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Anti-Human CD73 Monoclonal Antibody Inhibits Metastasis Formation in Human Breast Cancer by Inducing Clustering and Internalization of CD73 Expressed on the Surface of Cancer Cells

Mikkel G. Terp,*† Kristina A. Olesen,* Eva C. Arnspang,†‡ Rikke R. Lund,* B. Christoffer Lagerholm,†‡ Henrik J. Ditzel,*†§ and Rikke Leth-Larsen*

Recent studies have shown that Abs that target the cell-surface enzyme CD73 (ecto-5′-nucleotidase) reduce growth of primary tumors and metastasis in syngeneic mice by inhibiting the catalytic activity of CD73, and thus increasing the activity of cytotoxic T lymphocytes. In this article, we report another anticancer mechanism of anti-CD73 Abs and show that an anti-CD73 mAb (AD2) inhibits metastasis formation by a mechanism independent of CD73 catalytic activity and inhibition of primary tumor growth. This mechanism involves clustering and internalization of CD73, but does not require cross-linking of CD73, because both whole IgG anti-CD73 AD2 mAb and Fab′ fragments thereof exhibited this effect. Ex vivo treatment of different breast cancer cell lines with anti-CD73 AD2 mAb before i.v. injection into mice inhibited extravasation/colonization of circulating tumor cells and significantly reduced metastasis development. This effect was also observed when the cancer cell-surface expression of CD73 was significantly reduced by small interfering RNA knockdown. The antimitastatic activity is epitope specific, as another Ab that efficiently binds CD73-expressing live cancer cells did not lead to CD73 internalization and metastasis inhibition. Furthermore, anti-CD73 AD2 mAb inhibited development of metastasis in a spontaneous animal model of human metastatic breast cancer. Our study shows that some anti-CD73 mAbs cause cell-surface clustering of CD73 followed by internalization, thus inhibiting the ability of circulating tumor cells to extravasate and colonize, leading to inhibition of metastasis. Ab-based CD73 cancer therapy should include a combination of Abs that target the catalytic activity of CD73, as well as those with the characteristics described in this article. The Journal of Immunology, 2013, 191: 000–000.

Membrane proteins is CD73 (ecto-5′-nucleotidase), a 70-kDa GPI-anchored protein. In a global proteomic analysis of isogenic metastatic and nonmetastatic breast cancer cell lines, we showed that CD73 was more highly expressed in metastasizing versus nonmetastasizing cancer cells, and analysis of clinical breast cancer biopsies demonstrated a correlation between CD73 expression in primary tumors and risk for distant metastasis (2, 3).

CD73 is ubiquitously expressed in most normal tissues, including endothelial and subsets of hematopoietic cells (4); however, increased levels are present in a number of cancers (5). CD73 has been proposed to have several functions, including cell adhesion and enzymatic conversion of AMP to adenosine and inorganic phosphate (6–10). It was recently shown in a murine syngenic breast cancer model that inhibition of adenosine production using anti-murine CD73 mAb or α, β-methylene adenosine-5′-diphosphate (APCP) decreased tumor growth. Adenosine is known to inhibit the effect of CD8+ T cell–mediated response; thus, the mechanism behind the tumor growth inhibition was induction of such CD8+ T cell–mediated antitumor responses in the absence of adenosine (5, 8). Inhibiting adenosine production also inhibited metastasis development by decreasing migration of cancer cells (5). In addition, studies in CD73 knockout mice showed that lack of host-derived CD73 significantly inhibited both tumor growth and metastasis formation, indicating that not only CD73 expressed on cancer cells, but also CD73 expressed by adjacent normal tissues plays a critical role in the cancer process (7, 11). Treatment of cancer cells with APCP or RNA interference silencing of CD73 decreased adhesion to extracellular matrix proteins, laminin and fibronectin (12). Interestingly, CD73 RNA interference was shown to inhibit adhesion and invasion
of cancer cells more potently than APCP, suggesting that the capacity of CD73 to promote adhesion and invasion may relate to other mechanisms in addition to its catalytic activity (12).

Although most studies have analyzed murine CD73 in syngenic mouse models, in this study, we examined the effect of anti-human CD73 Abs in a human breast cancer xenograft model. Using advanced live animal bioimaging, we identified an anti-human CD73 Ab that significantly decreased spontaneous metastasis formation independent of CD73 catalytic activity. The anti-CD73 mAb (AD2) that exhibits this novel mechanism of action alters the normal distribution of CD73 from being evenly expressed in the membrane to forming clusters followed by internalization. Blocking cell-surface–expressed CD73 reduced the ability of cancer cells to extravasate from the circulation and colonize at distant sites.

Materials and Methods

Cell lines, culture, and transfection

The MDA-MB-231 and M4A4 LM3-2 cell lines (American Type Culture Collection) were transfected with luciferase 2 (LUC2)–expressing lentiviral particles (In Vivo Imaging Solutions) using a multiplicity of infection virus particle concentration of 15 according to the manufacturer’s guidelines, generating stably luciferase-expressing MDA-MB-231 LUC2 (MDA-MB-231) and M4A4 LM3-2 LUC2 (LM3) cell lines. The two cell lines were maintained in DMEM (Life Technologies, Invitrogen) supplemented with 10% FBS (Life Technologies), 5% penicillin/streptomycin (Life Technologies), and 10% heat-inactivated horse serum. Culures grew in humidified atmosphere of 5% CO2 at 37°C. All cell lines were routinely checked for Mycoplasma infection and morphology changes, and propagated no longer than 3 mo after resuscitation.

Abs

The mouse anti-human CD73 AD2 mAb (a gift from Prof. L. Thompson, Oklahoma Medical Research Foundation, Oklahoma City, OK) was produced at the Immunology Core Facility at the University of North Carolina, Chapel Hill. The mouse anti-human CD73 IE9 mAb was purchased from Santa Cruz Biotechnologies. Both mAbs were generated by immunization with full-length CD73 purified from human placenta (4). The isotype-matched control mAb was an IgG1κ α-Dilipophilic toxoid mAb (HYP 123-05; Bioporte). Alexa Flour 555 labeling was performed using Alexa Fluor 555 labeling Kit according to the manufacturer’s guidelines (Invitrogen). The anti-Cd4 Ab (AF2655) used for immunohistochemical analysis was purchased from R&D Systems.

Generation of Fab′ fragments of anti-CD73 AD2 mAbs

To generate Fab′ fragments, we initially diazylated anti-CD73 AD2 mAb against 100 mM acetic acid buffer, 1 mM EDTA, pH 4.5, and incubated them for 72 h with pepsin (5 μg/mg Ab; Sigma-Aldrich). The generated Fab′ fragments were separated by size exclusion chromatography using 100 mM acetic acid buffer, 1 mM EDTA, and 50 mM cysrate pH 5.5 on a Superdex 200 HiLoad 16/60 column (GE Healthcare) and incubated for 24 h with papain (10 μg/mg Ab; Sigma-Aldrich). The generated Fab′ fragments were separated by size exclusion chromatography using PBS, pH 7.4 on a Superdex 200 HiLoad 16/60 column (GE Healthcare).

CD73 knockdown

Human CD73 small interfering RNA (siRNA; #S9735 and #S9436; Life Technologies) and scrambled control siRNA (Qiagen, Denmark) was transfected into MDA-MB-231 LUC2 cells either in combination or as single siRNAs (300 nM) in 100 μl nucleofector solution V (Cell Line Nucleofector Kit V; Amaxa) using an Amaxa Nucleofector II according to the manufacturer’s guidelines (Lonza Europe). Knockdown was evaluated using flow cytometry as described later. Injection of the cancer cells in the tail vein was performed 96 h after transfection.

In vivo studies of anti-CD73 AD2 mAb treatment in a spontaneous metastasis xenograft model

Subcutaneous cells in culture were washed in PBS and harvested by a cell scraper (Almecco). Cells (1 × 10^6) were resuspended in a 1:1 mixture of extracellular matrix from the Engelbreth-Holm-Swarm sarcoma (Sigma-Aldrich) and DMEM before inoculation. Orthotopic transplantation of cancer cells was performed by injection into a surgically exposed mammary gland of anesthetized 8-wk-old female CB-17 SCID mice (Taconic). Anti-CD73 AD2 mAb (5 mg/kg) and control mAb (5 mg/kg) were injected i.p. 4 d after cancer cell transplantation into groups of five animals and subsequently biweekly for 5 wk. Primary tumors were removed from anesthetized mice using blunt instrument dissection. All animal experiments were approved by The Experimental Animal Committee, The Danish Ministry of Justice and were performed at the animal facility core at University of Southern Denmark. The mice were housed under specific pathogen-free conditions with ad libitum food and drinking water. The mice were euthanized if they showed any adverse signs or symptoms of disease including weight loss, paralysis, or general discomfort.

Ex vivo pretreatment of cancer cells with anti-human CD73 mAb

Cells were harvested by scraping, then were washed and incubated with anti-CD73 AD2 mAb (10 μg/ml), anti-CD73 IE9 mAb (10 μg/ml), or control mAb (10 μg/ml) at 37°C for 2 h. For flow cytometry analysis of CD73 expression, the cells were washed three times in ice-cold PBS and subsequently stained with Alexa Flour 647 goat anti-mouse Ab at 4°C or incubated with a second round of primary Ab at 4°C for 30 min before staining with Alexa Flour 647 goat anti-mouse Ab. Analysis of the re-expression of CD73 was performed by AD2 incubation at 37°C followed by washing three times and subsequent 3-h incubation in complete medium without AD2 at 37°C. Thereafter, the cells were washed three times and then incubated with a second round of AD2 Ab at 4°C for 30 min before Alexa Flour 647 goat anti-mouse Ab staining.

Data acquisition and analysis was performed as described in the Flow Cytometry section.

Before tail vein injections, the cells were resuspended in PBS at a concentration of 5 × 10^6/ml and 0.2 ml was injected into the tail vein of 6- to 8-wk-old female CB-17 SCID mice.

In vivo imaging

Relative quantification of tumor burden and metastasis development was performed weekly using whole-body bioluminescent imaging (in vivo imaging system [IVIS] spectrum; Caliper Life Science). Mice were injected with α-luciferin (150 mg/kg body weight) and then anesthetized with isoflurane gas. Images were acquired starting 10 min after luciferin injection. Regions of interest (ROI) were drawn around the mammary fat pad tumor. Further, ROIs were drawn encircling each of the two lungs to quantify metastases. The photon emission transmitted from the ROIs was quantified in photons/s/cm^2/5sr using Caliper Life Science Living image (version 4.0.0). The animals were imaged as above except for an intraperitoneal injection to image excised organs, and organs and were imaged 2 min later.

Flow cytometry

Subconfluent cells in culture were washed in PBS, harvested by scraping, and washed (300 × g, 5 min, 4°C) in DMEM supplemented with Complete Mini Protease Inhibitor mixture (complete DMEM; Roche Diagnostics). PBMCs were isolated using standard Ficoll-Faque according to manufacturer’s protocol (GE Healthcare). Cells (1 × 10^6) were incubated with 10 μg/ml anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or control mAb for 30 min at 4°C, washed twice in complete DMEM, and incubated with an Alexa Flour 647 goat anti-mouse Ab (Invitrogen) for 60 min at 4°C. The cells were then washed, resuspended in PBS, and analyzed on a FACsCalibur (Becton Dickinson). The collected data were analyzed using FlowJo software (Tree Star).

Inhibition of catalytic activity

Cells (5 × 10^6) were resuspended in 100 μl glycine buffer (75 mM glycine, 5 mM MgSO4, pH 7.4) containing various concentrations of anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, control mAb, or 0.1 mM APCP (Sigma Aldrich). Cells treated with Ab or APCP were analyzed with and without 0.2 mM AMP and incubated for 3 h at 37°C. When analyzing the catalytic activity of cancer cells in the setup resembling the tail vein injection experiments, cancer cells were incubated with anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or control mAb for 2 h, washed three times in TBS, resuspended in glycine buffer with and without 0.2 mM AMP, and incubated for 1 h at 37°C. After incubation with AMP, 50 μl of 0.5 M H2SO4 was added to each well, followed by 50 μl phosphate reaction solution (0.4% NH4-molybdate, 10% ascorbic acid), and incubated for 30 min at room temperature while shaking to measure the generated inorganic phosphate, that is, the conversion of AMP to adenosine. The color-reaction product (molybdenum blue) was colorimetrically measured at 560 nm using a Victor3 Multilabel Plate Reader (PerkinElmer Life Sciences). The catalytic activity was determined by subtracting the values in the absence versus the presence of AMP.
Anchor-independent growth

Soft agar assay was performed as previously described by Ke et al. (13) with minor modifications. In brief, a prelayer of 25 μl prewarmed 2% IMDM (Invitrogen) and 25 μl 1.2% agar (Sigma) was plated into 96-well microplates. MDA-MB-231 cells (5 × 10^3) were seeded on top of the prelayer in 20 μl 2% IMDM and 30 μl 0.8% agar containing anti-CD73, AD2 mAb or 10 μg/ml control mAb. Finally, a semisolid feeder top layer of 25 μl 2% IMDM and 25 μl 0.2% agar were seeded on top of the cell-containing layer. The cells were incubated in a humidified atmosphere of 5% CO₂ and 37˚C for 12 d. Anchored independent growth was quantified by bioluminescence using a Victor 3 Multilabel Plate Reader.

Cell migration assay

Migration assays were performed as previously described with minor modifications (14). After MDA-MB-231 cells (5 × 10^3) were seeded in six-well plates, a scratch was performed using a pipette tip. After washing for four times in PBS, 3 ml serum-free DMEM containing 10 μg/ml anti-CD73 AD2 mAb or control mAb was added. Snapshots of migrating cells were taken after 3 and 48 h using a Nikon Coolpix P5100 camera mounted on a Leica DMIL microscope and converted into segmented dark-field images in Photoshop. The comparison of migration after Ab treatment was performed by quantifying the segmented dark-field images using ImageJ. Each scratch was normalized to the quantification of the starting point 3 h postscraping.

Immunofluorescence microscopy

Cells (1 × 10^4) were seeded in 12-well plates on coverslips and, 24 h postseeding, incubated with anti-CD73 AD2 mAb (IgG or Fab', 10 μg/ml) or anti-CD73 IE9 mAb (10 μg/ml) for 2 h at 37˚C. After incubation, the cells were counterstained for 10 min with 5 μg/ml Alexa 633–conjugated wheat germ agglutinin (Invitrogen), washed in PBS, and fixed for 15 min with 4% formaldehyde. After permeabilization using 0.05% Triton-X 100 wheat germ agglutinin (Invitrogen), washed in PBS, and fixed for 15 min with 4% formaldehyde, the cells were blocked with 1% BSA (Sigma-Aldrich) with 4% formaldehyde. After permeabilization using 0.05% Triton-X 100 antigen retrieval, washed in PBS, and fixed for 15 min with 4% formaldehyde, cells were washed, incubated with Alexa Fluor 555–conjugated anti-CD73 mAb (Invitrogen) and 25 μg/ml control mAb. Finally, a semisolid feeder top layer of 25 μl 2% IMDM and 25 μl 0.2% agar were seeded on top of the cell-containing layer. The cells were incubated in a humidified atmosphere of 5% CO₂ and 37˚C for 12 d. Anchored independent growth was quantified by bioluminescence using a Victor 3 Multilabel Plate Reader.

Live cell fluorescence microscopy

Cells (1 × 10^4) were seeded in 12-well plates on coverslips and, 24 h postseeding, incubated with anti-CD73 AD2 mAb (IgG or Fab', 10 μg/ml) or anti-CD73 IE9 mAb (10 μg/ml) for 2 h at 37˚C. After incubation, the cells were counterstained for 10 min with 5 μg/ml Alexa 633–conjugated wheat germ agglutinin (Invitrogen), washed in PBS, and fixed for 15 min with 4% formaldehyde. After permeabilization using 0.05% Triton-X 100 antigen retrieval, washed in PBS, and fixed for 15 min with 4% formaldehyde, cells were washed, incubated with Alexa Fluor 555–conjugated anti-CD73 mAb (Invitrogen) for 30 min at 4˚C. The coverslips were mounted on glass slides in antifade before staining with Alexa Fluor 488–conjugated anti-mouse Ab (Invitrogen) (Bio-Rad Laboratories), the cells were blocked with 1% BSA (Sigma-Aldrich) with 4% formaldehyde. After permeabilization using 0.05% Triton-X 100 antigen retrieval, washed in PBS, and fixed for 15 min with 4% formaldehyde, cells were washed, incubated with Alexa Fluor 555–conjugated anti-CD73 mAb (Invitrogen) and 25 μg/ml control mAb. Finally, a semisolid feeder top layer of 25 μl 2% IMDM and 25 μl 0.2% agar were seeded on top of the cell-containing layer. The cells were incubated in a humidified atmosphere of 5% CO₂ and 37˚C for 12 d. Anchored independent growth was quantified by bioluminescence using a Victor 3 Multilabel Plate Reader.

Immunohistochemistry

Generation of tissue arrays of cell line–derived tumors and staining procedures were performed as previously described (2, 15).

Proliferation assay

Cells were incubated with anti-CD73 AD2 mAb (0.01–10 μg/ml) or control mAb (0.01–10 μg/ml) for 10 min and washed again before monitoring with spinning disc microscopy performed with a Nikon Eclipse Ti (Nikon Industries) microscope equipped with a Yokogawa CSU-X1 spinning disc unit (Yokogawa Electric Corporation), Andor Laser launcher, and an Andor EMCCD (Andor Technology) for detection. Imaging was performed using the 561 laser line for excitation and a 593/40 emission filter. The integration time was 40 ms, and a 60× oil objective was used.

Statistics

The statistical significance of bioluminescence measurements of tumor growth or metastasis in different groups was calculated using Student t test or random-effect models when appropriate. The p values >0.05 were considered nonsignificant.

Results

Anti-human CD73 mAb binds human, but not mouse, CD73 and detects human xenograft tumors in mice

Flow cytometry analysis showed that anti-CD73 AD2 mAb (AD2 mAb) and anti-CD73 IE9 mAb (IE9 mAb) both recognized CD73 on the human breast cancer cell lines LM3 and MDA-MB-231, but failed to detect the murine CD73 variant expressed on isolated mouse mononuclear hematopoietic cells (Fig. 1A). Furthermore, when cells were costained with AD2 and IE9 mAbs, the saturation signals increased compared with staining with each Ab alone, indicating that AD2 and IE9 recognize different epitopes on CD73 (Supplemental Fig. 1A). The expression of CD73 in LM3 is ~20% of that in MDA-MB-231 cells.

The specificity of AD2 mAb toward human CD73 was further tested by injecting Alexa Fluor 647–labeled AD2 mAb or control mAb into mice with xenograft tumors established from LM3 breast cancer cells. In vivo fluorescence bioimaging showed that only AD2 mAb detected the primary tumor, and that the AD2 mAb remained visible above background after 72 h (Fig. 1B). No accumulation of AD2 mAb was observed in any mouse organs, demonstrating that the AD2 mAb only recognizes human CD73, and not murine host CD73.

Anti-human CD73 mAb inhibits establishment of spontaneous metastasis, but not primary tumor growth

Next, we examined the ability of AD2 mAb to inhibit primary tumor growth in vivo. LM3 breast cancer cells were inoculated into the mammary fat pad of immunodeficient mice. Starting from day 4 after cancer cell implantation, mice received AD2 mAb (n = 5, 5 mg/kg) or control mAb (n = 5, 5 mg/kg) i.p. twice a week for 5 wk, whereas the primary tumor growth was simultaneously quantified using bioluminescence imaging. No difference in growth rate of
the tumors was observed between the two groups when the relative light emission was compared, indicating that AD2 mAb does not inhibit primary tumor growth (Supplemental Fig. 1B, 1C).

Furthermore, MTT proliferation assays showed that AD2 mAb had no effect on the proliferation of cancer cells in vitro (Supplemental Fig. 1D, 1E), demonstrating that the AD2 mAb is neither cytostatic nor cytotoxic, supporting the in vivo observations.

We next examined whether AD2 mAb had an effect on the ability of cancer cells to establish spontaneous metastasis in the lungs. The primary tumors were surgically removed 5 wk after LM3 cancer cell inoculation when they reached a size of 1.2 cm in diameter, and the metastatic burden in the lungs was quantified weekly for 5 wk using bioluminescence (Fig. 2A). At end point, 10 wk after LM3 cancer cell inoculation, the overall metastatic burden was 20 times lower in the mice treated with AD2 mAb (n = 5) than those treated with control mAb (n = 5; p < 0.05; Fig. 2B), demonstrating that the AD2 mAb inhibits spontaneous metastasis formation. Because the complement system is still active in CB-17 SCID mice and may be involved in the elimination of cancer cells, we examined the complement activation factor C3d deposits in the primary tumors from mice treated with AD2 mAb and control mAb by immunohistochemistry and found no indication of complement activation by the AD2 mAb or control mAb (Supplemental Fig. 1F, 1G).

Because migration of cancer cells is central in the metastatic process, we investigated whether AD2 mAb inhibited the migratory potential of cancer cells in vitro. However, comparing the migration of cancer cells in a wound-healing scratch assay showed no difference between cancer cells treated with AD2 mAb compared with those treated with control mAb (Supplemental Fig. 2A).

**FIGURE 2.** Anti-CD73 AD2 mAb inhibits metastasis in a spontaneous metastasis model. LM3 cells (10⁶) were transplanted into the mammary fat pad of SCID mice, and anti-CD73 AD2 mAb (5 mg/kg) or control mAb (5 mg/kg) were administered at day 4 and subsequently twice a week for 5 wk. (A) After surgical removal of the primary tumors, the animals were imaged weekly for potential lung metastasis by measuring luciferase activity in the lungs using an IVIS spectrum instrument. (B) Nonspecific reflection of light on paws was graphically removed. End-point measurements (5 wk after primary tumor removal) of photon radiance per area were compared between the two groups of five animals each using the Student t test (*p < 0.05).

Ex vivo preincubation of LM3 cells with anti-CD73 AD2 mAb inhibits colonization, extravasation, or both

To further study how the anti-human CD73 mAbs inhibit metastasis development, we pretreated LM3 (10⁶) cells with 10 µg/ml AD2 mAb or control mAb ex vivo and injected the cells into the tail vein of immunodeficient mice (n = 6 in each group) to study the process of extravasation, colonization, or both. Ex vivo incubation of LM3 cells with AD2 mAb significantly inhibited metastasis formation when compared with LM3 cells incubated with control mAb (p < 0.05; Fig. 3A). Furthermore, no visible metastasis was detected (0/6) in lungs from mice injected with AD2 mAb-treated LM3 cells examined ex vivo by bioluminescence, whereas metastases were present in five of six mice injected with control mAb-treated LM3 cells. Furthermore, ex vivo examination of excised rib cages from mice injected with LM3 cancer cells revealed that bone metastases were present in 100% (3/3) of mice treated with control mAb, but in none of those treated with AD2 mAb. This demonstrates that AD2 mAb inhibits bone and lung metastasis. It should be noted that bone metastases were present in animals with no detectable lung metastases, suggesting that the bone metastases were not secondary to lung metastases (Fig. 3B).

**FIGURE 3.** Ex vivo incubation of LM3 cells with anti-CD73 AD2 mAb, but not anti-CD73 IE9 mAb, inhibits metastasis formation in mice

To further study the anti-human CD73 mAb effect on metastasis development, we pretreated another human metastatic breast cancer cell line, MDA-MB-231 (10⁶) cells with 10 µg/ml AD2 mAb, IE9 mAb, or IgG control mAb ex vivo and injected the cells into the tail vein of immunodeficient mice (n = 6 in each group). AD2 mAb...
preincubation significantly reduced MDA-MB-231–derived lung metastasis formation compared with those preincubated with control mAb ($p < 0.05$; Fig. 4A, 4B). In contrast, IE9 mAb did not inhibit metastasis formation of MDA-MB-231 compared with control mAb-treated cells (Fig. 4D, 4E).

Immunohistochemical staining with an anti–pan-cytokeratin Ab of lungs from mice injected with MDA-MB-231 cells treated with either AD2 mAb or control mAb demonstrated a significantly lower number of metastatic foci in the lungs of mice injected with MDA-MB-231 cells treated with AD2 mAb compared with those injected with MDA-MB-231 cells treated with control mAb (Fig. 4C; $p < 0.001$). Furthermore, the size of the metastases was generally smaller in the AD2 mAb-treated versus control mAb-treated group, in agreement with the bioimaging results. The metastasis was located in the alveolar parenchyma, both in the alveolar wall and in the alveolar lumen, and did not differ between the two treated groups. No stromal reaction was observed, and the overall structure of the lung was normal.

Next, we reduced the MDA-MB-231 cell surface expression of CD73 by CD73 siRNA transfection and observed only 20–30% expression of normal levels (Supplemental Fig. 2B). Incubation of these cells with AD2 mAb followed by tail vein injection into immunodeficient mice ($n=6$ in each group) continued to inhibit metastasis formation in the lungs compared with controls, indicating that AD2 mAb treatment inhibits metastasis formation even for cancer cells with significantly reduced CD73 expression levels (Fig. 4F, 4G).

The growth rate of metastases already established in the lungs was equal in the two treated groups (AD2 mAb versus control mAb) in both the spontaneous and tail vein metastasis models, indicating that the Ab affected the establishment of the cancer cell’s ability to extravasate and/or colonize the lungs, but not the proliferation rate

![Image](https://example.com/image.png)
Treatment increased 5D). Interestingly, CD73 activity in LM3 cells after AD2 mAb subsequent internalization of CD73 commencing cells at 37˚C induced clustering of CD73 in the membrane and fluorescence microscopy. AD2 mAb incubation of MDA-MB-231 incubation was investigated using live cell confocal immunofluorescence microscopy. The distribution of CD73 in the plasma membrane upon AD2 mAb treatment with anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, induce alterations of CD73 distribution in catalytic activity was observed compared with untransfected cells (Fig. 5E).

Anti-CD73 AD2 mAb and Fab’ fragments of AD2 mab, but not anti-CD73 IE9 mab, induce alterations of CD73 distribution in the plasma membrane

The distribution of CD73 in the plasma membrane upon AD2 mab incubation was investigated using live cell confocal immunofluorescence microscopy. AD2 mab incubation of MDA-MB-231 cells at 37˚C induced clustering of CD73 in the membrane and subsequent internalization of CD73 commencing ~10 min after incubation began (Fig. 6A, Supplemental Video 1). In contrast, the IE9 mAb induced minimal, if any, clustering and internalization of CD73 in MDA-MB-231 cells, indicating that the clustering and internalization of CD73 is epitope dependent (Fig. 6B). Interestingly, Fab’ fragments of the AD2 mAb also induced clustering and internalization of CD73 upon incubation of MDA-MB-231 cells, indicating that the clustering and internalization of CD73 is not dependent on cross-linking of CD73 (Fig. 6B).

Internalization of anti-CD73 mAbs was further investigated using flow cytometry of live cancer cells. Analysis of CD73 expression after AD2 mab incubation at 37˚C and a subsequent round of AD2 incubation at 4˚C showed that the mean fluorescence intensity (MFI) of CD73 staining decreased in MDA-MB-231 and LM3 cells compared with cells incubated at 4˚C, whereas no decrease in CD73 MFI was observed after IE9 mAb incubation of MDA-MB-231 cells (Supplemental Fig. 3A). An increased MFI was observed after incubation of LM3 cells with IE9 mAb at 37˚C compared with 4˚C, which further supports that IE9 does not induce internalization of CD73 (Supplemental Fig. 3A).

Anti-Human CD73 mAb inhibits metastasis formation

Treatment of MDA-MB-231 and LM3 cells with AD2 mAb (up to 70 mg/ml) reduced the catalytic activity of CD73 in vitro by only 18 ± 4.35 (SD) and 49 ± 1.81%, respectively, compared with control mAb-treated cells (Fig. 5A, 5B). In contrast, the selective CD73 inhibitor APCP promoted a 93 ± 1.5 and 96 ± 3.14% inhibition of the catalytic activity of CD73 in MDA-MB-231 and LM3 cells, respectively (Fig. 5A, 5B).

To reiterate the setup of the tail vein injection experiments, we measured the catalytic activity of CD73 in MDA-MB-231 and LM3 cells when excess Ab was removed after preincubation of the cancer cells with AD2 mAb and IE9 mAb in vitro for 2 h at 37˚C. The catalytic activity after AD2 mAb and IE9 mAb treatment decreased 11 ± 6.64 and 40 ± 18.9%, respectively, in MDA-MB-231 (Fig. 5C). Preincubation of LM3 cells with AD2 mAb demonstrated a 52 ± 3.0% inhibition of CD73 catalytic activity, whereas preincubation of LM3 cells with IE9 mAb resulted in a remarkable 91 ± 4.85% inhibition of CD73 catalytic activity (Fig. 5D). Interestingly, CD73 activity in LM3 cells after AD2 mAb treatment increased ~20% only 3 h after removal of excessive Ab, demonstrating a short-lived effect of anti-CD73 mAbs, indicating the effect on the catalytic activity is functionally unimportant in an in vivo setting where a longer lasting effect is preferred (Fig. 5D). It should be noted that the effect of APCP was lost after washing the cells (Fig. 5C, 5D). Interestingly, upon knockdown of CD73 to ~50% in the highly expressing MDA-MB-231 cell line, no reduction in catalytic activity was observed compared with untransfected cells (Fig. 5E).

Anti-CD73 AD2 mAb and Fab’ fragments of AD2 mab, but not anti-CD73 IE9 mab, induce alterations of CD73 distribution in the plasma membrane

The distribution of CD73 in the plasma membrane upon AD2 mAb incubation was investigated using live cell confocal immunofluorescence microscopy. AD2 mAb incubation of MDA-MB-231 cells at 37˚C induced clustering of CD73 in the membrane and subsequent internalization of CD73 commencing ~10 min after incubation began (Fig. 6A, Supplemental Video 1). In contrast, the IE9 mAb induced minimal, if any, clustering and internalization of CD73 in MDA-MB-231 cells, indicating that the clustering and internalization of CD73 is epitope dependent (Fig. 6B). Interestingly, Fab’ fragments of the AD2 mAb also induced clustering and internalization of CD73 upon incubation of MDA-MB-231 cells, indicating that the clustering and internalization of CD73 is not dependent on cross-linking of CD73 (Fig. 6B).

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Discussion

CD73 has recently been proposed as a target for anticancer therapy (8, 9, 11), because treatment of mice with anti-murine CD73 mAb affects tumor progression by inhibiting the CD73-mediated pro-
duction of adenosine. In this study, we provide novel insight to the role of CD73 in metastasis development and a novel mechanism by which some anti-CD73 Abs can inhibit metastasis formation independent of the catalytic activity of CD73. Our study indicates that CD73 redistribution, induced by anti-CD73 AD2 mAb (AD2 mAb), is central in the inhibition of human breast cancer metastasis in the lungs. Our data support the growing evidence suggesting CD73 as an attractive candidate for targeted treatment in breast cancer, and we suggest using the dual role of CD73 in metastasis development to increase the efficacy of anti-human CD73 mAb treatment by targeting the different functions of CD73.

The functions of CD73 can be divided into those relating to its catalytic activity, converting AMP into adenosine, and those independent of such activity, for example, cell adhesion and signal transduction (10). Most studies on CD73, including those in cancer, have focused on the functions related to the catalytic activity showing that adenosine has a broad range of effects on tumor progression, including promoting angiogenesis, immunosuppression, and metastasis development (7–9, 11, 16). It was recently shown that CD73 on cancer cells promotes spontaneous metastasis by enhancing cancer-cell migration in an adenosinergic manner through the adenosine A2B receptor and downstream promigratory mechanisms (8). Our in vivo data confirmed a role for CD73 in spontaneous metastasis of tumors established from the human breast cancer line LM3, and although the AD2 mAb exhibited a moderate effect on adenosine production in the LM3 breast cancer cells, this was shown to be short-lived and, therefore, functionally negligible. Furthermore, we show that AD2 mAb treatment inhibits metastasis after tail vein injection of MDA-MB-231 cells, but has only a slight effect on the catalytic activity in vitro. Moreover, when 80% of adenosine generating CD73 was removed by siRNA knockdown in MDA-MB-231 cells, AD2 mAb continued to significantly inhibit metastasis, further suggesting that the antimetastatic effect of AD2 mAb is independent of catalytic activity. Taken together, our data add yet another mechanism of CD73 in metastasis formation in human breast cancer in addition to the catalytic activity. Moreover, the inhibitory effect of AD2 mAb on metastasis development was significant in the CD73-expressing LM3 and MDA-MB-231 cells, as well as the CD73 siRNA-treated MDA-MB-231 cells with reduced level of CD73, suggesting that the anti-metastatic effect of AD2 mAb is independent of CD73 expression levels.

Our study showed that AD2 mAb inhibited metastasis formation whereas having no effect on the growth of established primary tumors or metastasis. Furthermore, we showed that ex vivo preincubation of human cancer cells with AD2 mAb inhibited metastasis after tail vein injection, which circumvents the early steps of the metastatic process, suggesting that CD73 promotes an essential function in extravasation, colonization, or both. Together, this indicates that AD2 mAb inhibits metastasis formation independent of an effect on the primary tumor. This suggestion is supported by our previous study with isogenic cancer cell lines wherein we identified CD73 as one of the markers overexpressed in the cancer cell line that was capable of colonization in distant organs compared with its isogenic partner: a cancer cell line that could spread from the primary tumor but remained dormant in the distant organ (17).

Incubation of the breast cancer cells with AD2 mAb and AD2 Fab’ fragments, but not anti-CD73 IE9 mAb (IE9 mAb) in vitro, caused CD73 to cluster and subsequently internalize, indicating that the process is epitope specific, but does not depend on cross-linking of CD73. It is well recognized that although some cell-surface proteins are internalized only when cross-linked by one or two layers of Abs (primary and secondary Abs), other receptors are internalized by binding of Fab’ fragments of an Ab (18–20). IE9 and AD2 mAbs are both generated from mice immunized with full-length native CD73, however, as we showed IE9 and AD2 mAbs recognized different epitopes about which little is known other than the fact that IE9 mAb, in contrast with AD2, recognizes denatured CD73, suggesting that IE9 recognizes a linear epitope of CD73, whereas AD2 mAb recognizes a conformational epitope. Ex vivo preincubation of MDA-MB-231 tumor cells with IE9 did not significantly inhibit metastasis development compared with control mAb-treated tumor cells. Treatment of cancer cells with

FIGURE 6. Anti-human CD73 mAb causes clustering and subsequent internalization of CD73 at 37°C. Immunofluorescence imaging of the subcellular localization of CD73 after anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or anti-CD73 AD2 Fab’ fragment treatment. (A) Representative time points from live cell fluorescence microscopy imaging of MDA-MB-231 cells incubated with Alexa Fluor 555 anti-CD73 AD2 mAb (red) over 1.5 h at 37°C. (B) CD73 (green) distribution after treatment of MDA-MB-231 cells with anti-CD73 AD2 mAb, anti-CD73 IE9 mAb, or anti-CD73 AD2 Fab’ fragments for 2 h at 37°C. Phase-contrast images were merged with the fluorescence images to depict cellular location of CD73. Original magnification ×60.
AD2 mAb, but not IE9, might trigger alterations of CD73 distribution in the plasma membrane and internalization, disrupting prometastatic functions of CD73. The internalization of CD73 might occur concomitantly as a result of CD73 redistribution, and the lower CD73 expression resulting from internalization might not cause inhibition of metastasis development per se. Although the fluorescent microscopy and the flow cytometry data of AD2 mAb supported each other, the readouts were not directly proportional, likely because of technical rather than biological reasons. Re-expression of CD73 in MDA-MB-231 cells after 3-h incubation with AD2 mAb at 37˚C were shown to be only ∼50% expression of CD73 in MDA-MB-231 cells after 3-h incubation likely because of technical rather than biological reasons. Re-fluorescent microscopy and the flow cytometry data of AD2 mAb cause inhibition of metastasis development per se. Although the lower CD73 expression resulting from internalization might not be nonimportant in our model. However, an Ab designed to inhibit the metastasis formation and inhibition thereof observed in our model established by an equipment grant from the Danish Agency for Science Technology and Innovation, and by internal funding from the University of Southern Denmark.

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Disclosures

The authors have no financial conflicts of interest.

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