uPA and uPA-Receptor Are Involved in Cancer-associated Myeloid-derived Suppressor Cell Accumulation

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Abstract. Background: Myeloid-derived suppressor cells (MDSC) have been shown to play a critical role in tumor-induced immunosuppression, in many mouse and human cancers. The aim of this study was to show that MDSC accumulation is tumor burden-dependent, and to investigate the role of the tumor-derived urokinase plasminogen activator (uPA) and its receptor (uPAR) on MDSC recruitment. Materials and Methods: Levels of MDSC were assessed in tumor-bearers, and the ability to recruit MDSC by uPA was investigated in normal, tumor-bearers, uPAR⁻/⁻, and CD11b⁻/⁻ mice. uPAR expression in MDSC was also explored. Results: MDSC accumulate to dramatic levels in tumor-bearers, and tumor-derived factors such as uPA also increase to great levels in circulation. MDSC can be recruited by uPA, and uPAR but not CD11b are required for such recruitment. Conclusion: MDSC accumulation is tumor burden-dependent, and tumor-derived factors such as uPA and its receptor uPAR play a role in their recruitment.

The immune system plays an important role in preventing tumor development, and it is now widely accepted that tumor cells induce and are shaped by host anti-tumor immune responses. The theory that the immune system is involved in tumor surveillance was proposed by Paul Ehrlich in the early 1900’s and has since evolved into a very complex process. In fact the specific assessment of immune cell infiltration for a given tumor has been shown to serve as a better predictor of patient survival than histopathological staging (1). However, tumors are believed to exploit a chronic inflammatory environment, leading to the recruitment of immune cells with a regulatory phenotype, such as T-regulatory cells and myeloid-derived suppressor cells (MDSC). These cells have been shown to prevent tumor immunity (2), and their depletion has proven to be effective for immune cell therapies (3).

Over twenty years ago, our laboratory described accumulation of myeloid cells in the spleen due to tumor-derived factors (4). Furthermore, the laboratory reported that these CD11b⁺(MAC-1) cells, now recognized as MDSC, down-regulate T- and B-cell responses (5). MDSC in the mouse have been phenotypically identified as GR-1⁺CD11b⁺ (6), while in humans the markers are less definitive and described as LIN⁻HLA-DR⁻CD33⁺ or CD11b⁺CD14⁻CD33⁺ (7). MDSC accumulation has been associated with inflammation in the tumor microenvironment, and several tumor-derived cytokines have now been shown to influence their phenotypes and suppressive pathways (8-10). These cells have been shown to suppress T-cells (11), repress innate immune cells such as NK cells (12), and skew macrophage differentiation from M1 type to M2, normally associated with immunosuppression (13). We have shown that MDSC accumulate in tumor-bearers in various compartments such as the bone marrow, blood, spleen, and liver, and can interact with various immune cells in those organs (14).

Tumor cells have several means of evading the immune system, one of which is by the production of immune-modulating factors which can, in part, recruit immune regulatory cells such as MDSC. One such factor is urokinase plasminogen activator (uPA), a serine protease produced by many tumors at high levels. It is also increased in the serum of cancer patients, and correlates with the extent of disease. High uPA levels have been found in patients with various
cancers including breast cancer (15), prostate (16), melanoma (17), head and neck (18), and colorectal cancers (19). uPA aids tumor cells, as well as immune cells, in remodeling of the surrounding tissue to allow for migration (20-22), activates pro-tumorigenic factors such as TGF-β (23) and MMPs (24) into their biologically active forms, and can recruit MDSC as we have previously shown (25). Interestingly the uPA-receptor (uPAR) aids uPA function, being involved in cell migration by immune cells (26) and tumor cells (27), involvement in signaling cascades (28), and activation of pro-tumorigenic master-regulators such as signal transducer and activator of transcription 3 (Stat3) (29). uPA also cleaves uPAR from cell surfaces, and increased serum uPAR is also found to correlate with the extent of disease in cancer patients (30). In this manuscript we show that MDSC can accumulate to high levels in tumor-bearers, and that uPA produced by tumors can aid in their recruitment and accumulation, but requires the presence of uPAR, also shown to be correlated with progressive disease.

Materials and Methods

Animals and cell lines. BALB/c mice (H-2b) between 8 and 12 weeks of age were bred in our animal facility at the University of Miami according to guidelines of the NIH. C57BL/6 and CD11b+ mice were purchased from the Jackson Laboratory. uPAR-/- mice were kindly provided by Dr. Peter Carmeliet (Vesalius Research Center, Leuven, Belgium). The DA-3 mammary tumor cells were maintained as previously described (4). The 4T1 cell line was kindly provided by Dr. Fred Miller (Wayne State University, Detroit, MI). B16.F10 and LLC cells were purchased from ATCC (Manassas, VA). Tumor cells (1x10^6 DA-3, 1x10^4 4T1, 2.5x10^5 B16 or LLC) were injected s.c., and then 3-4-week-old tumor-bearing animals were used for indicated studies. Tumor volumes were calculated by measuring two diameters of the tumor (small diameter=x, large diameter=y) using digital calipers and entering measurements into the equation tumor volume=x^2y/2 (0.52), as previously published (64). For uPA recruitment studies, mice were injected i.p. with either 1μg recombinant murine uPA (Molecular Innovations, Southfield, MI), or 0.9% saline.

Cell harvesting and purification. Spleens were removed from animals, and then mashed through 70 μM-cell strainers (BD Biosciences, San Jose, CA) to obtain a single-cell suspension. Livers were removed from animals and processed to obtain a single-cell suspension, as previously described (14). Peritoneal lavage was performed by opening the abdominal skin of mice carefully as to not puncture the peritoneal cavity, and then injecting 5 mL of cold PBS into the cavity. The syringe is then removed, a hemostat is used to contain the PBS opening the abdominal skin of mice carefully as to not puncture the peritoneal cavity, and then injecting 5 mL of cold PBS into the cavity.

Statistical analysis. Error bars represent the standard error of the mean (SEM), and all p-values were two-sided (t-test).

Results

MDSC accumulate to high levels in various compartments in tumor-bearers. We and other laboratories have previously shown that MDSC accumulate in various tumor models, and that many tumor-derived factors can lead to their recruitment and other hematopoietic changes in these tumor-bearers (14, 25, 31). In fact the DA-3 mouse breast tumor, studied extensively in our laboratory, has been shown to produce factors such as PGE2 (32), granulocyte macrophage-colony stimulating factor (GM-CSF) (5), phosphatidyl serine (33), vascular endothelial growth factor (VEGF) (34), MMP-9 (34, 35), and uPA (36), all of which can alter the hematopoietic...
environment of the host. Since MDSC have been proposed to accumulate due to these and other tumor-derived factors we analyzed the levels of MDSC in relation to tumor volume, expecting that with increasing tumor burden there is likewise an increase in tumor-derived factors. BALB/c mice were implanted with $1 \times 10^6$ DA-3 cells s.c., and levels of CD11b$^+$GR-1$^+$ cells (MDSC) in the blood were analyzed by flow cytometry as the tumor burden progressed. Mice with tumor volumes less than 50 mm$^3$ had on average 38% of circulating white blood cells comprised by MDSC, mice with tumors 100-500 mm$^3$ had on average 63%, and mice with tumors 600-1000 mm$^3$ had 78% (Figure 1). Thus increasing tumor burden correlates with increasing circulating MDSC, presumably due to an increase in tumor-derived factors. The accumulation of MDSC is not only in circulation in the blood, but has been reported in other organs and in various other tumor models. We analyzed the levels of splenic MDSC in established tumors, using two BALB/c tumor models, DA-3 and 4T1, and two C57BL/6 tumor models, B16 melanoma and Lewis lung carcinoma (LLC). Unfortunately one cannot compare the levels among the strains/tumors, as the different tumors have different growth characteristics and metastatic potential. However, in all four models there is a dramatic accumulation of MDSC in the spleens of mice bearing established tumors (Figure 2A). Likewise, in all four models there is a striking accumulation of MDSC in the livers of these tumor-bearing mice (Figure 2B).

**Urokinase plasminogen activator (uPA) is elevated in the circulation of tumor-bearing.** We have previously reported that our breast tumor model DA-3 produces the protease urokinase plasminogen activator in vitro (36). Many reports in the literature indicate that serum uPA levels are elevated in cancer patients and correlate with the disease burden and poor prognosis. We analyzed if our DA-3 tumor leads to elevated uPA levels in vivo, by checking the circulating levels of uPA in tumor-bearers. BALB/c mice were implanted with $1 \times 10^6$ DA-3 cells s.c., and after 3-4 weeks blood was harvested from tumor-bearing mice and normal BALB/c mice, and plasma uPA levels were analyzed by ELISA. We found that plasma levels of uPA were elevated three-fold in DA-3 tumor-bearers relative to normal BALB/c controls (Figure 3). This elevation of uPA could be from both the tumor cells and host cells, but confirms that with increased tumor burden there is an elevation of uPA in circulation as has been reported in cancer patients.

**MDSC can be recruited by uPA.** We have previously reported that uPA, produced by DA-3 tumor cells expressing a pro-tumorigenic protein known as Mucin 1 (DA-3/TM) are able to recruit and accumulate MDSC, and that knockdown of uPA...
expression by those cells leads to decreased MDSC recruitment (25). We analyzed whether recombinant uPA injected into the peritoneal cavity could directly recruit MDSC into the cavity of tumor-bearing mice which had high circulating levels of MDSC, as shown in Figure 1. BALB/c mice were implanted with 1 × 10^6 cells of the parental DA-3 tumor cell line s.c., and after 3-4 weeks mice were injected i.p. with 1 μg of uPA recombinant protein, or saline as control. Two hours later mice were sacrificed, i.p. lavaged to analyze which cells were recruited, and spleens harvested for analysis of MDSC levels. While splenic MDSC levels were elevated but unchanged in DA-3 tumor-bearers, receiving either i.p. saline injections or uPA, there was a prominent recruitment of MDSC into the peritoneal cavity in mice that received uPA, 43.0%, relative to mice receiving saline, 2.9% (Table I). This recruitment is not specific to tumor-associated MDSC, as we have shown previously that uPA can recruit MDSC in normal mice (25). Normal BALB/c mice received eight injections of uPA or saline every other day, and then two hours after the last injection mice were sacrificed, and i.p. lavaged for MDSC recruitment analysis. Just as in tumor-bearers, there is a dramatic recruitment of MDSC into the peritoneal cavity of normal BALB/c mice receiving uPA injections i.p., but not in mice receiving saline injections i.p. (Table I).

**MDSC express uPA-Receptor (uPAR).** The receptor for uPA (uPAR) has been found on various immune cells, such as neutrophils (26), natural killer cells (37), macrophages (38), and T-cells (39), and is proposed to aid such cells in migration through tissues. This migration has been found to involve the binding of uPA to uPAR, and thus we suspected that MDSC may express uPAR, as they respond to and are recruited by uPA. We implanted C57BL/6 mice with B16 tumor cells, and BALB/c mice with DA-3 cells, and 3-4 weeks later-isolated BM and spleens from the tumor-bearers. MDSC were then purified from the BM and spleen cells, and total protein was extracted and analyzed by western blot for uPAR expression and β-actin was used as control. uPAR protein was expressed by splenic and BM MDSC from both tumors and mouse strain models (Figure 4A and 4B). Controlling protein expression to β-actin indicates however that MDSC from the spleen and BM express varying levels of uPAR (Figure 4B). To analyze whether uPAR was not just present in total cell protein from MDSC but actually present on the surface of MDSC, BALB/c mice were implanted with DA-3 tumor cells s.c., and after 3-4 weeks splenocytes and blood cells were harvested. By gating on MDSCs, cells were analyzed by flow cytometry for surface uPAR expression. Both splenic and blood MDSC expressed surface uPAR, and at similar levels (Figure 4C).

**MDSC recruitment by uPA requires uPAR, but not CD11b.** Since uPAR has been implicated in the migration of immune cells, and MDSC express uPAR and are recruited by uPA, we investigated whether uPA is capable of recruiting MDSC in uPAR+/− C57BL/6 mice. uPAR−/− mice and control C57BL/6 mice were injected with 1 μg of uPA i.p., and 2 h following the injection, mice were sacrificed and recruited cells i.p. were analyzed by flow cytometry. While C57BL/6 had 68% MDSC i.p., uPAR−/− mice only had 21% MDSC i.p. (Figure 5A), indicating that uPAR was essential for the dramatic recruitment of MDSC by uPA. Furthermore, this also confirms that the uPA recruitment of MDSC is not strain-specific to the BALB/c mouse. Additionally, one study reported that uPA forms a trimolecular complex with CD11b and uPAR to aid in the migration of human neutrophils (26). We, thus, investigated whether the CD11b integrin was critical for uPA-mediated MDSC recruitment using CD11b−/− mice. However, CD11b−/− and control C57BL/6 mice recruited the same levels of GR-1+ cells two hours following administration of uPA i.p. (Figure 5B). Thus, perhaps CD11b is not required for uPA recruitment of MDSC, or at least not in this mouse model. uPA has been shown to activate different proteins and enzymes involved in migration, such as matrix metalloproteases (MMPs) (24). Furthermore, it has previously been reported by several groups that MDSC express high levels of MMP-9 (40), and that MMP-9 made both by MDSC and BM progenitor cells, is partly responsible for the expansion of MDSC in tumor-bearing hosts (41). We previously analyzed uPA-mediated recruitment of MDSC in FVB MMP-9−/− mice, which showed that recruitment was not hindered by the absence of MMP-9 (25).

### Discussion

Immunosuppression in cancer has been widely investigated due to progressive disease in the light of identifiable anti-tumor immune responses, as well as failed immunotherapies. One of the key players has been found to be MDSC, which are a
heterogeneous cell population of immature myeloid cells able to mediate immunosuppression in cancer (6, 42, 43), traumatic stress (44), burns (45), and infection (46). Our laboratory has reported that these myeloid cells, accumulate in tumor-bearing mice due to tumor-derived factors (4), and that they hinder T- and B-cell responses via different mechanisms (5). Specifically in cancer, they have been found to expand in many mouse models (47), and in various human cancers, including melanoma (48), breast (49), lung (50), renal cell carcinoma (51), and hepatocellular carcinoma (52). Other laboratories have also shown that MDSC suppress T-cells (43) and NK cells (12), alter macrophages (13), and induce T-regulatory cells (53, 54). Furthermore, MDSC accumulation has been reported to correlate with tumor burden. We also observed in our DA-3 breast tumor model that MDSC levels in circulation rise dramatically in tumor-bearers, and also importantly correlate with tumor burden (Figure 1). We have previously reported that cancer-associated MDSC not only rise in circulation, but accumulate in the bone marrow, spleen, liver, and tumors (14). This dramatic increase in MDSC accumulation in the spleen and liver can be seen in mice harboring various tumor types, as well as various mouse strains (Figure 2A and 2B).

The correlation of cancer-associated MDSC accumulation with tumor-burden is purported to be a result of increased tumor-derived factors with increased disease burden. We have shown in the past that the DA-3 tumor model produces the pro-tumorigenic factor uPA (36), which is produced by various mouse and human tumors. uPA production by tumor cells can be seen not only in vitro, but is increased in the circulation of DA-3 tumor bearers (Figure 3), and has been reported to be increased in patients with various cancers.
uPA has been shown to be critical for macrophage (15-19) and basophil chemotaxis (55), migration and invasion by immature DC (56), and inflammatory cells in uPA−/− mice fail to recruit to sites of infections (57). Likewise, we have shown that uPA can recruit both cancer-associated as well as normal MDSC using our DA-3 tumor model (Table 1), as well as DA-3/TM tumor cells expressing a protumorigenic Mucin 1 (25). Additionally, DA-3/TM tumor cells with a knock down of uPA expression recruit less MDSC (25). uPA, thus, has been linked to immunosuppression at various levels, such as by activation of TGF-β for peripheral tolerance in the eye (23), inhibition of NK cell effector functions (58), and also recruitment of immune regulatory MDSC.

Interestingly, uPAR the receptor for uPA, is known to activate pro-tumorigenic signaling cascades (29), and associate with integrins to aid in migration of immune (26) and tumor cells (27). Expression of uPAR has, thus, also been correlated with more aggressive disease and poor prognosis (59-61). Additionally, uPAR can be cleaved from the cell surface by uPA and soluble forms have been reported to be elevated in cancer patients, usually with poor outcomes (61, 62). Soluble fragments of human uPAR have even been described to mobilize CD34+ mouse and human hematopoietic stem and progenitor cells (63). MDSC like other immune cells also express uPAR (Figure 4), and require its expression for recruitment by uPA (Figure 5A). This supports other reports that have described decreased intra-tumoral macrophages in uPAR−/− mice (38), which are required for maintaining the angiogenic and immunosuppressive microenvironment needed for tumor progression. However, uPAR may aid migration differently in different cells, species, or disease states, as the integrin CD11b was not required in our uPA-mediated recruitment of MDSC (Figure 5B), while some report forming of a trimolecular complex of uPA with uPAR and CD11b in human neutrophils as an example (26).

Understanding the biology of MDSC accumulation or recruitment by uPA in cancer can help us to better target therapies for their elimination in cancer, or potentially to exploit them for autoimmune disease states.

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References


Figure 5. uPAR, but not CD11b, are essential for uPA-mediated MDSC recruitment. (A) C57BL/6 and uPAR−/− mice were injected i.p. with 1 μg recombinant uPA, or saline as control. I.P. cells were harvested two hours after the injection and stained for MDSC and analyzed by flow cytometry. (B) C57BL/6 and CD11b−/− mice were injected i.p. with 1 μg recombinant uPA, or saline as control. I.P. cells were harvested two hours after the injection and stained for GR-1+ cells and analyzed by flow cytometry. Error bars representing SEM and p values are provided.


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