Proteomic Pathway Analysis Reveals Inflammation Increases Myeloid-Derived Suppressor Cell Resistance to Apoptosis*§

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Myeloid-derived suppressor cells (MDSC) accumulate in patients and animals with cancer where they mediate systemic immune suppression and obstruct immune-based cancer therapies. We have previously demonstrated that inflammation, which frequently accompanies tumor onset and progression, increases the rate of accumulation and the suppressive potency of MDSC. To determine how inflammation enhances MDSC levels and activity we used mass spectrometry to identify proteins produced by MDSC induced in highly inflammatory settings. Proteomic pathway analysis identified the Fas pathway and caspase network proteins, leading us to hypothesize that inflammation enhances MDSC accumulation by increasing MDSC resistance to Fas-mediated apoptosis. The MS findings were validated and extended by biological studies. Using activated caspase 3 and caspase 8 as indicators of apoptosis, flow cytometry, confocal microscopy, and Western blot analyses demonstrated that inflammation-induced MDSC treated with a Fas agonist contain lower levels of activated caspases, suggesting that inflammation enhances resistance to Fas-mediated apoptosis. Resistance to Fas-mediated apoptosis was confirmed by viability studies of MDSC treated with a Fas agonist. These results suggest that an inflammatory environment, which is frequently present in tumor-bearing individuals, protects MDSC against extrinsic-induced apoptosis resulting in MDSC with a longer in vivo half-life, and may explain why MDSC accumulate more rapidly and to higher levels in inflammatory settings. Molecular & Cellular Proteomics 10: 10.1074/mcp.M110.002980, 1–9, 2011.

Immunotherapies aimed at activating the host’s immune system are promising strategies for the treatment of cancer because of their potential for minimal toxicity to healthy cells and their ability to induce immune memory that may protect against metastatic disease (1). Disappointingly, clinical trials of most cancer vaccines or other active T-cell mediated immunotherapies have not yielded significant patient responses (2). Because most cancer patients are immune suppressed, these failures are most likely because of the inability of cancer patients to immunologically respond to the immunotherapy agents. Although multiple mechanisms contribute to immune suppression in individuals with cancer (3), myeloid-derived suppressor cells (MDSC)1 accumulate in virtually all cancer patients and are a major cause of tumor-induced immune suppression because of their inhibition of both adaptive and innate immunity (4). Because of their widespread presence and potent immune suppressive effects, identifying the cellular and molecular mechanisms responsible for MDSC accumulation and suppressive activity may facilitate the development of effective immunotherapy strategies.

Chronic inflammation frequently precedes tumor onset (5) and many cancer cells produce pro-inflammatory mediators, suggesting that chronic inflammation contributes to tumorigenesis and tumor progression (6). We and others have previously demonstrated that inflammation via the pro-inflammatory molecules interleukin (IL)-1β (7, 8), toll-like receptor 4 (TLR4) (9), IL-6 (10), prostaglandin E2 (11, 12), and S100A8/A9 proteins (13, 14) increases either the number or the suppressive potency of MDSC, or both. This causative relationship between inflammation and MDSC induction led us to hypothesize that MDSC not only are an obstacle to immunotherapy, but also contribute to the onset and progression of tumors by inhibiting immune surveillance of newly transformed cells and by blocking natural immunity to established tumors (15).

We are studying the effects of inflammation on tumor progression and MDSC development using the mouse BALB/c-derived, spontaneously metastatic 4T1 mammary carcinoma (16) transfected with the IL-1β gene (4T1(IL-1β)) (7). When

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1 The abbreviations used are: MDSC, myeloid-derived suppressor cells; DAPI, 4',6-diamidino-2-phenylindole; IL-1β interleukin-1β; IL-6 interleukin-6; MCF mean channel fluorescence; mAb monoclonal antibody; TCCM tumor cell conditioned medium; TGF-β transforming growth factor beta; TLR4 Toll-like receptor 4.
Inflamed into the mammary fat pad of syngeneic BALB/c mice, wild type 4T1 and 4T1/IL-1β tumor cells form a primary tumor at the site of injection and spontaneously metastasize to the lungs, liver, brain, lymph nodes (16), and bone marrow (17). Increasing tumor burden drives the accumulation of Gr1⁺CD11b⁺ MDSC in bone marrow, spleen, blood, and at the site of primary and metastatic tumor (13). Heightened inflammation in the form of high levels of IL-1β in the tumor microenvironment exacerbates tumor progression through various mechanisms. For example mice with 4T1/IL-1β tumors have a shorter survival time, and contain more MDSC that are more suppressive as compared with mice with 4T1 tumors (7, 8). IL-1β also increases the ability of MDSC to induce tumor-promoting macrophages through a Toll-like receptor 4 (TLR4)-dependent mechanism (9), and increases the in vivo half-life of MDSC (7, 8).

Pathways and proteins that differ between MDSC induced in highly inflammatory ("inflammatory" MDSC) versus less inflammatory ("conventional" MDSC) environments may be potential drug targets for eliminating or reducing MDSC activity. Therefore, we have used mass spectrometry based quantitative proteomic analysis followed by pathway analysis to identify activated pathways and proteins of inflammatory MDSC induced by 4T1/IL-1β tumor versus conventional MDSC induced by 4T1 tumor. Because TLR4 transmits signals that increase MDSC potency, we have also compared the protein and pathway profiles of MDSC induced in wild type BALB/c mice versus TLR4-deficient mice. Proteomic analysis revealed numerous regulated proteins, whereas pathway analysis identified several pathways that were up-regulated in inflammatory versus conventional MDSC, and in TLR4-deficient versus wild type mice. Interestingly, the Fas pathway and caspase network were up-regulated in inflammatory MDSC from BALB/c mice, and the caspase network was up-regulated in MDSC from TLR4⁻/⁻ mice. Because caspase network proteins are the effector molecules of Fas-mediated apoptosis, we hypothesized that MDSC survival and half-life in vivo may be regulated by apoptosis. We have confirmed this hypothesis and demonstrate that inflammatory MDSC have enhanced resistance to Fas-mediated apoptosis. These results suggest that inflammation protects MDSC against extrinsic-induced apoptosis resulting in MDSC with a longer in vivo half-life, and may explain why MDSC accumulate more rapidly and to higher levels in inflammatory tumor environments.

**EXPERIMENTAL PROCEDURES**

**Mice and Cell Lines**—TLR4⁻/⁻, TS1 (T-cell receptor transgenic for influenza hemagglutinin peptide 110–115 restricted to I-Eκ; (18)), DO11.10 (T-cell receptor transgenic for ovalbumin peptide 323–339 restricted to I-Aκ; (19)), and wild type BALB/c mice were obtained from The Jackson Laboratory and bred and maintained in the University of Maryland Baltimore County animal facility. All animal procedures were approved by the UMBC Institutional Animal Care and Use Committee. 4T1 and 4T1/IL-1β cell lines were maintained as described (7).

**Tumor Inoculations and MDSC Harvesting**—Female 6- to 10-week-old BALB/c and TLR4⁻/⁻ mice were inoculated in the abdominal mammary fat pad with 1 × 10⁵ 4T1 or 4T1/IL-1β cells. Approximately 3 weeks later when tumors were 9 to 11 mm in diameter and metastatic disease was established, mice were bled from the submandibular vein. Red blood cells were removed by lysis. Samples in which 90% or more of the remaining leukocytes were Gr1⁺CD11b⁺ MDSC as measured by flow cytometry were used.

**T-cell Activation**—CD4⁺ T-cell activation was done as described (20). Briefly, TS1 or DO11.10 splenocytes were cocultured with their cognate peptide and irradiated blood MDSC, pulsed on day four with ³H-thymidine, and harvested 24 h later. Samples were run in triplicate and were counted in a scintillation counter. Data are expressed as the percent suppression relative to transgenic splenocytes plus peptide without MDSC. Percent suppression = 100% × ([(cpm splenocytes + peptide) – (cpm splenocytes + peptide + MDSC)]/cpm splenocytes + peptide).

**Reagents and Antibodies**—All chemicals were purchased from Sigma unless noted otherwise. Fluorescently coupled monoclonal antibodies (mAbs) to Gr1, CD11b, Fas (CD95), activated caspase-3, and isotype control mAbs were obtained from BD Pharmingen (San Diego, CA), 4′,6-diamino-2-phenylindole (DAPI) and caspase-8 carboxyfluorescein reagent were purchased from Invitrogen. Activated caspase-3 polyclonal antibody for Western blot was purchased from Abcam (Cambridge, UK).

**Apoptosis Assay**—4T1 tumor cells were grown to confluence in 20 ml of culture medium in T-75 flasks and the supernatants were collected and used as tumor-cell conditioned media. MDSC were cultured in 1:1 tumor-cell conditioned media:culture medium for 12–20 h with Jo-2 (Fas agonist; BD Pharmingen) or isotype control mAb. Flow Cytometry and Confocal Microscopy—Intracellular and cell surface fluorescence staining for flow cytometry was performed as described (20). Gr1⁺CD11b⁺ cells were gated and analyzed for expression of caspase 3, caspase 8, or Fas using a Cyan ADP flow cytometer and Summit analysis software (Beckman/Coulter). Percent positive cells was determined by comparing the staining with specific mAb versus isotype control mAb. Numerical values shown are the mean channel fluorescence (MCF) for the indicated, gated populations. For microscopy, live MDSC were adhered to cover slips coated with Cell Tak (BD Pharmingen) for 20 min at room temperature, washed twice with excess phosphate buffered saline (PBS), and fixed for 10 min at 37 °C with 2% formaldehyde in PBS. Fixed cells were washed with excess PBS containing 10% fetal calf serum (FCS) (PBS-10% FCS) for 20 min. For cell surface markers, cells were then stained in the dark for 1 h at 4 °C with antibodies diluted in PBS-10% FCS, followed by washing with excess PBS-10% FCS. For intracellular staining antibodies were diluted in PBS-10% FCS containing 0.2% saponin, followed by a 10 min incubation with DAPI in PBS (5 μg/ml), and a final wash in PBS-10% FCS. Cover slips were mounted on slides with 10 μl of SlowFade (Invitrogen), visualized using a Leica TCS SP5 Broadband Tandem scanning confocal microscope fitted with an HCX PL APO 63 × 1.4 numerical aperture objective, and analyzed using Leica Image Browser software. Fluorescent images were collected sequentially. Approximately 16 optical slices through the z-plane were collected for each field. A minimum of 20 cells in multiple fields was counted for each sample.

**Western Blots**—Western blots were performed according to the manufacturer’s (Abcam) protocol except blocking was performed overnight. Bands were quantified using ImageJ software and measuring the mean density of the activated caspase-3 and β-actin bands. Normalized density of activated caspase 3 = (activated caspase-3 mean density/β-actin mean density).

**Sample Preparation and Liquid Chromatography (LC)-Tandem MS (MS/MS)**—MDSC (≥90% Gr1⁺CD11b⁺ cells; 5 × 10⁶ - 10⁷/mouse)
were lysed at a final concentration of 0.1% Rapigest acid-cleavable detergent (Waters, Denver, CO) in 100 mM NH₄HCO₃, pH 8.4. Cell lysates were digested with sequencing grade modified trypsin (Promega, Madison, WI) (1:50 trypsin:protein ratio; cleaves after arginine or lysine) for 1 h at 37 °C, following which trifluoroacetic acid was added to a final pH of ~3. The resulting material was incubated for 1 h at 37 °C, then freeze-thawed at -80 °C to ensure detergent precipitation, and microfuged at 13,200 rpm for 5 min (Eppendorf 5415 D microfuge). The acid-precipitated material was then discarded (21). Supernatant containing tryptic peptides was collected, brought to pH 7, and peptide concentration was measured by OD 280. Thirty micrograms of peptides were desalted using C18 spin cartridges (Pierce), and analyzed using a linear-trap quadrupole-Fourier transform (LTQ-FT) Ultra mass spectrometer (ThermoFischer) interfaced with an Agilent 1100 nanoLC system. A 15-cm fused silica emitter (75-μm inner diameter, 375-μm outer diameter; Proxeon Biosystems) was used as a nanocolumn. The emitter was packed in-house with methanol slurry of reversed-phase, fully end-capped Reprosil-Pur C18-AQ 3 μm resin (Maisch, GmbH) using a pressurized “packing bomb” operated at 50–60 bars (Proxeon Biosystems, Thermo Fisher). The sample aliquots were injected into the nanocolumn and peptide elution was performed at a flow rate of 200 nL/min with mobile phases A (water with 0.5% acetic acid) and B (89.5% acetonitrile, 10% water, and 0.5% acetic acid). A 100-min gradient from 2% B to 50% B followed by a washing step with 98% B for 5 min was used. Mass spectrometric analysis was performed using unattended data-dependent acquisition mode, in which the mass spectrometer automatically switches between acquiring a high resolution survey mass spectrum in the FT detector (resolving power 100,000) and consecutive low-resolution, collision-induced dissociation of up to five most abundant ions in the linear ion trap. MDSC samples from an individual mouse were analyzed twice by LC-MS/MS. One biological sample per group was analyzed by Pathway Search Engine.

**Database Searching and Criteria**—Acquired data (RAW-files) were converted to the mgf format using an in-house program (C++, +) and submitted to the database search engine Mascot (version 2.1.3, Matrix Science, Torrance, CA). Database search (NCBI database 20070109; 4966331 sequences; 1512170332 residues) was performed with the following conditions: trypsin specificity; peptide mass tolerance set to 10 ppm; fragment mass tolerance at 1.5 Da; a maximum of two allowed missed cleavages; carbamidomethyl, deamidation, methionine oxidation, and disulfide as the variable modifications. Proteins that were identified by two or more peptides with a Mascot molecular weight search score above 30 were considered as reliable identifications. Protein identification probability was derived from Mascot Mowse score (22). Mascot output peptide list in the html-format and the corresponding mgf and RAW files of each sample were used as input files for quantification using an in-house program package (C++, +). The identified unique (“bold red” in Mascot) peptides were then searched in each LC-MS/MS dataset using their accurately known molecular mass and approximately known retention time.

**Label-free Quantification**—Quantification of the peptide expression levels was based on the chromatographic peak areas and was carried out using an in-house program. The area of the chromatographic peak was considered the peptide abundance. Sum of the abundances of all “bold red” nonidentical peptides was attributed to the protein expression level (>2 peptides per protein). Normalization was done as previously described (23).

**Proteomic Pathway Analysis**—Pathway analysis was done as described (24). Briefly, lists of protein identifiers, namely International Protein Index entry names together with their relative abundance values and the highest Mascot score the protein received were loaded into ExPlain™ 1.3 tool (BIOSBASE). Upstream key-nodes most relevant for the input gene products were then identified. Key-nodes (also known as bottle-necks) are signaling molecules found on pathway intersections in the upstream vicinity of the genes from the input list. Each key-node was given a score reflecting its connectivity, i.e. how many input-list genes were reached and the proximities to those genes. The score calculation also included the abundances of the downstream proteins detected in the proteomics experiment. Changes in the key node scores thus reflected the changes in the activation levels of the corresponding signaling networks.

**Statistical Analysis**—Percent suppression (Fig. 1C) and flow cytometry for caspase 3 expression (Fig. 4) were analyzed by the SIGN test. Values for percent dead cells (Fig. 4D) and for suppressive activity of MDSC (Fig. 1C) were analyzed by Student’s t test. MS pathway analysis and statistical scoring were done using Pathway Search Engine (24).

**RESULTS**

**MDSC Accumulate in Tumor-bearing Mice and Suppress T-cell Activation**—BALB/c and TLR4−/− mice were inoculated in the mammary fat pad with 4T1 or 4T1/IL-1β cells on day 0 and bled on day 21–24 when primary and metastatic tumors were established (Fig. 1A). Red blood cells were removed by lysis, and the remaining leukocytes were assayed by flow cytometry to ascertain their identity as MDSC. Greater than 92% of the remaining leukocytes in all groups had the Gr1+CD11b+ phenotype characteristic of MDSC (Fig. 1B). In agreement with previous studies and indicative of the heterogeneity of MDSC, Gr1 phenotypes ranged from Gr1med to Gr1hi (7, 9, 10), with inflammation-induced (IL-1β-induced) MDSC displaying the greatest heterogeneity (25). To ensure that the Gr1+CD11b+ cells were functional MDSC, they were cocultured at varying ratios with naïve splenocytes from transgenic mice in the presence of cognate peptide (Fig. 1C). All populations of Gr1+CD11b+ cells suppressed T-cell activation, with 4T1-induced MDSC from BALB/c mice being the least suppressive. Therefore, the cell populations used in subsequent experiments consist of >92% immune suppressive MDSC.

**Proteomic and Pathway Analysis of Inflammation-induced Proteins in MDSC**—To elucidate the effects of inflammation on MDSC we compared conventional MDSC (4T1-induced MDSC from BALB/c mice) versus inflammatory MDSC (4T1/IL-1β-induced MDSC from BALB/c mice). Because of its role in enhancing MDSC-macrophage cross-talk, we also compared MDSC capable of signaling through the TLR4 receptor (4T1-induced MDSC from BALB/c mice) versus MDSC unable to signal through TLR4 (4T1-induced MDSC from TLR4−/− mice). MDSC characterized in Fig. 1 were prepared for LC-MS/MS analysis using Rapigest and digested by trypsin, and peptide samples were analyzed using a LTQ-FT Ultra mass spectrometer (Fig. 2). Seven hundred and eighty-nine proteins were present in both 4T1 and 4T1/IL-1β BALB/c MDSC, and were identified and quantified using a label free approach. 4T1-induced MDSC from TLR4−/− and BALB/c mice shared 749 proteins, which were similarly identified and quantified.
derived protein identification probability for these proteins. Resulting protein identifications and relative protein abundances were loaded into ExPlain, converted into gene identifications, and the gene identifications were then mapped to upstream key nodes in cellular pathways. Each key-node was given a score reflecting its connectivity, i.e. how many input-list genes were reached and the proximities to those genes, as well as abundances of the downstream proteins measured by LC-MS/MS. Changes in the key node scores thus reflected the changes in the activation levels of the corresponding signaling networks. The pathway score was represented by the sum of the scores of all key nodes that are part of that pathway.

Two pathway analyses comparisons were conducted: 4T1-induced MDSC from BALB/c mice (control) versus 4T1/IL-1β-induced MDSC from BALB/c mice (experimental); and 4T1/IL-1β-induced MDSC from TLR4−/− mice (control) versus 4T1-induced MDSC from TLR4−/− mice (experimental). Fourteen significantly up-regulated pathways and nine significantly down-regulated pathways were detected in 4T1/IL-1β-induced MDSC from BALB/c mice (Fig. 3A). Predominant pathways included the caspase network, Fas, TGF-β, and IL-1 pathways. Among the most highly up-regulated pathways were the caspase network and Fas pathways. For 4T1 TLR4−/− MDSC there were 2 significantly up-regulated pathways and 8 significantly down-regulated pathways (Fig. 3B).

The Fas pathway is the extrinsic apoptosis pathway and is activated when Fas ligand (CD95L) on an effector cell binds to Fas on the plasma membrane of the target cell. These interactions result in the cleavage of caspase 8 and the subse-
quent cleavage and activation of caspase 3, which in turn leads to cell apoptosis (26). Multiple proteins associated with the Fas pathway were identified by MS in all MDSC samples, including caspase-3, caspase-8, lamin B1, and Rho GDP inhibitor beta (Rho GDI β) (Fig. 3C). The convergent identification of Fas pathway-associated proteins in MDSC samples suggests that MDSC accumulation, maintenance, and survival in vivo may be regulated by apoptosis. This hypothesis provides a mechanistic explanation for our previous reports that inflammation increases the rate of accumulation of MDSC in tumor-bearing mice (7, 10), and led us to validate the MS findings with biological experiments.

**Inflammation Increases MDSC Resistance to Apoptosis**

To test this possibility, BALB/c mice were inoculated with 4T1/IL-1β or 4T1 cells and blood MDSC were collected 21–24 days later. MDSC were incubated overnight with or without the Fas agonist Jo2 mAb, labeled for Gr1, CD11b, and activated caspase 3 or caspase 8, and the gated Gr1/CD11b cells analyzed by flow cytometry. Jo2-treated conventional (4T1-induced) BALB/c MDSC have consistently less activated caspase-3 as compared with 4T1-induced MDSC (percent caspase 3 cells: 58.6% versus 65.6%; mean channel fluorescence (MCF) of caspase 3 cells: 76.9 versus 87.9, respectively) (Fig. 4A). Caspase 8 expression was similarly reduced in inflammatory versus conventional MDSC following Jo2 treatment (61.6% versus 64.9% caspase 8 cells; 160.5 versus 191.4 MCF of caspase 8 cells, respectively) (Fig. 4B). These data indicate that inflammatory MDSC are less susceptible to Fas-mediated apoptosis as compared with conventional MDSC.

**Inflammation-induced MDSC are More Resistant to Fas Mediated Apoptosis**—To determine whether inflammation increases MDSC by reducing their susceptibility to Fas-mediated apoptosis, we assessed the expression of activated caspase-3 and caspase-8 in conventional versus inflammatory MDSC. If inflammation increases MDSC levels by reducing their apoptotic rate, then treatment of inflammatory MDSC with a Fas agonist will yield lower levels of activated caspase 3 and caspase 8 relative to treatment of conventional MDSC.
Inflammation Increases MDSC Resistance to Apoptosis

Fig. 4. Inflammatory MDSC from BALB/c mice have increased resistance to Fas-mediated apoptosis. A, Inflammatory (4T1/IL-1β-induced) MDSC contain less activated caspase-3 as compared with conventional (4T1-induced) MDSC. Blood leukocytes from BALB/c mice with 4T1 or 4T1/IL-1β tumor were cultured overnight with the Fas agonist mAb Jo2 or a control mAb, and subsequently stained with fluorescent mAbs to Gr1, CD11b, and activated caspase-3. Gr1+CD11b+ cells were gated and analyzed for activated caspase-3 by flow cytometry. Control and Jo2-treated samples are shown as blue and red histograms, respectively. The corresponding blue and red bars and their associated numbers indicate the MCF and percent positive cells for each sample. The first peak of each color represents activated caspase 3- cells; the second peak represents activated caspase 3+ cells; p < 0.03 for Jo2-treated 4T1-induced versus 4T1/IL-1β-induced MDSC. Data are from one of five independent experiments with five individual mice. B, Inflammatory (4T1/IL-1β-induced) MDSC contain less activated caspase 8 as compared with conventional (4T1-induced) MDSC. Blood leukocytes from BALB/c mice induced by 4T1 or 4T1/IL-1β tumor cells were cultured as in panel A, except they were stained for activated caspase 8. Data are from one of three independent experiments with three individual mice. C, Inflammatory MDSC contain less activated caspase 3 than conventional MDSC. Lysates of Jo2-treated conventional and inflammatory MDSC were electrophoresed by SDS-PAGE and Western blotted with mAbs to activated caspase 3. Bands were quantified by densitometry. Normalized density of conventional and inflammatory MDSC treated with Jo2 were 1.31 and 0.74, respectively. Data are from one of two independent experiments with MDSC pooled from 1–2 mice per group. D, Conventional and inflammatory MDSC express similar levels of Fas. Blood leukocytes from BALB/c mice with 4T1 (top panel) or 4T1/IL-1β (bottom panel) tumors were stained with fluorescent mAbs to Gr1, CD11b, and Fas. Gr1+CD11b+ cells were gated and analyzed for cell surface expression of Fas by flow cytometry. Bars indicate the percent Fas+ cells and the MCF of the Fas+ cells. E, Inflammatory MDSC are more viable than conventional MDSC following treatment with a Fas agonist. Blood leukocytes from BALB/c mice with 4T1 or 4T1/IL-1β tumors were cultured overnight with the Fas agonist mAb Jo2 and assessed the next day for viability by trypan blue dye exclusion. Leukocytes were >90% Gr1+CD11b+. Data are from one of five independent experiments with MDSC from individual mice.
Inflammation increases MDSC resistance to apoptosis.

**DISCUSSION**

The association of chronic inflammation and tumor onset and progression was first observed more than 100 years ago (27). However, it is only recently that the mechanisms linking inflammation and cancer, and particularly the role of the immune system, are being clarified (28). An individual’s immune system has the capacity to reduce or eliminate malignant cells; however, tumors frequently evade antitumor immunity. Paradoxically, many of the evasive mechanisms involve co-opting the function of immune cells (3). MDSC are a major contributor to tumor immune escape because of their potent suppression of innate and adaptive antitumor immunity and their widespread presence in most cancer patients (29).

Inflammation limits the activation of caspase 3 and protects MDSC against Fas-mediated apoptosis.

In addition to the caspase and Fas pathways, other pathways were also up-regulated in inflammation-induced versus conventional MDSC, and these pathways are also likely to regulate MDSC maintenance as well as function. For example, TGFβ is a suppressive cytokine that is produced by other suppressive cells as well as by MDSC (30) and its increased expression in inflammation-induced MDSC could contribute to heightened suppressive activity. Likewise, stress is associated with the induction of MDSC (31), and increased expression of the stress pathway is therefore likely to regulate MDSC levels. Interestingly, the caspase network is also up-regulated in 4T1-induced MDSC from TLR4−/− versus BALB/c mice, suggesting that MDSC generated in TLR4−/− mice may turnover more quickly than MDSC in wild type BALB/c mice. Additional biological studies are needed to determine if the TGFβ and stress pathways significantly facilitate the survival of inflammation-induced MDSC and whether the half-life of MDSC from TLR4−/− mice is controlled by proteins of the caspase pathway.

The lower number of activated pathways observed in MDSC from TLR4−/− versus TLR4+/+ mice is not unexpected. In addition to their suppressive effects on T lymphocytes, MDSC also facilitate tumor growth by polarizing macrophages toward a tumor-promoting type 2 phenotype (32). Previous studies established that MDSC from TLR4−/− mice are less able to induce macrophage polarization (9). This characteristic is consistent with the observation that fewer pathways are activated in TLR4−/− MDSC, and suggests that the pathways that are absent in TLR4−/− MDSC may be responsible for MDSC-mediated macrophage polarization. Activation of the caspase network in TLR4−/− MDSC is also
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not unreasonable and indicates that MDSC turnover is regulated independently of how MDSC are activated.

There are several potential mechanisms by which inflammation could increase MDSC resistance to apoptosis. Fas-mediated apoptosis is inhibited by the intracellular protein c-FLIP which prevents the activation of caspase 8 (33). However, c-FLIP was not over-expressed in our proteomic screen and there is no difference in c-FLIP levels between conventional and inflammatory MDSC treated with the Fas agonist (data not shown), so c-FLIP does not regulate resistance of 4T1/IL-1β-induced MDSC. Fas-mediated apoptosis is also regulated by the mitogen-activated protein kinase pathway (34). When activated by phosphorylation, p38 kinase inhibits the activation (auto-cleavage) of caspase-3 and caspase-8 (35). Multiple stimuli induce p38 activation, including transforming growth factor beta (TGF-β (36)), which directly down-regulates Fas-mediated apoptosis (37). Interestingly, our pathway analysis identified both TGF-β and p38 pathways as up-regulated in inflammatory MDSC as compared with conventional MDSC (Fig. 3A), suggesting that elevated levels of TGF-β in inflammatory MDSC may either directly or indirectly through the MAPK pathway, protect MDSC from apoptosis.

Regardless of the mechanism by which inflammation exacerbates MDSC survival and function, our proteomic and pathway analysis and validation studies demonstrate that inflammation facilitates MDSC survival by increasing MDSC half-life. In previous studies we observed that surgical removal of primary 4T1 mammary tumors resulted in a significant decrease in circulating MDSC, whereas circulating MDSC levels remained elevated in mice whose 4T1/IL-1β-induced tumors were removed (7). We have also reported that inflammation increases the rate of accumulation of MDSC in tumor-bearing mice (7, 10). We proposed that both of these effects could be because of either increased production of MDSC and/or an increase in the half-life of MDSC. The current studies support the concept that these effects are at least in part the result of an increase in half-life because of increased resistance to apoptosis.

Our studies not only provide a mechanistic explanation for the longevity of inflammation-induced MDSC, but they also identify proteins and pathways that are potential drug targets for perturbing MDSC viability and facilitating active immunotherapies. In addition, these studies are another example of the usefulness of quantitative pathway analysis and the new analytical tool, Pathway Search Engine for analyzing proteomics data and generating knowledge-based predictions of signal transduction pathways.

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This article contains supplemental Tables 1–2.

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