Targeting tumor-associated macrophages in an orthotopic murine model of diffuse malignant mesothelioma

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Abstract

Tumors are a mixture of neoplastic and host stromal cells, which establish a microenvironment that contributes to tumor progression. In this study, the contribution of tumor-associated macrophages (TAMs) to tumor growth and metastasis was examined using an orthotopic, immuno-competent murine model of diffuse malignant peritoneal mesothelioma. The expression profile of cytokines and chemokines in solid tumors was consistent with a M2-polarized, TAM-mediated immunosuppressive microenvironment. TAMs were targeted using liposome-encapsulated clodronate (CLIP). Exposure of tumor spheroids to CM-DiI-labeled CLIP in situ confirms targeting of macrophages and not mesothelioma cells. Intraperitoneal (i.p.) delivery of CLIP produced apoptosis in tumor spheroids and solid tumors in contrast to delivery of liposome-encapsulated PBS or PBS. Mice received an i.p. injection of mesothelioma cells with CLIP delivered i.p. every 5 days. This treatment protocol produces a 4-fold reduction in the number of tumors, a 17-fold reduction in the relative tumor burden, and a 5-fold reduction in invasion and metastasis when compared with mice exposed to liposome-encapsulated PBS or PBS. Following transplantation of tumor spheroids and treatment with CLIP, mice showed a 4-fold reduction in the number of tumors and a 15-fold reduction in relative tumor burden. Mice bearing established tumors showed a 2-fold reduction in the number of tumors and relative tumor burden when exposed to half the previous dose of CLIP delivered by repeated i.p. injection. These reductions in tumor burden are statistically significant and identify TAMs as an important host-derived cell that contributes to growth, invasion, and metastasis in diffuse malignant peritoneal mesothelioma. [Mol Cancer Ther 2008;7(4):788–99]

Introduction

The interaction between tumor cells and host-derived cells establishes a microenvironment that promotes tumor progression (1). One of the most prominent immune cells identified in solid epithelial cancers is the macrophage (2). In >80% of epithelial cancers, increased macrophage density correlates with tumor stage and poorer prognosis (3, 4). This prognostic correlation has elicited investigation of the contribution of macrophages to tumor development and progression.

During inflammation, circulating monocytes are recruited to the site of inflammation where they adopt a macrophage phenotype dictated by the presence of specific cytokines and growth factors (5). Mature macrophages are divided into two populations, M1 or “classically activated” and M2 or “alternatively activated.” Recent studies indicate that tumor-associated macrophages (TAMs) show a M2 phenotype (6). The tumor environment is characterized by elevated IL-10, which stimulates alternatively activated macrophage differentiation. The profile of chemokines produced by TAMs recruits additional host cells that contribute to an immunosuppressive environment allowing tumor establishment and avoidance of immunodetection. Recruitment of T\textsubscript{H}2 lymphocytes by CCL17 and CCL22 inhibits T\textsubscript{H}1 cell function and the absence of CXCL9 and CXCL10, which recruit T\textsubscript{H}1 cells, is important to establishing an immunosuppressive environment (7).

In addition to their role in regulation of tumor immunity, TAMs are a source of angiogenic factors. In many cancers, there is a close association between TAM density, microvessel density, and expression of angiogenic factors (3, 8, 9). High levels of vascular endothelial growth factor in tumors are strongly linked to tumor size and density of macrophages (9–12). TAM-derived factors also stimulate tumor cell survival, invasion, and metastasis. The growth factors fibroblast growth factor and epidermal growth factor produced by TAMs stimulate tumor cell proliferation and survival in many cancers, including diffuse malignant mesothelioma (13).

Multiple factors recruit monocytes to the tumor providing a continuous source of TAMs. The macrophage chemotactic protein family of chemokines and macrophage colony-stimulating factor recruit monocytes to many tumor types (4, 5). The level of MCP-1 (CCL2) expression in tumors has been correlated with increased infiltration of macrophages and tumor grade (14–16). Both human and
murine malignant mesotheliomas show macrophages diffusely distributed throughout solid tumors (17–19), suggesting that macrophages contribute to tumor growth and progression and are a potential target for novel therapies (1, 4, 20, 21).

To determine if TAMs play a vital role in tumor growth and progression, the effect of macrophage depletion has been examined using a murine orthotopic transplanted tumor model. A chemical-based method has been developed to target macrophages based on their role as active phagocytes. I.p. administration of the amino-containing bisphosphonate clodronate encapsulated in a phospholipid liposome has been described (22, 23) and is used to produce prolonged depletion of macrophages (24) as well as to eliminate TAMs (25, 26). Liposomes are phagocytosed, and after internalization, the liposomes are disrupted within lysosomes releasing free clodronate into the cytoplasm. Intracellular clodronate is incorporated into a nonhydrolyzable analogue of ATP resulting in apoptosis (27–30).

This study examines the contribution of macrophages to growth and progression of murine peritoneal malignant mesothelioma with the goal of assessing whether TAMs are a potential therapeutic target. Solid tumors and tumor spheroids established in this model system were examined to confirm macrophage depletion and reduction in tumor growth, invasion, and metastasis following repeated i.p. injections of liposome-encapsulated clodronate (CLIP). Our findings also identify TAMs as a potential therapeutic target in diffuse peritoneal malignant mesothelioma.

Materials and Methods

Cell Culture

The 40L mesothelioma cell line was maintained at 37°C in DMEM with 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10 units/mL penicillin, 10 µg/mL streptomycin, 10 µg/mL gentamycin, and 10% fetal bovine serum in a humidified atmosphere of 94% air/6% CO2. Cultures were passaged by trypsinization and all experiments were done using cells that were <20 passages removed from the original explant.

Mice

C57BL/6 [wild-type (WT)] and C57Bl/6 TgN (bACT-eGFP) 10sb [enhanced green fluorescence protein (eGFP)] mice were purchased from The Jackson Laboratory and used between 2 and 12 months of age. Mice were housed in sterile HEPA-ventilated microisolators in an approved facility meeting all federal and state animal care guidelines.

Intraperitoneal Transplantation of Mesothelioma Cells

The malignant mesothelioma cell line used in this study, 40L, was derived from lung micrometastases developed in a C57Bl/6 mouse following subcutaneous (s.c.) injection of the primary, asbestos-induced mesothelioma cell line, 40, reported in Goodglick et al. (31). I.p. injection of 40L cells (2 × 10^5) into syngeneic mice reproducibly produces free floating spheroids by 14 days and solid tumor masses by 21 days. Mice were euthanized by CO2 asphyxiation to harvest spheroids and solid tumors. The peritoneum of eGFP mice was perfused with 4% paraformaldehyde for 1 h followed by 4% formalin, frozen into OCT blocks for cryosectioning and immunofluorescent labeling, or flash-frozen in liquid nitrogen for RNA isolation. All organs from the thorax and abdomen were removed including the reproductive tract, urinary bladder, intestines and mesentery, kidneys, spleen, pancreas, stomach, liver with gall bladder, diaphragm, heart, lungs, and mediastinum. Tissues fixed in 10% formalin were paraffin embedded, sectioned at 5 µm, and stained with H&E. Bright-field micrographs were taken using an Eclipse E800 with a Spot RT color camera and software. Total RNA was isolated from tumor tissue and spheroids with TRI-REAGENT (Molecular Research Center) according to the manufacturer’s protocol.

Reagents for Immunofluorescence and Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assays

Tissue sections or cells attached to glass coverslips were fixed in 4% paraformaldehyde, rehydrated in PBS, and blocked in 10% normal serum/0.1% bovine serum albumin/0.1% Tween 20. After incubation in primary antibody solution containing: rabbit anti-eGFP (Chemicon), rat anti-CD68, rat anti-F4/80 (ABD Serotec), or hamster anti-CD11c (BD PharMingen) samples were incubated in a secondary antibody solution containing Alexa 594–conjugated rabbit anti-rat, Alexa 488–conjugated donkey anti-rabbit IgG (Invitrogen), or FITC-conjugated goat anti-Armenian hamster IgG (eBioscience). Samples were mounted in Vectashield hardset containing 4',6-diamidino-2-phenylindole and fluorescent micrographs were taken using a Nikon Eclipse E800 microscope with a Spot RT color camera and software or an Axiovert 200M microscope with a coolscan HQ camera and Metamorph 6.0 software.

RT² Profiler PCR Arrays for Mouse Cytokines and Receptors

Total RNA (1 µg) from solid mesenteric tumors or mesothelioma cells grown in vitro was converted to cDNA and used to screen inflammatory cytokines and receptors using quantitative real-time PCR arrays according to the manufacturer’s instructions (SuperArray Bioscience). Reactions were cycled in an ABI Prism 7500 FAST sequence detector (Applied Biosystems) and acquired data were analyzed using the ΔΔCt method to determine the expression level of each transcript normalized to the expression level of housekeeping gene controls. A gene-wise, two-sample, t-test was done for each transcript to identify statistical differences in expression between solid tumors and the cell line in vitro.

Liposomes

CLIP was prepared by the laboratory of Dr. Nico van Rooijen as described previously (32) and diluted in sterile PBS for i.p. injection. Chloromethylbenzamido-octadecyl
(C_{18}) indocarbocyanine (CM-DiI; Invitrogen) was used according to the manufacturer’s recommendations to fluorescently label CLIP as well as liposome-encapsulated PBS (PLIP) and PBS-only control. Chromogenic Limulus assay of CLIP, PLIP, and PBS control preparations showed each to be essentially endotoxin-free (Cogen Laboratories).

**Targeting Macrophages Using CLIP Ex vivo**

WT or eGFP mice were injected i.p. with $2 \times 10^6$ mesothelioma cells and tumor spheroids were harvested after 25 days by peritoneal lavage. The spheroids were collected by centrifugation, transferred to glass coverslips, and incubated with CM-Dil-labeled CLIP, CM-Dil-labeled PLIP, or mock CM-Dil-labeled PBS for 24 h. The coverslips were then centrifuged at 2,000 rpm for 15 min in a Sorvall Legend Mach 1.6R centrifuge, washed with PBS, and fixed with 4% paraformaldehyde. Immunofluorescent labeling for CD68, CD11c, and GFP was used to identify macrophage, dendritic cells, and host cells, respectively. Digital photographs were taken using an Axiovert 200M microscope.

**Mesothelioma Cell Phagocytosis of Apoptotic Macrophages**

WT mice were injected i.p. with 4.05% thioglycollate, and on the fourth day, mice were euthanized and peritoneal lavage was done. Half of the peritoneal exudate cells were labeled with CM-Dil and both labeled and unlabeled cells were plated separately and incubated overnight. The liposome containing media were removed after 6 h and replaced with untreated media, and after 18 h, the conditioned medium from CLIP-exposed CM-DiI-labeled macrophages or unlabeled macrophages or PLIP-exposed CM-DiI-labeled macrophages was transferred to cultures of mesothelioma cells grown on glass coverslips. After a 24-h exposure, the coverslips were washed with PBS and fixed with 4% paraformaldehyde. Fluorescent micrographs were taken using a Nikon Eclipse E800 microscope.

**CLIP Treatment of Thioglycollate-Elicited Macrophages**

Peritoneal exudate cells were isolated by peritoneal lavage 4 days after injection with thioglycollate and 24 h after exposure to 50, 75, 100, or 125 µL CLIP. The number of leukocytes in each lavage sample was counted using a hemacytometer and stained with May Grünwald-Giemsa. Data are expressed as the mean ± SE calculated from at least five mice per CLIP dose. Significance was assessed using a Student’s two-tailed t-test to compare each dose to the PBS-injected control.

**CLIP Distribution In vivo**

WT mice were injected i.p. with $2 \times 10^6$ mesothelioma cells. After 21 to 24 days, mice were injected i.p. with 100 µL CM-Dil-labeled CLIP, CM-Dil-labeled PLIP, or mock CM-Dil-labeled PBS. Twenty-four hours later, mice were sacrificed, the peritoneal cavity was perfused with 4% paraformaldehyde for 1 h to preserve CM-DiI fluorescence, and the spleen, liver, intestines with mesentery, pancreas, kidneys, and lungs were frozen into OCT blocks. Following cryosectioning, tissues were counterstained with 4',6-diamidino-2-phenylindole and each organ was examined for CM-Dil labeling using fluorescence microscopy.

**Induction of Apoptosis by CLIP In vivo**

Spheroids were harvested by peritoneal lavage from animals treated with CM-Dil-labeled liposomes separated by centrifugation, frozen into OCT as a cell block, cryosectioned, and examined for apoptosis using the *in situ* cell death detection terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) kit (Roche Applied Science). To quantify TUNEL labeling, tumor-bearing mice were exposed to CLIP, PLIP, and PBS beginning 21 days following tumor cell injection with booster injections every 5 days until day 40. Pelvic mesenteric tumors and testes were examined for apoptosis using the TUNEL assay to determine the mean number of apoptotic cells counted in four random fields at ×200 magnification in solid tumor. Data were compiled according to treatment and are presented as the mean ± SE ($n \geq 4$). One ×100 field of testes was assessed for TUNEL-labeled cells per mouse ($n \geq 4$) and averaged by treatment.

For transmission electron microscopy, WT mice were injected i.p. with $2 \times 10^6$ mesothelioma cells and after 27 days were exposed to 200 µL CLIP, PLIP, or PBS by i.p. injection. Twenty-four hours later, mice were sacrificed, peritoneal lavage was collected, and tumor spheroids were isolated by centrifugation. Samples were fixed with 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) and postfixed with 1% osmium tetroxide. After rinsing with buffer, the specimens were dehydrated through a series of graded ethyl alcohols and infiltrated overnight with a 1:1 solution of 100% ethanol and Spurr embedding medium. Samples were infiltrated with fresh Spurr medium for 4 h, embedded in molds, and polymerized at 60°C. Blocks were sectioned at a thickness of 80 nm on a Reichert Ultramicrotome, placed on copper grids, stained with lead and uranyl acetate, and viewed on a Phillips 410 transmission electron microscope equipped with an Advantage HR CCD camera with Advanced Microscopy Techniques imaging software.

**CLIP Treatment In vivo**

Three *in vivo* models were used to assess the effect of CLIP on mesothelioma growth. First, WT mice were injected i.p. with 100 µL CLIP, PLIP, or PBS. The following day, $2 \times 10^6$ mesothelioma cells were transplanted orthotopically by i.p. injection. On every fifth day, mice received an additional injection of CLIP, PLIP, or PBS alone. Mice were sacrificed 28 to 30 days after injection of mesothelioma cells and the tissues were fixed, paraffin embedded, sectioned, and stained with H&E for histopathologic examination. Data were compiled according to mouse treatment and are presented as the mean ± SE ($n \geq 14$ mice per treatment group). Representative tissues were examined using an Axioplan microscope and the number and size of tumors and the presence and location of metastasis were recorded.

In a second experiment, mice were injected i.p. with $2 \times 10^6$ mesothelioma cells. After 25 days, animals were euthanized and a peritoneal lavage was done. Tumor spheroids were isolated by centrifugation, diluted 2-fold in PBS, and transplanted by i.p. injection into WT mice after...
mixing with 100 μL CLIP, PLIP, or PBS. On every fifth day, mice received an additional injection of CLIP, PLIP, or PBS. Twenty days after injection of tumor spheroids or 40 days after injection of mesothelioma cells, mice were sacrificed and examined as described above.

In a third experiment, mice were injected i.p. with 2 × 10⁶ mesothelioma cells. After 21 days, mice bearing solid tumors were injected i.p. with CLIP, PLIP, or PBS. Liposome dosage was reduced to half (50 μL) due to the greater sensitivity of tumor-bearing animals to repeated CLIP injections. On every fifth day, mice received an additional injection of CLIP, PLIP, or PBS. On the 40th day, mice were sacrificed and tissues were fixed and stained with H&E for histopathologic examination as described above; additionally, the testes and pelvic mesenteric tumors were analyzed using the TUNEL assay to identify apoptotic cells.

**Statistical Analysis**

All data, except assessment of metastasis and PCR arrays, were calculated as the mean ± SE for each treatment group. The number and area of tumors were determined on representative tissue sections from seven sites of the peritoneal organs and one site on the pleura. The areas of all tumors within each mouse were added to determine the total tumor burden. Statistical significance was assessed using a two-tailed, unpaired t-test to compare the differences between groups. The percentage of mice displaying extensive invasion and metastases was calculated for each treatment group. Statistical significance was assessed using a Fisher’s exact test. Differences with P < 0.05 were considered statistically significant.

**Results**

**TAM in Spheroids and Solid Tumors**

Spheroids and tumors established in eGFP-expressing mice contain host-derived stromal cells as identified by green eGFP fluorescence (Fig. 1A and B, left) in spheroids and solid tumor masses. Macrophages expressing F4/80 antigen are abundant within tumor spheroids and solid tumors (Fig. 1A and B, middle); abundant host stromal and immune cells are also evident on histologic sections (Fig. 1A and B, right).

A real-time PCR array was used to profile expression of cytokines, chemokines, and their receptors in the tumor microenvironment in comparison with mesothelioma cells grown in vitro (Fig. 1C). This cytokine profile is consistent with a M2 macrophage phenotype characteristic of TAMs. Dual-antigen immunofluorescent labeling for F4/80 and arginase 1 confirms a M2 macrophage phenotype in both transplanted solid tumors and tumor spheroids (data not shown). Expression of IL-10 is up-regulated 107-fold; this up-regulation is characteristic of a TAM-mediated immunosuppressive environment and results in decreased expression of TGFβ cell-recruiting chemokines, such as CXCL9 and CXCL10 (33–35). TAMs show elevated expression of CCL22 and CCL24 chemokines in addition to elevated expression of CXCL13 (36). In addition, the macrophage chemokine CCL12 is highly up-regulated in solid tumors.

The chemokine profile of transplanted malignant mesotheliomas and tumor spheroids (data not shown) is consistent with a tumor microenvironment characterized by the recruitment of TAMs and suppression of a Th1 immune response. We hypothesize that TAMs contribute to tumor growth and progression of murine malignant peritoneal mesothelioma.

**CLIP Targets Macrophages Ex vivo**

Spheroids exposed to CM-DiI-labeled liposomes ex vivo reveal a subpopulation of cells exhibiting both eGFP and CM-DiI fluorescence (Fig. 2A, left and middle, arrows), identifying host stromal cells that have phagocytosed CM-DiI-labeled liposomes. In spheroids exposed to CM-DiI-labeled PLIP, cells expressing eGFP are more numerous and show intense green fluorescence. The lower intensity of eGFP green fluorescence in spheroids exposed to CM-DiI-labeled CLIP is most likely due to reduced eGFP expression following intracellular accumulation of clodronate. Treatment with CM-DiI mock-labeled PBS shows no CM-DiI labeling and no reduction in eGFP fluorescence.

The majority of CM-DiI-labeled cells in explanted spheroids are macrophages (Fig. 2B, left and middle, arrows). CLIP has also been reported to target subsets of dendritic cells (26, 37). Only a few dendritic cells are identified in explanted tumor spheroids (arrows) and these cells did not show CM-DiI red fluorescence (Fig. 2C). The infrequent labeling of mesothelioma cells with CM-DiI (Fig. 2B, left) may be due to phagocytosis of apoptotic macrophages. To investigate this possibility, thioglycollate-elicited macrophages were labeled with CM-DiI and exposed to CLIP. After 6 h, the liposome-containing media were removed and replaced. The resulting conditioned media were transferred to cultures of mesothelioma cells. Exposure to the conditioned media of CLIP-treated macrophages produces CM-DiI labeling in mesothelioma cells (Fig. 2D, left). In contrast, no labeling is observed when macrophages were labeled with CM-DiI and exposed to PLIP or when unlabelled macrophages were exposed to CLIP (Fig. 2, middle and right).

**CLIP Reduces Recruitment of Thioglycollate-Elicted Macrophages**

Injection of thioglycollate broth stimulates monocyte recruitment to the peritoneal cavity (38, 39). Cells recovered by peritoneal lavage 24 h following injection of CLIP show characteristic apoptotic nuclear morphology (Fig. 3A, left, arrows) and an increase in neutrophils. In contrast, cells recovered following treatment with PBS alone show the characteristic morphology of elicited macrophages (Fig. 3A, middle). All doses of CLIP greater than 50 μL produce a statistically significant reduction in the number of cells recovered by peritoneal lavage compared with injection of PBS alone (Fig. 3A, right). A dose of 100 μL was selected because it produced a 2-fold reduction in elicited cells, similar to the percentage of macrophages induced to undergo apoptosis in vitro, in the absence of systemic toxicity.

**CLIP Distribution In vivo**

I.p. injection of CLIP has been shown to distribute to the draining lymph nodes, spleen, and liver (40). Mice exposed...
to CM-DiI-labeled CLIP show extensive labeling in the marginal zone of the spleen with scattered labeling of individual cells in the white pulp (Fig. 3B, left). Histopathology shows extensive apoptosis (white arrows) and focal congestion (black arrows) in the red pulp of spleen (Fig. 3B, right). The liver shows uptake by Kupffer cells and focal liver cell injury (Fig. 3C). Solid tumors show extensive labeling at the periphery following exposure to either CM-DiI-labeled CLIP or CM-DiI-labeled PLIP (Fig. 3C and D) and CLIP decreases tumor cell density and increases apoptosis within mesenteric tumors (Fig. 3D, right, black arrows).

**CLIP Induces Apoptosis In vivo**

TUNEL assay shows that exposure to CLIP induces apoptosis in a fraction of the cells within tumor spheroids (Fig. 4A, left, arrows), whereas PLIP and PBS do not (Fig. 4A, middle and right). This assay was confirmed using transmission electron microscopy to identify apoptotic cells induced by injection of CLIP in vivo. Figure 4B (left, A) identifies an apoptotic cell within a tumor spheroid.

### Figure 1.

A, solid tumors formed in eGFP mice contain host-derived stromal cells (left, green fluorescence) including macrophages that express F4/80 (middle, red fluorescence). H&E stain confirms the presence of infiltrating stromal and immune cells (right, arrows). B, tumor spheroids show host stromal and immune cells (left, green fluorescence) and TAMs (middle, red fluorescence). May Grünwald-Giemsa stain identifies host immune cells (right, arrows). Fluorescent micrographs are counterstained with 4',6-diamidino-2-phenylindole. C, cytokine and chemokine expression profile of transplanted murine malignant mesothelioma solid tumors was determined by real-time PCR array. *, P > 0.06 (not significant); **, P < 0.05; ***, P < 0.005.
adjacent to a mesothelioma cell that has phagocytosed an apoptotic body (arrow). Exposure to PLIP produced vacuolated but viable macrophages (Fig. 4B, middle, V) and exposure to PBS showed no evidence of cell death within tumor spheroids (Fig. 4B, right, M). The mean number of apoptotic cells was determined from pelvic mesenteric tumors and testes following a TUNEL assay (Fig. 4C). These results show a statistically significant 2.5-fold increase in apoptosis.

Figure 2. CLIP targets macrophages ex vivo. A, tumor spheroids isolated from eGFP mice exposed to CM-DiI-labeled liposomes or PBS ex vivo for 24 h. Stromal cells express eGFP (green fluorescence) and cells have endocytosed liposomes (red fluorescence). Yellow or orange fluorescence, colocalization of lipid uptake in host stromal cells (arrows). B, tumor spheroids from a C57Bl/6 mouse exposed to CM-DiI-labeled liposomes ex vivo and labeled for expression of CD68 (green fluorescence). Yellow or orange fluorescence, colocalization of CM-DiI and CD68 (arrows). C, spheroids exposed as described in B labeled for CD11c (green fluorescence, arrows). D, CM-DiI-labeled or unlabeled macrophages were treated with CLIP, PLIP, and PBS for 6 h and the conditioned medium was transferred to mesothelial cells for 24 h. Mesothelioma cells incubated in conditioned medium from CM-DiI-labeled CLIP-treated macrophages (left, arrows); mesothelioma cells incubated in conditioned medium from CM-DiI-labeled, PLIP-treated macrophages (middle) or unlabeled, CLIP-treated macrophages (right). All cells were counterstained with 4',6-diamidino-2-phenylindole (blue nuclear fluorescence).
following CLIP exposure. In comparison, the testes of these animals show a low number of apoptotic cells that did not significantly change with these treatment protocols. These data confirm that i.p. injection of CLIP induces apoptosis in both tumor spheroids and in established solid tumors \textit{in vivo}.

**CLIP Reduces Tumor Growth, Invasion, and Metastasis**

To explore the role of TAMs in tumor growth and progression, mice were injected i.p. with mesothelioma cells and treated with CLIP, PLIP, or PBS. Mice treated with CLIP had small residual tumors infiltrating into the skeletal muscle of the diaphragm and on the surface of the mesenteries (Fig. 5A and B, left). In contrast, >90% of mice injected with PLIP or PBS had extensive tumor growth and invasion on the diaphragm (Fig. 5A, middle and right).

These mice had large tumor masses invading the pancreas and intestinal mesentery (Fig. 5B, middle and right). Mice injected with CLIP showed a 4-fold reduction in tumors with a mean of only 12.3 tumors compared with 48.6 tumors following injection of PLIP and 57.3 tumors following injection of PBS alone (Fig. 5C, left). In addition to a lower tumor number, treatment with CLIP resulted in a 17-fold reduction in tumor burden (Fig. 5C, right).

In this orthotopic, syngeneic model of murine malignant peritoneal mesothelioma, 58% to 69% of mice treated with PLIP or PBS developed lung or pleural metastases 30 days following injection of mesothelioma cells. In contrast, only 1 of 13 mice showed a single lung metastasis (Fig. 5D, top, left, arrow) following CLIP therapy. A statistically significant 5-fold reduction in metastases and invasion in the liver

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**Figure 3.** CLIP targets macrophages \textit{in vivo}. A, nuclear morphology of apoptotic macrophage (left) and normal macrophages (middle). CLIP exposure produces a dose-dependent reduction in leukocyte recruitment to thioglycollate (right). Bar, 25 \textmu m. *, $P < 0.01$; **, $P < 0.001$. Mice with established tumors exposed to CM-DiI-labeled CLIP, CM-DiI-labeled PLIP, or mock CM-DiI-labeled PBS to identify sites of liposome uptake (red fluorescence) in (B) the marginal zone of the spleen, (C) Kupffer cells in the liver sinusoids, and (D) the periphery of a solid tumor.
as well as in the lungs was produced by this targeted treatment protocol (Fig. 5D, bottom).

To determine if CLIP can prevent tumor progression when therapy was initiated at more advanced stages, mice were injected i.p. with CLIP, PLIP, or PBS-treated tumor spheroids. Tumor growth is inhibited by treatment with CLIP (Fig. 6A and B, left) with two mice showing a complete absence of tumors at necropsy. No lung metastases were observed because mice were sacrificed after only 20 days. The average number of tumors per mouse is 13.1 following treatment with CLIP and is a 4-fold reduction from 54.4 and 55.8 tumors per mouse following treatment with PLIP or PBS, respectively (Fig. 6C, left). Superficial tumors on the mesentery show extensive necrosis and apoptosis following CLIP treatment, whereas tumors from mice treated with PLIP or PBS form large, infiltrating masses (Fig. 6B, middle and right). Mice injected with tumor spheroids and treated with CLIP had an average tumor burden of 5.3 mm² showing a 15-fold reduction when compared with mice treated with PLIP (157 mm²) or PBS (82 mm²; Fig. 6C, right).

To see if CLIP would be effective in treating established solid tumors, mice were injected with mesothelioma cells and, following tumor establishment, were treated with CLIP, PLIP, or PBS. Tumor growth and invasion into the diaphragm is observed in all treatments (Fig. 6D, top, T).

**Figure 4.** CLIP induces apoptosis in vivo. **A.** TUNEL labeling in CM-DiI-labeled CLIP (left, green fluorescence, arrows), CM-DiI-labeled PLIP (middle), and PBS-exposed tumor spheroids (right). **B.** Transmission electron micrographs of tumor spheroids from mice treated with CLIP (left, A), PLIP (middle, V), or PBS (right, M). **C.** Frequency of apoptosis in pelvic mesentery tumors and testes as determined by TUNEL labeling. *, P < 0.01; **, P < 0.006.
Figure 5. CLIP-induced reduction of tumor growth, invasion, and metastasis following injection of mesothelioma cells. Tumor (T) growth and invasion in the diaphragm (A) and mesentery (B; arrow). C, tumor frequency (left) and tumor burden (right) in mice treated with CLIP, PLIP, and PBS (n ≥ 14). D, metastases in the lung (top). Percent of animals showing tumor invasion and metastasis in the lung and liver (bottom). *, P < 0.02; **, P < 0.009; ***, P < 0.0007.

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Figure 6. CLIP-induced reduction in tumor growth in mice bearing tumor spheroids or established tumors. Tumors invading the diaphragm (A) and intestinal mesentery, hilum of the liver, and pelvic mesentery, respectively (B). C, tumor frequency (n = 9; left) and tumor burden (n ≥ 6; right) of spheroid-injected mice. *, P = 0.004; **, P < 0.0007; ***, P < 0.00000003. D, top, tumor burden in the diaphragm of mice bearing established tumors following treatment with CLIP (left), PLIP (middle), or PBS (right). Bottom, tumor frequency (left) and burden (right) of mice bearing established tumors (n ≥ 5). *, P = 0.06 (not significant); **, P < 0.009; ***, P < 0.0004.
Mice injected with CLIP have a mean of 32.8 tumors compared with 68.8 tumors following PLIP and 79.1 tumors following PBS exposure (Fig. 6D, bottom, left). The >2-fold reduction in number of tumors produced by injection of CLIP is significant. Mice treated with CLIP have an average tumor burden of 44.4 mm² after day 40. This is a statistically significant 2-fold reduction when compared with mice treated with PLIP (98 mm²) but not PBS (95.4 mm²; Fig. 6D, bottom, right). CLIP treatment of mice bearing solid tumors does not reduce lung metastases or prolong survival (data not shown).

**Discussion**

The specific contribution of macrophages to the initiation and progression of diffuse malignant mesothelioma has not been evaluated. In this transplanted murine malignant peritoneal mesothelioma model, the presence of macrophages in spheroids suggests that they may contribute to tumor cell survival and proliferation under anchorage-independent conditions. In the natural history and progression of malignant mesothelioma, tumor spheroids implant on the serosal lining facilitating dissemination and growth in the pleural and peritoneal cavities. The widespread distribution of macrophages within solid tumors is consistent with their role in contributing to the tumor microenvironment via immunosuppression (7), angiogenesis, or as a source of proinflammatory cytokines and proteases (41) that promote tumor invasion and metastasis (42).

The expression profile of chemokines and cytokines in solid tumors using real-time PCR arrays suggest that TAMs in transplanted mesotheliomas display a M2 phenotype. Elevated IL10 and IL-10RA expression was detected as well as expression of CXCL13, CCL22, and CCL24 and their respective receptors. When these results are considered in conjunction with the high level of transforming growth factor-β expression seen in these and other murine malignant mesotheliomas (17) using microarray analysis (data not shown), this tumor microenvironment would be predicted to suppress T lymphocyte-mediated Th1 responses (36).

CLIP was used to eliminate macrophages from the peritoneal cavity following tumor transplantation in a murine syngeneic, orthotopic model system. **In situ** studies examining spheroids confirmed that CLIP targets and induces apoptosis in macrophages but not mesothelioma cells. Additionally, immunofluorescence shows that phagocytic dendritic cells are not targeted by CLIP treatment **ex vivo**. The TUNEL assay and transmission electron microscopy also shows that i.p. injection of CLIP induces apoptosis in tumor spheroids and solid tumors **in vivo**.

Injection of mice with tumor cells or tumor spheroids followed by repeated injections of CLIP produced a significant reduction in tumor growth and invasion. Treatment of mice bearing established tumors also shows a significant reduction in tumor growth but no inhibition of invasion or metastasis. These results are consistent with previous studies assessing the role of macrophages in tumor development and progression. Transgenic mice that lack expression of CSF-1 and possess a predisposition to develop breast cancer show a delay in development of invasive and metastatic tumors when compared with WT mice (43). CSF-1 antisense RNA reduced growth of embryonic carcinoma and colon carcinoma xenografts accompanied by reduced expression of angiogenic factors and inhibition of tumor vascularity (44). Reduced tumor growth and vascularity was also reported following treatment of s.c. murine models of teratocarcinoma and rhabdomyosarcoma with CLIP (26). Finally, these results show that macrophages contribute to tumor progression in immunocompetent animals as well as immunodeficient mice bearing ovarian cancer xenografts (45).

Both this and previous studies suggest that TAMs participate in tumor growth and angiogenesis via secretation of growth factors; however, additional mechanisms could explain the reduction in tumor growth reported in this study. Targeting TAMs using CLIP may modulate the immunosuppressive tumor microenvironment by allowing dendritic cells and naïve T lymphocytes to mature stimulating an adaptive immune reaction that would lead to tumor rejection. Alternatively, exposure to CLIP might indirectly induce apoptosis in mesothelioma cells via uptake of macrophage-derived apoptotic bodies containing clodronate.

In summary, we have shown that targeting peritoneal macrophages compromises the ability of mesothelioma cells to survive as single cells or as free-floating multicellular spheroids, to establish transplanted solid tumors, to invade locally, and to metastasize. This novel therapeutic approach may warrant clinical trials in selected patients who are unable to tolerate currently used multimodality therapies (46).

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**References**


Targeting tumor-associated macrophages in an orthotopic murine model of diffuse malignant mesothelioma


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