-CSF (50 U/mL), or Hsp27 + M-CSF. Monocytes were always preincubated with rhHsp27 for 1 hour before the addition of M-CSF.

Human tissue samples
Normal and tumor-containing breast tissue samples were collected from preconsented healthy volunteers undergoing breast reduction and breast cancer patients undergoing scheduled surgery, respectively. For the isolation of tissue interstitial fluid, breast tumor and normal mammary tissue were first minced into small pieces (<1 mm3), then weighed and suspended in PBS (1 ml of PBS for each gram of tissue), and finally centrifuged at 3,000 rpm for 5 minutes. The supernates were further centrifuged at 10,000 rpm for 5 minutes for interstitial fluid collection. For the isolation of cells, tissue samples were digested with a collagenase/hyaluronidase cocktail followed by treatment with trypsin, dispase, and DNase and the isolation of CD3+ epithelial cells with a PE selection kit (Stem Cell Technologies). Normal primary breast epithelial cells and breast tumor cells were employed for assessing the intracellular expression of Hsp27 by flow cytometry and the release of Hsp27 by culturing the cells (5 × 105 cells/mL) for 24 hours and testing soluble Hsp27 by ELISA.

For immunohistochemistry studies, tissue samples were fixed in buffered formalin and embedded in paraffin wax. Consecutive sections (4 μm) were prepared for the assessment of different proteins in similar cell types. Sections were pretreated with a citrate buffer (pH 6.0) at 99°C for 20 minutes...
before staining with anti-human CD68, PD-L1, or CD86 MAb delayed followed by horseradish peroxidase–conjugated secondary antibodies and detection by EnVision reagents (Dako). Slides were checked by an inverted light microscope. Photographs were taken at 400× magnification.

**Human mammary cell lines**

Normal HMECs and breast tumor cell lines were maintained according to the suppliers’ instructions. Their culture supernatants were prepared as mentioned for primary breast epithelial cells. Culture supernatants of both primary breast tumor cells and breast tumor cell lines were further concentrated (>50-fold) with the Amicon 3K filter. Concentrated culture supernatants were utilized for assessing the phosphorylation of extracellular Hsp27 by Western blotting and compared with the phosphorylation status of rhHsp27. Gel band intensities were analyzed with Bio-Rad Image lab software.

**Assay for testing tolerogenic activity of MACs**

Harvested MACs were cocultured (1:5 ratio) with autologous T cells in the presence of immobilized anti-CD3 (iCD3; 1 μg/100 μL/well for 2 hours at RT) for 6 days. T cells were re-isolated by rosetting with sheep red blood cells (SRBC) and rested for 24 hours. T cells were then restimulated with iCD3 plus soluble CD28 (sCD28; 2 μg/mL) in the presence or absence of IL-2 (100 U/mL) for 4 days. The culture supernatants were assessed for cytokine levels by a Luminex multiplex assay, whereas T-cell proliferation was assessed by 3H-TdR incorporation and the data are expressed as counts per minute (mean ± SEM). In a selected experiment, M-CSF–induced monocyte to MAC differentiation was done in the presence of concentrated (>50-fold) ZR-75-1 culture supernatants corresponding to a final Hsp27 concentration of 100 ng/mL (tested by ELISA). Hsp27 antibody or control IgG was added at 5 μg/mL for the neutralization of Hsp27 present in the ZR-75-1 culture supernatants. MACs were then co-cultured with autologous T cells followed by restimulation, as mentioned above.

**Cytotoxicity assay**

Monocyte-derived MACs were stimulated overnight with IFN-γ (100 U/mL) and cocultured with CFSE-labeled SK-BR-3 for 4 hours. Different effector to target ratios were used to determine the optimal cytotoxic activity. The cells were then stained with 7-AAD and fixed, and percentage of cytotoxicity was analyzed by flow cytometry. Spontaneous cell death was subtracted from each experimental data set.

**Angiogenesis assay**

MACs (2 × 10⁴) were cocultured with 1 × 10⁶ HUVEC cells (lower chamber coated with fibronectin) in trans-well plates (0.4-μm membrane) for 20 hours, and then HUVEC cells were cultured for 14–16 hours (5 × 10⁴ to 1 × 10⁵ cells/well in 96-well plates containing polymerized ECMatrix; BD Biosciences). Neovascularization in HUVEC cells (assessed by network formation) was recorded as arbitrary pixel units using an inverted light microscope at 10×–200× magnification.

**Quantitative reverse transcription PCR**

Total RNA was isolated using an RNeasy mini kit. One to 2 micrograms of total RNA was reverse-transcribed using an iScript cDNA Synthesis kit. For a quantitative mRNA measurement, real-time RT-PCR was done with the Stratagene MX3005 machine and the SYBR green jumpstart PCR kit, following the manufacturer’s protocol. Data are normalized to GAPDH and expressed as mean ± SEM.

**Flow cytometric assessment of phenotypic markers**

Cells were stained and analyzed by flow cytometry with BD FACSCalibur (Cell Quest Pro software). Data are represented as a percentage of positive cells and net mean fluorescence intensity (MFI; calculated by subtracting geometric MFI of isotype control from that of specific antibody).

**Statistical analysis**

Data are presented as mean ± SEM. Student’s t-test was done for assessing the significant differences between 2 different data sets. Two-tailed hypothesis was considered, and the difference was considered statistically significant if P < 0.05. Nonparametric statistics (Wilcoxon) were also done where the data were either not normally distributed or had unequal variance.

**Results**

**Hsp27 levels are moderately increased in breast cancer patients’ sera, and their tumor microenvironments have highly elevated soluble Hsp27 levels**

Serum levels of Hsp27 in untreated breast cancer patients (n = 32) with localized disease were compared with that of age-matched healthy volunteers (n = 26). We simultaneously tested for intracellular expression of Hsp27 and its release from primary human breast tumor cells and normal breast epithelial cells. Serum levels of Hsp27 were significantly (P < 0.001) higher in breast cancer patients (1,038.38 ± 135.37; n = 32) compared with healthy volunteers (256.29 ± 54.01; n = 26; Fig. 1A). Primary breast tumor cells expressed higher levels of intracellular Hsp27 compared with normal primary mammary epithelial cells isolated from healthy volunteers (Fig. 1B). Because of the difficulties in the availability of healthy volunteers for primary breast epithelial cells, we also tested intracellular Hsp27 expression in a normal HMEC and compared it with that of human breast tumor cell lines (MCF-7, ZR-75-1). Similar to primary cells, established breast cancer cell lines expressed higher levels of intracellular Hsp27 compared with that of human breast tumor cell lines (MCF-7, ZR-75-1). To test whether the tumor-released Hsp27 is phosphorylated, we concentrated (>50-fold) the tumor culture supernates and tested for phosphorylation at Ser82. Compared with rhHsp27, tumor-released Hsp27 was less phosphorylated (49% for the primary tumor, 26% for MCF-7, and 36% for ZR-75-1; Fig. 1D). We also compared Hsp27 levels released by patients’ tumor cells with that present in their serum. Tumor cell–released Hsp27 (24,220 ± 4,796 pg/10⁶ cells/mL; n = 7) were much...
higher \((P < 0.0001)\) than that simultaneously present in the same patients’ serum \((1,459 \pm 471 \text{ pg/mL}; n = 7)\). These data indicated markedly elevated levels of soluble Hsp27 in the HBTM. To confirm this possibility, we measured soluble Hsp27 levels in the interstitial fluid isolated from primary breast tumor and also from normal mammary tissue. Hsp27 levels in the tumor interstitial fluid \((2,615,428 \pm 566,442 \text{ pg/mL}; n = 7)\) was >2,500-fold higher than we detected in patients’ serum, >100-fold higher than patients’ breast tumor culture supernates, and >25-fold higher than the Hsp27 level detected in the normal breast tissue interstitial fluid \((103,600 \pm 35,702 \text{ pg/mL}; n = 3)\), indicating highly elevated levels of soluble Hsp27 in the HBTM.

Hsp27 induces both immunosuppressive and tumor growth–supporting soluble factors in human monocytes

The exposure of human monocytes to soluble Hsp27 induced very high levels of immunosuppressive cytokines, IL-10 and IL-6 (ref. 23; Fig. 2). In addition, Hsp27 induced high levels of prostaglandin \(E_2\) (PGE\(_2\)), a highly immunosuppressive and proangiogenic factor (Fig. 2; refs. 21, 24). Hsp27 also induced VEGF-A, IL-8, IL-1β, and TNF-α, all of which are highly proangiogenic (refs. 18, 20, 25, 26; Fig. 2). The addition of M-CSF, an important MAC-differentiating cytokine that is also essential for mammary tumor progression (27), did not alter the cytokine induction by Hsp27 (Fig. 2). Hsp27 also induced highly elevated levels of monocyte chemotactic protein-1 (MCP-1), the primary chemokine known to recruit monocytes to the breast tumor site (Fig. 2; refs. 28–30). In contrast to inducing several soluble factors that support tumor growth, Hsp27 failed to induce IL-12, the most important antiangiogenic cytokine that is efficient in inhibiting tumor progression (Fig. 2; ref. 31).

Hsp27 induces the differentiation of human monocytes to TAM-like MACs

We then assessed whether the exposure of circulating monocytes to Hsp27 would differentiate them to TAM-like MACs with immunosuppressive/tolerogenic phenotypes. A majority of the monocytes cultured for 3–4 days in either medium alone (adherence-stimulated) or in the presence of Hsp27 and/or M-CSF were differentiated to MACs, as evidenced by the simultaneous expression of CD14 and CD16 (Supplementary Fig. S1). Hsp27-differentiated MACs expressed reduced levels of MHC class II (e.g., HLA-DR) and costimulatory molecules (e.g., CD86) but highly elevated levels of coinhibitory molecules [e.g., PD-L1 (32, 33), ILT2, and ILT4 (refs. 34, 35; Fig. 3A)]. The reduced expression of MHC class II and costimulatory molecules concomitant to the increased expression of coinhibitory molecules indicate tolerogenic phenotypes in these MACs. Since mouse TAMs have been...
characterized as alternatively activated MACs (M2; refs. 17, 21, 25), we also tested for any increase in M2 markers, such as CD206 and CD163, in these MACs. Although Hsp27 alone did not induce any change in the expression of these molecules, the combination of Hsp27 and M-CSF substantially increased the expression of both molecules (Fig. 3A). We then assessed the presence of MACs in tissue sections of patients’ breast tumor and control human breast. The presence of MACs was minimal in the control normal breast tissue and several tumor and control human breast. The presence of MACs was increased. Hsp27 or M-CSF induced severe tolerance in autologous T cells, as indicated by the >95% inhibition of their proliferation as compared with the M-CSF group alone (Fig. 4B). The extent of inhibition of T-cell proliferation induced by tumor-culture supernatant–differentiated MACs was much greater than that induced by rhHsp27-differentiated MACs. The addition of Hsp27 antibody, but not of control IgG, partially abrogated the T-cell proliferation defect, and IL-2 almost completely restored T-cell proliferation.

**Hsp27-differentiated MACs are not cytotoxic but are highly proangiogenic**

As depicted in Figure 5A and B, Hsp27-differentiated MACs exhibited significantly reduced cytotoxic activity against SKBR3, a human breast cancer cell line, as compared with control MACs (differentiated by adherence alone; Fig. 5A and B). Cytotoxic potential was highly reduced in MACs differentiated by M-CSF, a known M2 MAC inducing cytokine (21). Therefore, the addition of Hsp27 to M-CSF did not further reduce cytotoxicity (Fig. 5A and B).

Since TAMs are extremely proangiogenic supporting neovascularization and enhancing tumor growth (17–20), we tested whether Hsp27-differentiated MAC-induced angiogenesis was increased. Hsp27 or M-CSF + Hsp27-differentiated MACs significantly induced angiogenesis (found by matrigel network formation assay; Fig. 5C and D). Since VEGF is a potent cytokine inducing angiogenesis, we assessed VEGF both at mRNA and protein levels. Hsp27 or M-CSF + Hsp27-differentiated MACs expressed higher levels of VEGF-A, as compared with control or M-CSF–differentiated MACs, respectively (Supplementary Fig. S2A). Similar results were observed at the protein level (Supplementary Fig. S2B).

**Discussion**

Although several immune escape mechanisms induced by tumor cells have been identified in murine mammary and other cancers, many of the mechanisms are not yet established in human breast cancer. The critical role of TAMs in malignant progression in mouse models of breast cancer have been well documented (17–20). The increased frequency of TAMs in breast cancer patients correlates to a negative clinical outcome (14, 15), indicating the possible link between human TAM development and breast tumor progression. Here, we report that human breast tumor cells release a small...
molecular weight protein, Hsp27, and its concentration in the HBTM is extremely high. Moreover, the exposure of human monocytes to Hsp27 induces several factors that can recruit monocytes to the tumor microenvironment, cause immune suppression, and/or support tumor growth. Finally, we demonstrate that Hsp27 can induce the differentiation of human circulating monocytes to TAM-like immunosuppressive, proangiogenic MACs that may support human tumor progression. Recently, Chalmin and colleagues reported that Hsp72 extracted from tumor-derived exosomes can also mediate the immunosuppressive functions of myeloid-derived suppressor cells (39).

The role of MCP-1 in TAM infiltration and breast tumor progression is well documented (28–30). Our data show a high level of MCP-1 induction by Hsp27, indicating that Hsp27 may be responsible for increased TAM recruitment in human...
breast cancer via MCP-1 production. Strong association of IL-10 expression with the loss of T-cell activation proteins is reported in breast cancer patients (17, 40). Increased IL-10 production concomitant with a lack of IL-12 induction is a hallmark of TAMs (19, 21). Interestingly, Hsp27 induced high levels of IL-10 and many other well-known immunosuppressive and tumor growth–promoting soluble factors but failed to induce IL-12, indicating the possible role of soluble Hsp27 in monocyte to TAM differentiation in human breast cancer.

Although TAMs have been well characterized in mice, their phenotypic and/or functional properties in human solid tumors are not clear. TAMs are predominantly of type II MACs (M2) that have immunosuppressive phenotypes and release cytokines that promote a Th2 response (17, 21, 25). Interestingly, Hsp27 induced high levels of IL-10 and many other well-known immunosuppressive and tumor growth–promoting soluble factors but failed to induce IL-12, indicating the possible role of soluble Hsp27 in monocyte to TAM differentiation in human breast cancer.

Role of Hsp27 in Human Breast Cancer

Figure 4. Hsp27-differentiated MACs induce anergy in T cells. A, C, and D, monocyte macrophages were cocultured (1:5 ratio) with autologous T cells in the presence of iCD3 for 6 days. T cells were re-isolated, rested for 24 hours, and tested for anergy by restimulation of the T cells (1 × 10^5/well) with iCD3 + sCD28 in the presence or absence of IL-2 (100 U/mL) for 4 days for assessing proliferation (n = 6; B, C; *, P < 0.05; ***, P < 0.0005, compared with adherence alone group; †, P < 0.05; ‡, P < 0.0005, compared with M-CSF group). B, monocytes were cultured in the presence of M-CSF alone or in the presence of ZR-75-1 culture supernate [added to monocyte culture at 20% (v/v) at a final concentration of 100 ng Hsp27/mL, as determined by ELISA]. The tumor culture supernate was also incubated for 1 hour with Hsp27 antibody or control IgG (each 5 µg/mL) before their addition to the monocyte culture. rhHsp27 (250 ng/mL) was also added to the M-CSF group to serve as positive control. Differentiated MACs were then cocultured with autologous T cells, and the proliferation of re-isolated T cells was assessed as described above. Cul Sup, culture supernate.

We report that both rhHsp27 and breast tumor cell–released extracellular Hsp27 differentiated human monocytes to tolerogenic MACs that induce T-cell anergy, a common dysfunction of TILs. Partial abrogation of T-cell tolerance by Hsp27 antibody strongly suggests the presence of one or more immunosuppressive factors other than Hsp27 in the tumor culture supernates. In addition to T-cell anergy, a shift from Th1 to Th2, particularly the presence of IL-13 (a Th2 cytokine) in the breast tumor microenvironment is reported to facilitate tumor development (41). IL-13–regulated M2 MACs can block immune surveillance against metastasis in a murine mammary tumor model (42). A recent study demonstrates the critical role of Th2 cells in mouse breast cancer progression (metastasis) that is mediated by the protumor properties of MACs (43). Our data showed a reduction of both Th1 (IFN-γ) and Th2 (IL-13) cytokine production by Hsp27-differentiated MACs. However, when the anergy was broken by the exogenous addition of IL-2, the restoration of IL-13 production was more predominant.
than that of IFN-γ. These data indicate that Hsp27-differentiated MACs may have induced the preferential differentiation of naive T cells to IL-13–producing T cells (Th2) prior to tolerance induction. Although a Th1–Th2 shift, as reported in breast cancer (44, 45), is not confirmed in our current experiments, they certainly indicate that Th1 cells are more affected than Th2 cells during a coculture of Hsp27 or M-CSF + Hsp27-differentiated MACs with autologous T cells.

Taken together, our data strongly suggest that Hsp27-mediated immune suppression is a novel immune escape and tumor growth–supporting mechanism that is likely to be operative in human breast cancer. Our discovery opens a new avenue for treating breast cancer patients by targeting extracellular (released) Hsp27.

Figure 5. The loss of antitumor cytotoxic activity but significant increase in proangiogenic activities of Hsp27–differentiated MACs. A and B, monocyte-derived MACs were stimulated with IFN-γ for 24 hours and tested for cytotoxic activity against CFSE-labeled breast cancer cells (SK-BR-3) at different effector to target ratios by coculturing them for 4 hours. The cells were then stained with 7-AAD to distinguish dead target cells from live target cells, fixed, and analyzed by flow cytometry. Spontaneous cell death was subtracted from each experimental data set. Cytotoxicity data are shown for 20:1 effector to target ratios (n = 5; *, P < 0.05; ***, P < 0.0005 as compared with adherence alone group; a — representative data; b — all data). C and D, MACs (2 × 10⁵) were tested for their ability to induce neovascularization by coculturing with 1 × 10⁶ HUVECs in a trans–well plate. The endothelial cells were then harvested and cultured onto the surface of the polymerized ECMatrix in 96-well plates for the network formation assay. Neovascularization in HUVECs was recorded with an inverted light microscope at 10× to 200× magnification as an arbitrary unit of pixels. C and D, representative and combined data, respectively (n = 6; *, P < 0.05; ***, P < 0.001 compared with HUVEC culture alone; ***, P < 0.001 compared with HUVEC + adherent MAC culture. $$$, P < 0.001 compared with HUVEC + M-CSF–treated MAC culture).

Disclosure of Potential Conflicts of Interest
The authors have no potential conflicts of interest.

Acknowledgments
We sincerely thank Ms. Loralee A. McMahon for technical help with immunohistochemistry experiments.

Grant Support
American Cancer Society Research Scholar Grant RSG-06-266-01-LIB awarded to A.K. De.

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Received May 18, 2010; revised October 14, 2010; accepted October 28, 2010; published OnlineFirst January 11, 2011.

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www.aacrjournals.org
Cancer Res; 71(2) January 15, 2011
OF9

Published OnlineFirst January 11, 2011; DOI: 10.1158/0008-5472.CAN-10-1778

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Cancer Res  Published OnlineFirst January 11, 2011.

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