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

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Abbreviations: BM, bone marrow; *i.p.*, intraperitoneally; I.P., intraperitoneal lavage; MDSC, myeloid-derived suppressor cells; uPA, urokinase plasminogen activator; uPAR, uPA-receptor.

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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-2^d) 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 (1×10^6 DA-3, 1×10^4 4T1, 2.5×10^5 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=$x^2y(0.52)$, 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 intraperitoneally and to shake the cavity back and forth for 30 s. Then the needle is re-inserted to remove the PBS-containing intraperitoneal (I.P.) cells. For submandibular blood samples, mice were punctured with an animal lancet (Medipoint, Mineola, NY, USA), and blood was collected into microfuge tubes containing 0.008% heparin (Sigma-Aldrich, St. Louis, MO, USA) in PBS. Bone marrow (BM) was harvested by cutting femur ends, and flushing BM with RPMI media using a 25-gauge needle. Red blood cells were lysed from samples using ACK lysing buffer (Invitrogen, Carlsbad, CA, USA) for 5 min. Purification of MDSCs was performed by staining cells using anti–GR-1 (RB6-8C5) antibody (BD Biosciences), followed by magnetic antibody cell separation (MACS), using goat anti–rat microbeads (MiltenyiBiotec, Auburn, CA) (25).

Flow cytometry. Cells were stained in FACS buffer (PBS, 0.5% BSA, 0.1% sodium azide) with antibodies for 30-45 min, then washed in FACS buffer, and resuspended in FACS buffer for analysis on an LSR II flow cytometer (BD Biosciences). The antibodies used were anti-GR-1 (RB6-8C5) APC and anti-CD11b (M1/70) PE from BD biosciences. Anti-uPAR (M17) and anti-goat FITC were used for surface uPAR staining (Santa cruz biotechnology, Santa Cruz, CA, USA).

Plasma uPA analysis. Plasma was collected by bleeding into heparinized microvettes (Braintree Scientific, Braintree, MA, USA), then by spinning tubes at 3,000 ×g for 15min, 4˚C. uPA in plasma was analyzed by loading 100 μl of plasma per well in a Total uPA ELISA (Molecular Innovations).

Western blot. Spleen and BM MDSCs from B16 and DA-3 tumor-bearing mice were used. MDSCs were lysed using cold RIPA assay buffer supplemented with protease inhibitor cocktail tablets (Roche, Indianapolis, IN, USA) and sodium vanadate (Roche, 1mmol/L final concentration). Protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA) before analyzing the samples by western blot. 25 μg of protein were subjected to 10% SDS-PAGE followed by semi-dry electrotransfer onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was blocked with 5% milk powder, 0.05% Tween 20 in PBS, followed by incubation with rabbit anti-uPAR IgG (1:100; FL-290/sc-10815, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in blocking buffer. The levels of β-actin were detected by a rabbit anti-mouse polyclonal antibody (1:1000; Sigma). Protein-immune-complexes were detected by HRP-conjugated goat anti-rabbit IgG diluted in 1:5000 (sc-2004, Santa Cruz) in blocking buffer. Visualization of complexes was done with chemiluminescent method (West Pico; Pierce Chemical) and membrane was exposed on X-ray film. Films were scanned and data was subjected to densitometric analysis using Scion image software (NIH). Protein levels were normalized to the hybridization signals of b-actin, and reported as relative intensity.

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 PGE_2 (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

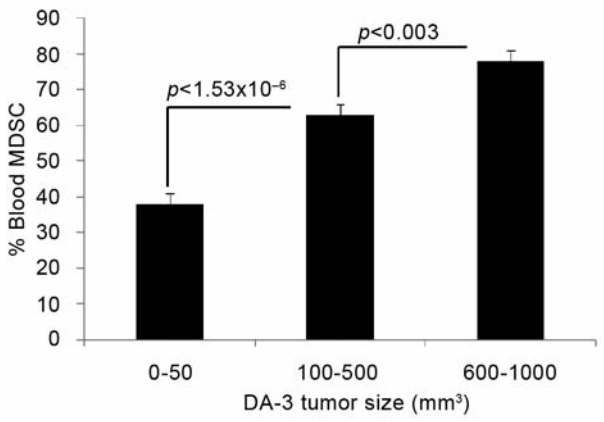

Figure 1. *MDSC accumulation is tumor burden-dependent. BALB/c mice were implanted with 1×10^6 DA-3 tumor cells s.c., and blood from mice at different stages in tumor development was collected. White blood cells were stained and analyzed by flow cytometry for MDSC markers (CD11b$^+$GR-1$^+$). Error bars representing SEM and p-values are provided.*

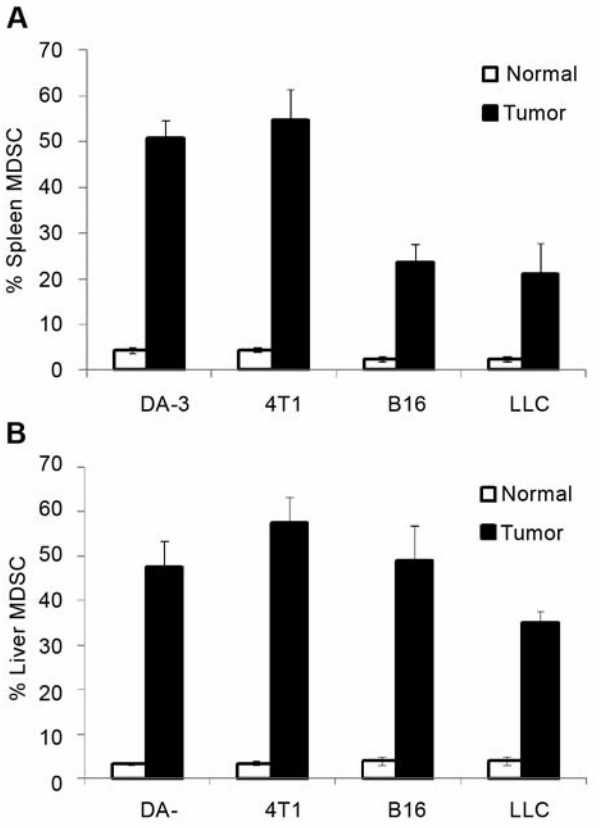

Figure 2. *Mice developing tumors of various types accumulate MDSC in several organs. BALB/c mice were implanted with DA-3 or 4T1 breast tumor cells s.c., and C57BL/6 mice were implanted with B16 melanoma or LLC lung tumor cells. (A) Spleen and (B) liver cells were isolated from the various tumor-bearing animals and normal controls, and were stained for MDSC (CD11b and GR-1) and analyzed by flow cytometry. Error bars representing SEM are provided.*

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 1×10^6 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-bearers. 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 1×10^6 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 analylzed 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).

Table I. *uPA can recruit tumor-associated MDSC and normal MDSC. DA-3 tumor-bearing mice were injected i.p. with saline as control or 1μg recombinant uPA. Two hours later peritoneal lavages (I.P.) were performed and spleens were harvested for staining MDSC and for analyzing by flow cytometry. Normal BALB/c mice were injected i.p. eight times every other day with either saline as control, or 1μg recombinant uPA. Two hours after the last injection the spleen and I.P. cells were harvested and stained for MDSC analysis by flow cytometry. SEM and p values are provided.*

	uPA	Control	*p*-Value
Tumor-bearer			
Spleen	37.82±2.32	43.44±4.44	0.322
I.P	43.04±5.0	22.94±0.31	<0.001
Normal BALB/c			
Spleen	6.23±0.63	3.93±0.66	<0.036
I.P	36.20±4.88	2.00±0.33	$<3.6\times10^{-5}$

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 b-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 b-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 tri-molecular 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 $GR\text{-}1^+$ 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 $MMP\text{-}9^{-/-}$ 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

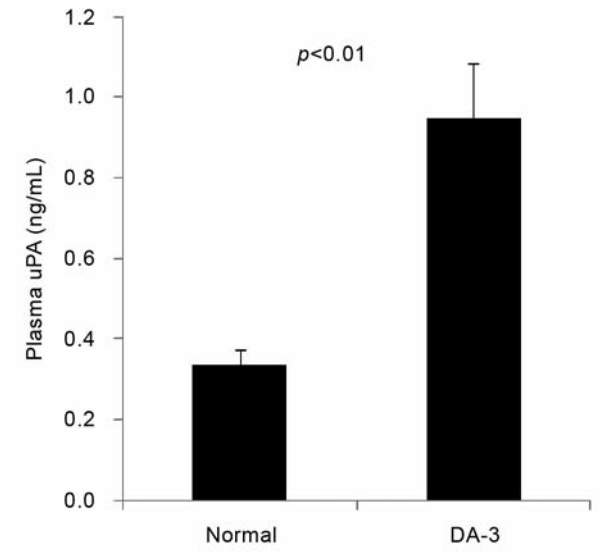

Figure 3. *uPA is up-regulated in the circulation of tumor-bearing mice. BALB/c mice were implanted with DA-3 tumor cells s.c., and after 3 to 4 weeks of established tumor development, blood was harvested from the tumor-bearers, and the normal BALB/c mice as controls. Plasma was assayed for urokinase plasminogen activator (uPA) using ELISA. Error bars representing SEM and p-value are provided.*

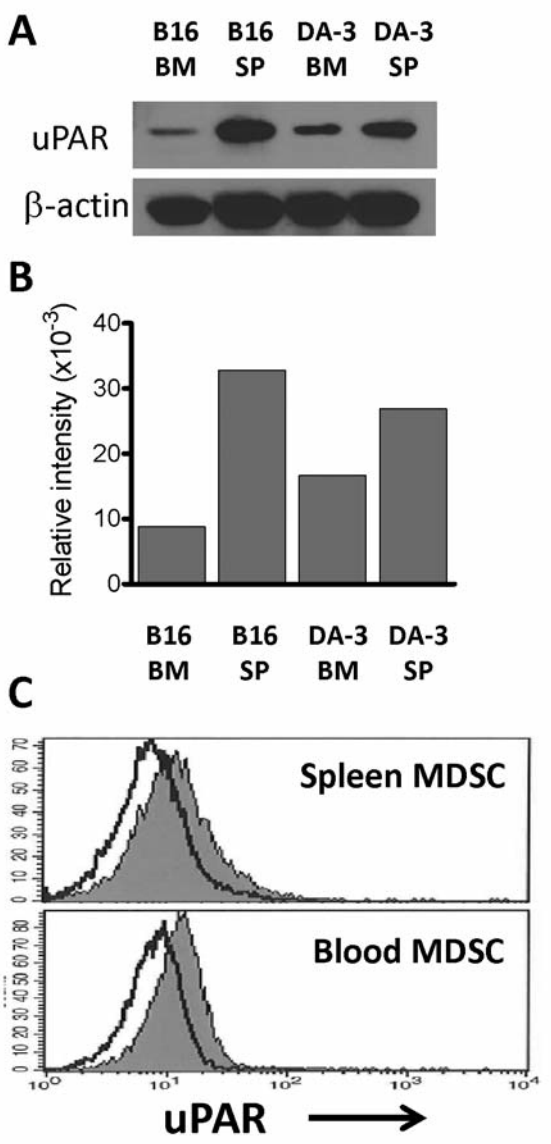

Figure 4. *uPA-Receptor (uPAR) is expressed by MDSC. (A) C57BL/6 mice and BALB/c mice were implanted with B16 and DA-3 tumor cells, respectively, and after 3 to 4 weeks spleen and BM MDSC were purified from tumor-bearing mice. MDSC total cell protein was then isolated and analyzed by western blot for uPAR and b-actin expression as a control. (B) Relative expression of uPAR controlled to b-actin protein expression from western blot seen in (A). (C) BALB/c mice were implanted with DA-3 tumor cells s.c., and after 3 to 4 weeks of established tumor development blood and splenocytes were isolated and stained for MDSC markers and uPAR. White histograms represent MDSC stained with isotype control, relative to shaded histograms representing MDSC stained with anti-uPAR antibody.*

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

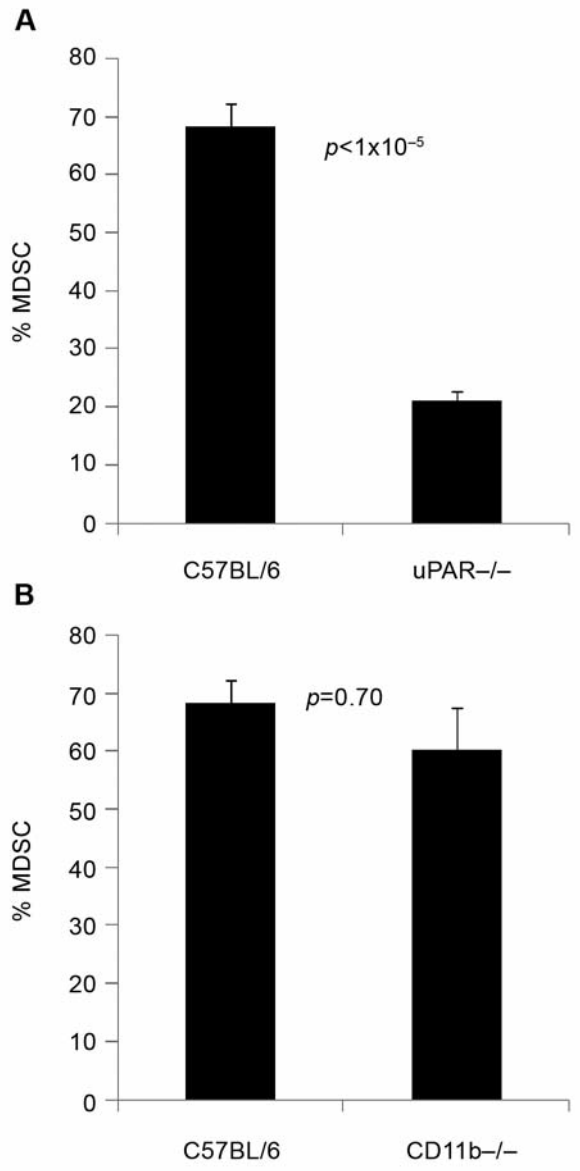

Figure 5. *uPAR, but not CD11b, are essential for uPA-mediated MDSC recruitment. (A) C57BL/6 and uPAR$^{-/-}$ mice were injected i.p. with 1 μg recombinant uPA, or saline as control. I.P. cells were harvested two hours after the injection and stained for MDSC and analyzed by flow cytometry. (B) C57BL/6 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.*

(15-19). uPA has been shown to be critical for macrophage (21) 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 I), as well as DA-3/TM tumor cells expressing a pro-tumorigenic 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 trimoleular 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

1 Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C *et al*: Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science *313*: 1960-1964, 2006.

2 Turk MJ, Guevara-Patino JA, Rizzuto GA, Engelhorn ME, Sakaguchi S and Houghton AN: Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. J Exp Med *200*: 771-782, 2004.

3 North RJ: Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. J Exp Med *155*: 1063-1074, 1982.

4 Fu YX, Watson G, Jimenez JJ, Wang Y and Lopez DM: Expansion of immunoregulatory macrophages by granulocyte-macrophage colony-stimulating factor derived from a murine mammary tumor. Cancer Res *50*: 227-234, 1990.

5 Watson GA, Fu YX and Lopez DM: Splenic macrophages from tumor-bearing mice co-expressing MAC-1 and MAC-2 antigens exert immunoregulatory functions via two distinct mechanisms. J Leukoc Biol *49*: 126-138, 1991.

6 Serafini P, Borrello I and Bronte V: Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. Semin Cancer Biol *16*: 53-65, 2006.

7 Gabrilovich DI and Nagaraj S: Myeloid-derived suppressor cells as regulators of the immune system. Nat Rev Immunol *9*: 162-174, 2009.

8 Bunt SK, Yang L, Sinha P, Clements VK, Leips J and Ostrand-Rosenberg S: Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. Cancer Res. 67: 10019-10026, 2007.

9 Movahedi K, Guilliams M, Van den Bossche J, Van den Bergh R, Gysemans C, Beschin A *et al*: Identification of discrete tumor-induced myeloid-derived suppressor cell subpopulations with distinct T-cell suppressive activity. Blood. 2008.

10 Rodriguez PC, Hernandez CP, Quiceno D, Dubinett SM, Zabaleta J, Ochoa JB *et al*: Arginase I in myeloid suppressor cells is induced by COX-2 in lung carcinoma. J Exp Med *202*: 931-939, 2005.

11 Bronte V and Zanovello P: Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol *5*: 641-654, 2005.

12 Liu C, Yu S, Kappes J, Wang J, Grizzle WE, Zinn KR *et al*: Expansion of spleen myeloid suppressor cells represses NK cell cytotoxicity in tumor-bearing host. Blood *109*: 4336-4342, 2007.

13 Sinha P, Clements VK, Bunt SK, Albelda SM and Ostrand-Rosenberg S: Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. J Immunol *179*: 977-983, 2007.

14 Ilkovitch D and Lopez DM: The liver is a site for tumor-induced myeloid-derived suppressor cell accumulation and immunosuppression. Cancer Res *69*: 5514-5521, 2009.

15 Grondahl-Hansen J, Agerlin N, Munkholm-Larsen P, Bach F, Nielsen LS, Dombernowsky P *et al*: Sensitive and specific enzyme-linked immunosorbent assay for urokinase-type plasminogen activator and its application to plasma from patients with breast cancer. The Journal of laboratory and clinical medicine *111*: 42-51, 1988.

16 Gupta A, Lotan Y, Ashfaq R, Roehrborn CG, Raj GV, Aragaki CC *et al*: Predictive Value of the Differential Expression of the Urokinase Plasminogen Activation Axis in Radical Prostatectomy Patients. European urology. 2008.

17 Stabuc B, Markovic J, Bartenjev I, Vrhovec I, Medved U and Kocijancic B: Urokinase-type plasminogen activator and plasminogen activator inhibitor type 1 and type 2 in stage I malignant melanoma. Oncology reports *10*: 635-639, 2003.

18 Leung KC, Tung Y, Ali M and Lau WN: Measurement of urokinase-type plasminogen activator activity in sera of nasopharyngeal carcinoma patients by an immunocapture assay. Cancer letters *58*: 233-240, 1991.

19 Huber K, Kirchheimer JC, Sedlmayer A, Bell C, Ermler D and Binder BR: Clinical value of determination of urokinase-type plasminogen activator antigen in plasma for detection of colorectal cancer: comparison with circulating tumor-associated antigens CA 19-9 and carcinoembryonic antigen. Cancer Res 53: 1788-1793, 1993.

20 Bezerra JA, Currier AR, Melin-Aldana H, Sabla G, Bugge TH, Kombrinck KW *et al*: Plasminogen activators direct reorganization of the liver lobule after acute injury. Am J Pathol *158*: 921-929, 2001.

21 Bryer SC, Fantuzzi G, Van Rooijen N and Koh TJ: Urokinase-type plasminogen activator plays essential roles in macrophage chemotaxis and skeletal muscle regeneration. J Immunol *180*: 1179-1188, 2008.

22 Luikart SD, Levay-Young B, Hinkel T, Shearer J, Mills C, Caldwell MD *et al*: Mactinin treatment promotes wound-healing-associated inflammation in urokinase knockout mice. Wound Repair Regen *14*: 123-128, 2006.

23 Sonoda KH, Nakamura T, Young HA, Hart D, Carmeliet P and Stein-Streilein J: NKT cell-derived urokinase-type plasminogen activator promotes peripheral tolerance associated with eye. J Immunol *179*: 2215-2222, 2007.

24 Carmeliet P, Collen D: Development and disease in proteinase-deficient mice: role of the plasminogen, matrix metalloproteinase and coagulation system. Thrombosis Res *91*: 255-285, 1998.

25 Ilkovitch D and Lopez DM: Urokinase-mediated recruitment of myeloid-derived suppressor cells and their suppressive mechanisms are blocked by MUC1/sec. Blood *113*: 4729-4739, 2009.

26 Pluskota E, Soloviev DA and Plow EF: Convergence of the adhesive and fibrinolytic systems: recognition of urokinase by integrin alpha Mbeta 2 as well as by the urokinase receptor regulates cell adhesion and migration. Blood *101*: 1582-1590, 2003.

27 Blasi F and Carmeliet P: uPAR: a versatile signalling orchestrator. Nat Rev Mol Cell Biol *3*: 932-943, 2002.

28 Crippa MP: Urokinase-type plasminogen activator. The international journal of biochemistry & cell biology *39*: 690-694, 2007.

29 Shetty S, Rao GN, Cines DB and Bdeir K: Urokinase induces activation of STAT3 in lung epithelial cells. American J Physiol *291*: L772-780, 2006.

30 Stephens RW, Nielsen HJ, Christensen IJ, Thorlacius-Ussing O, Sorensen S, Dano K *et al*: Plasma urokinase receptor levels in patients with colorectal cancer: relationship to prognosis. J Natl Cancer Inst *91*: 869-874, 1999.

31 Ilkovitch D and Lopez DM: Immune modulation by melanoma-derived factors. Experimental dermatology *17*: 977-985, 2008.

32 Handel-Fernandez ME, Cheng X, Herbert LM and Lopez DM: Down-regulation of IL-12, not a shift from a T helper-1 to a T helper-2 phenotype, is responsible for impaired IFN-gamma production in mammary tumor-bearing mice. J Immunol *158*: 280-286, 1997.

33 Calderon C, Huang ZH, Gage DA, Sotomayor EM and Lopez DM: Isolation of a nitric oxide inhibitor from mammary tumor cells and its characterization as phosphatidyl serine. J Exp Med *180*: 945-958, 1994.

34 Owen JL, Iragavarapu-Charyulu V, Gunja-Smith Z, Herbert LM, Grosso JF and Lopez DM: Up-regulation of matrix metalloproteinase-9 in T lymphocytes of mammary tumor bearers: role of vascular endothelial growth factor. J Immunol *171*: 4340-4351, 2003.

35 Handel-Fernandez ME, Ilkovitch D, Iragavarapu-Charyulu V, Herbert LM and Lopez DM: Decreased levels of both Stat1 and Stat3 in T lymphocytes from mice bearing mammary tumors. Anticancer Res *29*: 2051-2058, 2009.

36 Ilkovitch D, Handel-Fernandez ME, Herbert LM and Lopez DM: Antitumor effects of Mucin 1/sec involves the modulation of urokinase-type plasminogen activator and signal transducer and activator of transcription 1 expression in tumor cells. Cancer Res *68*: 2427-2435, 2008.

37 Al-Atrash G, Kitson RP, Xue Y, Mazar AP, Kim MH and Goldfarb RH: uPA and uPAR contribute to NK cell invasion through the extracellular matrix. Anticancer Res *21*: 1697-1704, 2001.

38 Zhang J, Sud S, Mizutani K, Gyetko MR and Pienta KJ: Activation of urokinase plasminogen activator and its receptor axis is essential for macrophage infiltration in a prostate cancer mouse model. Neoplasia, New York, NY *13*: 23-30, 2011.

39 Bianchi E, Ferrero E, Fazioli F, Mangili F, Wang J, Bender JR *et al*: Integrin-dependent induction of functional urokinase receptors in primary T lymphocytes. J Clin Investigation *98*: 1133-1141, 1996.

40 Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y *et al*: Expansion of myeloid immune suppressor Gr^+CD11b^+ cells in tumor-bearing host directly promotes tumor angiogenesis. Cancer cell *6*: 409-421, 2004.

41 Melani C, Sangaletti S, Barazzetta FM, Werb Z and Colombo MP: Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. Cancer Res *67*: 11438-11446, 2007.

42 Kusmartsev SA, Li Y and Chen SH: $Gr\text{-}1^+$ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation. J Immunol *165*: 779-785, 2000.

43 Sinha P, Clements VK and Ostrand-Rosenberg S: Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. J Immunol *174*: 636-645, 2005.

44 Makarenkova VP, Bansal V, Matta BM, Perez LA and Ochoa JB: $CD11b^+/Gr\text{-}1^+$ myeloid suppressor cells cause T cell dysfunction after traumatic stress. J Immunol *176*: 2085-2094, 2006.

45 Noel JG, Osterburg A, Wang Q, Guo X, Byrum D, Schwemberger S *et al*: Thermal injury elevates the inflammatory monocyte subpopulation in multiple compartments. Shock Augusta, Ga. 28: 684-693, 2007.

46 Delano MJ, Scumpia PO, Weinstein JS, Coco D, Nagaraj S, Kelly-Scumpia KM *et al*: MyD88-dependent expansion of an immature GR-1(+)CD11b(+) population induces T cell suppression and Th2 polarization in sepsis. J Exp Med *204*: 1463-1474, 2007.

47 Youn JI, Nagaraj S, Collazo M and Gabrilovich DI: Subsets of myeloid-derived suppressor cells in tumor-bearing mice. J Immunol *181*: 5791-5802, 2008.

48 Filipazzi P, Valenti R, Huber V, Pilla L, Canese P, Iero M *et al*: Identification of a new subset of myeloid suppressor cells in peripheral blood of melanoma patients with modulation by a granulocyte-macrophage colony-stimulation factor-based antitumor vaccine. J Clin Oncol *25*: 2546-2553, 2007.

49 Diaz-Montero CM, Salem ML, Nishimura MI, Garrett-Mayer E, Cole DJ and Montero AJ: Increased circulating myeloid-derived suppressor cells correlate with clinical cancer stage, metastatic tumor burden, and doxorubicin-cyclophosphamide chemotherapy. Cancer Immunol Immunother *58*: 49-59, 2009.

50 Almand B, Clark JI, Nikitina E, van Beynen J, English NR, Knight SC *et al*: Increased production of immature myeloid cells in cancer patients: a mechanism of immunosuppression in cancer. J Immunol *166*: 678-689, 2001.

51 Kusmartsev S, Su Z, Heiser A, Dannull J, Eruslanov E, Kubler H *et al*: Reversal of myeloid cell-mediated immunosuppression in patients with metastatic renal cell carcinoma. Clin Cancer Res *14*: 8270-8278, 2008.

52 Hoechst B, Ormandy LA, Ballmaier M, Lehner F, Kruger C, Manns MP *et al*: A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4(+)CD25(+) Foxp3(+) T cells. Gastroenterology *135*: 234-243, 2008.

53 Serafini P, Mgebroff S, Noonan K and Borrello I: Myeloid-derived suppressor cells promote cross-tolerance in B-cell lymphoma by expanding regulatory T cells. Cancer Res *68*: 5439-5449, 2008.

54 Huang B, Pan PY, Li Q, Sato AI, Levy DE, Bromberg J *et al*: $Gr\text{-}1^+CD115^+$ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host. Cancer Res *66*: 1123-1131, 2006.

55 de Paulis A, Montuori N, Prevete N, Fiorentino I, Rossi FW, Visconte V *et al*: Urokinase induces basophil chemotaxis through a urokinase receptor epitope that is an endogenous ligand for formyl peptide receptor-like 1 and -like 2. J Immunol *173*: 5739-5748, 2004.

56 Ferrero E, Vettoretto K, Bondanza A, Villa A, Resnati M, Poggi A *et al*: uPA/uPAR system is active in immature dendritic cells derived from $CD14^+CD34^+$ precursors and is down-regulated upon maturation. J Immunol *164*: 712-718, 2000.

57 Gyetko MR, Chen GH, McDonald RA, Goodman R, Huffnagle GB, Wilkinson CC *et al*: Urokinase is required for the pulmonary inflammatory response to Cryptococcus neoformans. A murine transgenic model. J Clin Investigation *97*: 1818-1826, 1996.

58 Van Den Broeck T, Stevenaert F, Taveirne S, Debacker V, Vangestel C, Vandekerckhove B *et al*: Ly49E-dependent inhibition of natural killer cells by urokinase plasminogen activator. Blood *112*: 5046-5051, 2008.

59 Grondahl-Hansen J, Peters HA, van Putten WL, Look MP, Pappot H, Ronne E *et al*: Prognostic significance of the receptor for urokinase plasminogen activator in breast cancer. Clin Cancer Res *1*: 1079-1087, 1995.

60 Meijer-van Gelder ME, Look MP, Peters HA, Schmitt M, Brunner N, Harbeck N *et al*: Urokinase-type plasminogen activator system in breast cancer: association with tamoxifen therapy in recurrent disease. Cancer Res *64*: 4563-4568, 2004.

61 Rasch MG, Lund IK, Almasi CE and Hoyer-Hansen G: Intact and cleaved uPAR forms: diagnostic and prognostic value in cancer. Front Biosci *13*: 6752-6762, 2008.

62 Sorio C, Mafficini A, Furlan F, Barbi S, Bonora A, Brocco G *et al*: Elevated urinary levels of urokinase-type plasminogen activator receptor (uPAR) in pancreatic ductal adenocarcinoma identify a clinically high-risk group. BMC Cancer *11*: 448, 2011.

63 Selleri C, Montuori N, Ricci P, Visconte V, Baiano A, Carriero MV *et al*: *In vivo* activity of the cleaved form of soluble urokinase receptor: a new hematopoietic stem/progenitor cell mobilizer. Cancer Res *66*: 10885-10890, 2006.

64 Carlsson G, Ekelund L, Stigsson L and Hafstrom L: Vascularization and tumour volume estimations of solitary liver tumours in rats. Ann Chir Gynaecol *72*: 187-191, 1983.

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