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Mannose Receptor–positive Macrophage Infiltration Correlates with Prostate Cancer Onset and Metastatic Castration-resistant Disease

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Abstract

Background: M2 tumor-associated macrophages (M2-TAMs) can suppress inflammation in the tumor microenvironment and have been reported to modulate cancer progression. We and others have previously reported M2-TAM infiltration in metastatic castration-resistant prostate cancer (mCRPC).

Objective: To determine whether the extent of M2-TAM infiltration correlates with PC aggressiveness.

Design, setting, and participants: Normal prostate tissue, localized PC, and mCRPC samples from 192 patients were retrospectively analyzed.

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Outcome measurements and statistical analysis: We analytically validated an immunohistochemistry assay for detection of the human mannose receptor (CD206) to assess M2 macrophage involvement.

Results and limitations: Multiplex immunofluorescent staining showed that a small fraction of CD206 staining co-localized with the endothelial cells of lymphatic vessels, while the vast majority of staining occurred in CD68-positive macrophages. The area fraction of staining for CD206-positive macrophages increased in a stepwise fashion from normal (ie, no inflammation) prostate tissue, to primary untreated carcinomas, to hormone-naïve regional lymph node metastases, to mCRPC. Complementary studies using flow cytometry confirmed CD206-positive M2-TAM infiltration. Limitations include the small number of rapid autopsy samples and the lack of neuroendocrine PC samples.

Conclusions: Our results revealed a progressive increase in CD206-positive macrophages from normal prostate to mCRPC. Given the immunosuppressive nature of macrophages and the lack of clinical success of immunotherapy for PC patients, our results provide a rationale for therapeutic targeting of macrophages in the PC microenvironment as a potential method to augment immunotherapeutic responses.

Patient summary: In this report we used 192 prostate cancer samples to determine if M2 macrophage infiltration is correlated with castration resistance in prostate cancer.

Keywords

Mannose receptor; Prostate cancer; Castration-resistant prostate cancer; M2 tumor-associated macrophages

1. Introduction

Prostate cancer (PC) is one of the most commonly diagnosed cancers among men in the USA [1]. Although several newer supra-castration agents can extend life for men with metastatic castrate-resistant PC (mCRPC), a majority of men stop responding to these agents and acquire additional androgen pathway resistance [2]. In addition, mCRPC has largely been unresponsive to the newer immunotherapeutic anti-CTLA4 and anti-PD-L1 therapies as monotherapy treatments [3–5]. Furthermore, mCRPC lesions typically exhibit poor infiltration of cytotoxic T cells and are known as “cold” tumors [6]. The paucity of T cell infiltration remains a challenge for checkpoint blockade immunotherapy in mCRPC.

There is increasing evidence that immune cells in the tumor microenvironment can significantly contribute to cancer progression, therapeutic circumvention, and sub-sequently shorter survival. A subset of infiltrating immune cells found in the mCRPC tumor microenvironment is associated with the reactive stroma that is enriched in M2 macrophages [7]. M2 macrophages have wound-healing functions and are found in abundance in several solid tumor types [8,9] and are therefore known as M2 tumor-associated macrophages (M2-TAMs) [10–14]. Recent studies have shown that M2-TAMs co-cultured with PC cells elicit epithelial-to-mesenchymal transition in the tumor cells in vitro [15] and an increase in tumor cell proliferation [16]. Furthermore, M2 macrophages can secrete pro-metastatic factors and cytokines that prevent immune responses initiated by cytotoxic T cells, causing

immunological silence. Lastly, work by members of our group and others has shown that growth suppression of M2-TAMs leads to prostate tumor regression and a decrease in metastatic potential in preclinical models [17–20].

Our most recent study demonstrated that the mannose receptor (CD206), an endocytic receptor that is characteristically expressed on M2 macrophages, was N-glycosylated on the cell surface of human M2 macrophages and expressed in CRPC samples [21]. While it is known that M2-TAMs aid in tumorigenesis in several tumor types, there have been no comprehensive pathologic studies across the spectrum of PC to date to determine M2-TAM infiltration levels in prostate tissue and PC using immunohistochemistry (IHC) against a specific N-glycosylated marker enriched on the surface of M2 macrophages. Therefore, we conducted a comprehensive study to determine the extent of CD206-positive macrophage infiltration across patient samples and various stages of PC, including organ-confined PC, hormone-naïve metastatic PC, and castration-resistant metastases obtained at autopsy. Our findings demonstrate a potential role of M2 macrophages in multiple stages of PC. Given the known immunosuppressive nature of M2 macrophages, these findings provide a potential link between the tumor microenvironment and resistance to novel immunotherapies.

2. Patients and methods

2.1. Patient samples, tissue microarrays, whole tissue sections, and IHC

A total of five high-density tissue microarrays (TMAs) containing tissue samples from three different patient groups were used ($n = 192$ patients in total). The first cohort represented on three TMAs consisted of a collection of primary prostatic tumors with matched benign regions, each multiply sampled and enriched in patients with high-grade tumors from Johns Hopkins ($n = 120$; age range 41–72 yr) who underwent surgery from 2000 to 2002. The second was a single TMA containing specimens from matched radical prostatectomy and pelvic lymph node metastases from lymph nodes removed at the same surgery ($n = 52$ patients; age range 46–71 yr) from men who underwent surgery between 1993 and 2009. The third TMA consisted of samples from multiple metastatic sites taken during rapid autopsies for men with mCRPC ($n = 15$ patients; age range 51–88 yr) who underwent autopsy between 1995 and 2002. All excised normal prostate tissue, localized PC, and mCRPC tissues acquired during rapid autopsy were fixed in 10% neutral buffered formalin and then embedded in paraffin. TMAs were constructed as described by Fedor and De Marzo [22]. TMA or standard slides were deparaffinized with xylene (Fisher, Fair Lawn, NJ, USA) twice for 10 min, rehydrated using a graded alcohol series, and subjected to antigen retrieval using $1 \times$ EDTA pH 8.0 (ThermoFisher, Waltham, MA, USA) for 45 min. Slides were cooled, washed three times with $1 \times$ phosphate-buffered saline-Tween (PBST), and blocked for 5 min in Dual Endogenous Enzyme Block (Dako, Carpinteria, CA, USA) at room temperature. Slides were then incubated with a primary antibody against CD206 (LSBio, Seattle, WA, USA; 1:400) at room temperature for 45 min and washed three times in PBST. After that, the slides were incubated with PowerVision⁺ Poly-HRP (Leica Biosystems, Buffalo Grove, IL, USA) for 1 h at room temperature, washed three times with $1 \times$ Tris-buffered saline-Tween (TBST), and then incubated in an aqueous solution of 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO) for 20 min. After washing three times

with TBST, the slides were stained with Mayer's hematoxylin stain (Dako). Chromogenic IHC slides were scanned using Aperio Images and a Zeiss Axio Imager microscope (Zeiss, Thornwood, NY, USA). Freshly obtained mCRPC tissue was collected during rapid autopsy for one patient with mCRPC.

2.2. Multiplex immunofluorescent staining

Multiplex staining was performed following the Opal Tyramide amplification signal protocol for the markers D2-40 (D2/40; Abcam, Cambridge, MA, USA) labeled with fluorophore 520 (Perkin Elmer, Akron, OH, USA); CD68 (KP1; Agilent, Santa Clara, CA, USA) labeled with fluorophore 520; and CD206 (5C11; LSBio, Seattle, WA, USA) labeled with fluorophore 570 (Perkin Elmer). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI; Perkin Elmer) and cover slips were applied to the slides with ProLong Gold (Life Technologies, Grand Island, NY, USA). The markers were distributed between two different panels of three colors each. The first panel included D2-40 and CD206; the second, CD68 and CD206. Slides containing prostate sections were deparaffinized in xylene and rehydrated as described above. For antigen retrieval, slides were microwaved with citrate H25 pH 6.0 buffer at 100 W for 1 min and 20 W for 15 min. After cooling for 15 min, they were treated with dual horseradish peroxidase block for 10 min, and the primary and secondary antibodies and fluorophores were applied according to their respective dilutions and incubation periods in a humid chamber. For the second round of primary antibody application, slides were first subjected to the same microwave antigen retrieval steps described above.

2.3. Scoring

All TMAs were scanned using ScanScope AT and ScanScope CS systems (Aperio, Vista, CA, USA). TMA digital images were imported into the TMAJ images manager module (<http://tmaj.pathology.jhmi.edu>) for histologic diagnoses. For each TMA core, regions of interest were created using Lasso Mask and the annotation tool in TMA/FrIDA [23]. Staining artifacts and glands that did not correspond with the annotation diagnosis were excluded. In TMAJ/FrIDA, HSV color space segmentation was used to define the brown (positive IHC) and tissue area (all stained pixels) masks; meta-masks were also created to combine the color and lasso masks for analysis. The CD206 ratio was defined as the brown area in the region of interest divided by the tissue area in the same region. Regions of lymphatic staining for CD206 (see the Results section) were excluded.

2.4. Statistical analysis

Statistical analyses for TMA data were performed using Stata/SE 14.1 (StataCorp, College Station, TX, USA) and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Data were tested for normal distributions using histograms and Kolmogorov-Smirnov and Shapiro-Wilk tests. Comparisons of the MRC1 mask area ratio among normal and other tissues were performed using the Wilcoxon rank-sum test (Mann-Whitney). Correlation analysis for the CD206 IHC nuclear area ratio was performed by calculating Pearson's correlation coefficient. Box-and-whisker graphs are bounded by the 25th and 75th percentiles, and whiskers extend to the minimum and maximum data values. Error bars depict the standard deviation.

2.5. Flow cytometry

PC tissues samples obtained during rapid autopsy were subjected to single cell homogenization using a gentleMACS tissue dissociator (Miltenyi, Auburn, CA, USA). Cells were then added to collected media to be washed. Suspended cells were centrifuged and washed ($1 \times$ PBS, 0.5% bovine serum albumin, 2 mM EDTA) twice, counted, and then incubated with fluorophore-conjugated primary antibodies against CD206 (FITC) and CD163 (PE-Cy7) in the dark for 45 min at 4 °C. Fluorescence was detected using an S3TM cell sorter (BioRAD, Hercules, CA, USA).

3. Results

3.1. Analytical validation of CD206 IHC staining

In our prior study, we performed immunoblotting using the same CD206 antibody as in the present study and reported a single band at the correct molecular weight using human CD14⁺ monocytes that were differentiated in vitro into M2 macrophages [21]. To establish the analytical validity of our IHC assay, we similarly differentiated human CD14⁺ monocytes into macrophages for 9 d in vitro [24] and subjected them to formalin fixation and paraffin embedding as previously described [25]. Human PC cells (PC3) and human CD14⁺ monocytes similarly prepared as negative controls were negative for CD206 according to immunoblots (data not shown); M1 macrophages did not express CD206 according to immunoblotting, as we have reported previously [21]. Figure 1A,B shows IHC staining for the positive and negative control cells, with robust membrane staining of M2 macrophages and negative staining of PC3 cells, which confirms the specificity of our staining assay..

3.2. Local and distant PCs exhibit progressive increases in CD206-positive macrophages

To determine the extent of CD206-positive macrophage infiltration in normal/benign prostate tissues, standard slides from radical prostatectomy specimens were subjected to CD206 IHC (Fig. 1). We found scattered CD206-positive cells with the morphological appearance characteristic of tissue macrophages within the stroma of normal prostate tissues (Fig. 1C–E), and at times just beneath the glandular basement membrane. In benign regions with somewhat higher levels of inflammatory infiltrates (predominantly mononuclear), there was a clear increase in CD206-positive cells, including some in the epithelial compartment (Fig. 1D). Such inflamed regions were not included in the TMAs below. We noted at times that structures morphologically identifiable as thin-walled vascular structures, without the presence of red blood cells in the lumen, stained positively for CD206, although this staining was generally weaker than in cells with a macrophage appearance. To determine whether these structures were lymphatic vessels and could interfere with our quantification of areas of CD206 macrophage staining, we performed multiplex fluorescent staining against CD206 and D240, which is known to be restricted to lymphatic vessels. Figure 2 shows an example of one such vascular structure co-labeled with anti-CD206 and the D240 antibody; tumor cells were visible on DAPI staining. Despite this co-staining of lymphatic vessels, we performed multiplex immunofluorescence for CD206 and CD68, a pan macrophage marker, and found that the majority of CD206-positive cells were also positive for CD68 (Fig. 2A,B). After performing both sets of multiplex assays on a number of standard slides and

TMA, we found that the vast majority of CD206 staining did not occur in the vascular structures and that these could almost always be avoided in our quantitative assessments.

3.3. Mannose receptor expression is increased in metastatic and CRPC

To determine the relative number of CD206-positive macrophages across normal prostate tissues and various stages of PC, we stained prostate and PC TMAs. For each region of interest (eg, benign-appearing epithelium without significant inflammation) and primary and metastatic tumor, we performed IHC staining and selected regions of interest for quantitative image analysis to determine the relative area of macrophage staining. Given their highly dendritic nature and the fact that tissue sections taken at 5 μ m often do not contain whole cells, we performed quantitative image analysis of the area of staining rather than attempting to determine cell numbers. As compared to benign normal-appearing epithelial regions, we found a greater extent of CD206 staining in prostate carcinoma and in hormone-naïve metastatic lesions found in pelvic lymph nodes at the time of radical prostatectomy (Fig. 3). There was a further increase in the levels of CD206 macrophage infiltration in CRPC samples taken at autopsy (Fig. 3B). While CD206 is considered a marker expressed on M2 macrophages, our aim was to further characterize the phenotype of these cells in patients with mCRPC (Fig. 4). We performed dual antibody labeling for CD206 and the scavenger receptor (CD163), another marker of M2 macrophages, in rapid autopsy samples including two soft-tissue metastases and one bone lesion from a patient with mCRPC. Figure 4 demonstrates by flow cytometry mCRPC tissue obtained from the first right rib has a vast majority of CD163-positive cells that were also positive for CD206, which provides further evidence of M2 macrophage infiltration in mCRPC tumors.

4. Discussion

It has long been believed that M2 macrophages contribute to cancer cell growth by promoting a permissive growth environment via secretion of multiple pro-metastatic growth factors [12,13,26]. One study identified low macrophage infiltration at the time of prostate biopsy as a good prognostic marker [27]. Another study analyzed 332 PC specimens stained with CD68 [28]. As we and others have shown, use of CD68 alone cannot distinguish between M1 and M2 macrophages, as in some cases staining of fibroblasts and other cell types has also been observed [29]. Our in vitro macrophage models facilitated the discovery of enriched N-glycosylation of CD206 via solid-phase extraction of glycoproteins and subsequent analysis [21]. Using the mannose receptor as a measure of the presence of M2 macrophages, our study provides a comprehensive view of M2 macrophage infiltration in normal prostate tissues and in the tumor microenvironment for the first time. Our findings could be relevant for other solid tumor types such as breast, cervical, lung, bladder, and brain tumors for which high M2-TAM infiltration correlates with poorer prognosis [26,30]. The limitations of this study include the number of samples from the rapid autopsy and the lack of neuroendocrine PC samples. Future research could address these limitations by performing an extensive study that includes neuroendocrine samples and rapid autopsy samples together with biobanked primary tumor samples from the same patients. Such an analysis would provide further clinical insight into the lack of positive clinical responses to immunotherapy treatment.

5. Conclusions

PC and mCRPC samples are infiltrated with mannose receptor–positive M2 macrophages relative to normal prostatic tissue. M2 macrophages have the ability to suppress effector T-cell tumor infiltration, activation, and proliferation [10]. Our findings suggest that the presence of M2 macrophages in PC may be related to the mCRPC resistance to immunotherapy.

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Fig. 1 -
Analytical validation of immunohistochemistry detection of CD206 expression. (A) Human M2 macrophages were stained for CD206 as a positive control and (B) PC3 cells were stained as a negative control. (C–H) Normal human prostate tissue and prostate cancer tissue were assessed for CD206 positivity. (C) Normal prostate tissue. (D) In benign regions with somewhat higher levels of inflammatory infiltrates (predominantly mononuclear) there was an increase in CD206-positive cells. (E) Normal prostate. (F) Gleason 3 + 4 + 7 core. (G) Lymph node metastasis. (H) Distant metastasis.

Fig. 2 -

Immunofluorescent staining for CD206-positive macrophages. Deparaffinized prostate sections were stained for three markers. (A) Infiltration of cells with dual CD206/CD68 positivity cells (arrows) within the prostatic stromal compartment and the lumen (Lu); CD206-negative cells (short arrow) were also present. (B) D240 was used as a marker for the lumen of lymphatic vessels. Cells with dual CD206/D240 positivity (arrows) surround the lymphatic lumen; CD206-positive/D240-negative cells (short arrows) were present but not fused to the lymphatic vessel wall. 4',6-Diamidino-2-phenylindole (DAPI) staining was also used for nuclei in (A) and (B).

Fig. 3 -

Mannose receptor expression was greater in prostate cancer metastasis relative to normal benign prostate tissue. Using prostate cancer tissue microarrays and immunohistochemistry, MRC1/CD206 expression was quantified and compared for normal human prostate tissue and prostate cancer tissue. Comparisons were performed using a Mann-Whitney test and/or *t* test as appropriate. Mets = metastases.

Fig. 4 -

Dual mannose receptor and scavenger receptor expression in metastatic castration-resistant prostate cancer (mCRPC) rapid autopsy samples obtained 0–4 h after death. M2 macrophage infiltration was determined via flow cytometry. Coronal lymph node in mCRPC with (A) CD163 staining and (B) CD206 staining. Paratracheal lymph node in mCRPC with (C) CD163 staining and (D) CD206 staining. First right rib in mCRPC with (E) CD163 staining, (F) CD206 staining, and (G) dual CD206 and CD163 staining.