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ORIGINAL ARTICLE

Chronic Inflammation Contributes to Tumor Growth: Possible Role of L-Selectin-Expressing Myeloid-Derived Suppressor Cells (MDSCs)

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Abstract—Recent data have demonstrated that chronic inflammation is a crucial component of tumor initiation and progression. We previously reported that immature myeloid-derived suppressor cells (MDSCs) with immunosuppressive activity toward effector T cells were expanded in experimental chronic inflammation. We hypothesized that elevated levels of MDSCs, induced by chronic inflammation, may contribute to the progression of tumor growth. Using the Ehrlich carcinoma animal model, we found increased tumor growth in mice with chronic adjuvant arthritis, which was accompanied by a persistent increase in the proportion of splenic monocytic and granulocytic MDSCs expressing CD62L (L-selectin), when compared to tumor mice without adjuvant arthritis. Depletion of inflammation-induced MDSCs resulted in decreased tumor growth. *In vitro* studies demonstrated that increased expression of CD62L by MDSCs was mediated by TNFα, elevated concentrations of which were found in tumor mice subjected to chronic inflammation. Moreover, the addition of exogenous TNFα markedly enhanced the suppressive activity of bone marrow-derived MDSCs, as revealed by the ability to impair the proliferation of $CD8^+$ T cells *in vitro*. This study provides evidence that chronic inflammation may promote tumor growth *via* induction of CD62L expression by MDSCs that can facilitate their migration to tumor and lymph nodes and modulation of their suppressor activity.

KEY WORDS: chronic inflammation; cancer; G-MDSC; M-MDSC; L-selectin; TNFα.

INTRODUCTION

Numerous studies have shown that chronic inflammation promotes development of cancer through a number of mechanisms. According to clinical and epidemiological data, chronic inflammation is a risk factor for oncological pathologies in the liver, pancreas, stomach, and larynx, as well as in the intestines, breast, *etc.* [1]. It is believed that a chronic inflammatory process generates an immunosuppressive microenvironment that favors tumor initiation and progression through production of proinflammatory mediators and the accumulation and activation of immune suppressor cells [2]. Among such cells, special attention has been recently paid to so-called myeloid-derived suppressor cells (MDSCs).

Mouse MDSCs are characterized as cells with $CD11b^+Gr1^+$ phenotype and represented by two main subpopulations: granulocytic (G-MDSCs) with the $CD11b^+Ly6G^{high}Ly6C^{low}$ phenotype and monocytic

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(M-MDSCs) with the $CD11b^{+}Ly6G^{-}Ly6C^{high}$ phenotype. According to the phenotypes, these cell subpopulations are similar to terminally differentiated neutrophils and monocytes, respectively, but, in contrast to them, MDSCs possess an ability to suppress functions of immunocompetent cells through multiple mechanisms [3]. MDSCs are most studied in oncological pathologies, but recent studies demonstrated that these cells play an important role in chronic inflammatory diseases. Depending on the location, nature, and the stage of the ongoing chronic inflammatory process, MDSCs exert beneficial or detrimental effects on a disease outcome. Thus, it was shown that obesity in mice was accompanied by an increase in the liver and adipose tissue $CD11b^{+}Gr1^{+}$ MDSCs, which reduced peripheral inflammation and maintained inflammatory homeostasis through suppression of $CD8^{+}$ cell activity and macrophage polarization toward M2 phenotype [4]. A protective role of MDSCs was demonstrated in the liver and kidney fibrosis models [5]. Resveratrol-induced MDSCs reduced splenic $CXCR3^{+}$ T cells and proinflammatory cytokines in $IL10^{-/-}$ mice with chronic colitis, which correlated with diminished intestinal inflammation [6]. On the other hand, it was shown that chronic tuberculosis in mice [7] and uncontrolled progressive HIV infection in humans [8] were associated with an increase in circulating MDSCs, which correlated with a decrease in $CD4^{+}$ T lymphocytes and a decrease in the proliferative capacity of $CD8^{+}$ T cells. Antiretroviral therapy of HIV infection resulted in the reduced levels of MDSCs. Host detrimental effects of MDSCs were demonstrated in a number of chronic infections that are associated with unresolved chronic inflammation [9]. MDSCs were shown to significantly expand in the elderly, which correlated with an increase in the serum levels of proinflammatory cytokines IL-1 and IL-6 and the risk of cancer [10]. Low-grade inflammatory responses independent of infection in naturally aged mice were accompanied by a significant increase in NF-κB-expressing cells in the bone marrow and spleen; the majority of which were represented by $CD11b^{+}Gr1^{+}$ MDSCs [11]. The proinflammatory role of MDSCs was also demonstrated in several mouse experimental models of chronic inflammation [12–14]. Treatment of chronic inflammation with IFNγ reduced the arginase activity of M-MDSCs [15].

Several studies using experimental models suggested that MDSCs, induced by a chronic inflammatory process, could contribute to tumor growth and progression through the blockade of immune surveillance and antitumor immunity, thereby removing the mechanisms that could eliminate transformed cells. Depletion of MDSCs from the inflamed intestine reduced colitis-associated tumorigenesis [16]. By contrast, adoptive transfer of MDSCs stimulated chronic inflammation in the colon and colitis-related tumor formation, growth, and progression *via* suppression of antigen-specific $CD8^{+}$ T cells [17]. Adoptive transfer of MDSCs obtained from chronically inflamed mice inhibited T cell-mediated rejection of tumor transplants [18]. Activation and recruitment of MDSCs from the bone marrow are shown to be driven by a number of proinflammatory mediators, such as IL-1β, IL-6, PGE2, S100 protein, GM-CSF [19], and TNFα [20, 21]. Despite significant progress in the identification of the factors that regulate MDSC accumulation in chronic inflammation, the exact mechanisms by which chronic inflammation contributes to tumor development *via* MDSC activation are not well understood.

The aim of the present study was to analyze the contribution of MDSCs induced by chronic inflammation into growth of a transplanted tumor. We therefore applied a previously described mouse model of sterile chronic inflammation, which results in the expansion of MDSCs [12, 13], for assessment of inflammation-related tumor progression.

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Abbreviations: AA, Arthritic animals; Ab, Antibody; APC, Allophycocyanin; Arg-1, Arginase-1; ATA, Arthritic animals with tumor; BM, Bone marrow; CA, Control animals; CFA, Complete Freund's adjuvant; CFSE, 5(6)-Carboxyfluorescein diacetate N-succinimidyl ester; ConA, Concanavalin ACXCR4; C-X-C, chemokine receptor type 4; DCFDA, 2′-7′-dichlorofluorescein diacetate; EDTA, Ethylenediaminetetraacetic acid; ELISA, Enzyme-linked immunosorbent assay; FBS, Fetal bovine serum; FITC, Fluorescein isothiocyanate; GHVD, Graft-*versus*-host disease; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HIV, Human immunodeficiency virus; IFNγ, Interferon gamma; IL, Interleukin; iNOS, Inducible nitric oxide synthase; LBS, Lysing buffer solution; MCP-1, Monocyte chemoattractant protein-1; MDSC, Myeloid-derived suppressor cells; MIP-1α, Macrophage inflammatory protein 1 alpha; NF-κB, Nuclear factor kappa B; NO, Nitric oxide; PE, Phycoerythrin; PerCP, Peridinin chlorophyll protein complex; PGE2, Prostaglandin E2; RAGE, Receptor for advanced glycation end products; ROS, Reactive oxygen species; SDF-1α, Stromal cell-derived factor one alpha; TA, Tumor animals; TGFβ, Transforming growth factor beta; TLR, Toll-like receptors; TNFα, Tumor necrosis factor alpha; VEGF, Vascular endothelial growth factor

METHODS

Mice

CD1 mice (males) were purchased from Charles River and bred to the age (5–6 weeks old, 23–27 g body weight) at the vivarium of Kazakh Research Institute of Oncology and Radiology. Animal studies were approved by the Ethical Committee of M.A. Aitkhozhin's Institute of Molecular Biology and Biochemistry. The experiments included healthy mice (control animals, CA), tumor bearing mice (tumor animals, TA), mice with chronic inflammation (arthritic animals, AA), and inflamed animals with inoculated tumor (arthritic animals with tumor, ATA). Adjuvant arthritis was induced by a single subdermal injection of 100 μl of complete Freund's adjuvant (CFA) containing heat-inactivated *Mycobacterium tuberculosis* (Sigma-Aldrich, USA) into a hind limb footpad. Ehrlich ascites carcinoma cells were maintained by weekly intraperitoneal transplantations of 2.5×10^6 cells/mouse. 5×10^5 Ehrlich carcinoma cells were inoculated per mouse subcutaneously 2 weeks after the injection of CFA (ATA) or PBS (TA). Each treatment group involved at least six mice. On day 14 after tumor inoculation or on day 28 after CFA administration, mice were sacrificed by cervical dislocation. Surgically obtained tumors and spleens were weighted. Blood samples were collected from the orbital sinus.

For MDSC depletion, mice with adjuvant arthritis received an intraperitoneal injection of gemcitabine (Eli Lilly) at a dose of 75 mg/kg on days 7 and 10 after initiation of adjuvant arthritis. 5×10^5 Ehrlich carcinoma cells were inoculated per mouse subcutaneously in 4 days after the last injection of gemcitabine. Mice were sacrificed in 14 days after tumor inoculation.

Splenocytes were obtained by homogenization of spleen in PBS by tissue grinder; contaminating erythrocytes were lysed with lysing buffer solution (LBS), containing 0.83% NH_4Cl, 0.1% $KHCO_3$, 0.003% EDTA (pH = 7.2–7.4), for 4 min at room temperature; then cells were washed, filtered through 30 μm pre-separation filters (Miltenyi Biotec, Germany), and re-suspended in PBS.

Flow Cytometry

Mouse FcR Blocking Reagent (Miltenyi Biotec, Germany) was used for blockage of Fc receptors before staining. The following anti-mouse antibodies (Abs) were used for surface staining: APC anti-CD11b (Invitrogen, USA), PerCP Cy5.5 anti-Ly6G, AF 488 anti-Ly6C, PE anti-Ly6C, PE anti-CD282 (eBioscience, USA), PE anti-CD49b, PE anti-CD49d (BD Biosciences, USA), FITC anti-CD62L (Miltenyi Biotec, Germany), AF-488 anti-CD49d, PerCP anti-CD4, APC anti-CD3 (Biolegend, USA), PerCP anti-CD8, and PE anti-CD184 (R&D Systems, USA). PE anti-Arginase-1 (R&D Systems, USA) was used for intracellular staining.

Briefly, 10^6 cells were stained with Abs according to the manufacturer's protocols and analyzed by FACS Calibur (Becton Dickinson Biosciences). For detection of ROS, splenocytes were incubated in serum-free medium with 2′-7′-dichlorofluorescein diacetate (DCFDA) (Sigma-Aldrich, USA) (2 μM) for 30 min at 37 °C. The reaction was stopped by RPMI-based culture medium containing 10% FBS (Sigma-Aldrich, USA). Cells were then washed with cold PBS and labeled with surface Abs. After incubation on ice for 20 min, cells were washed, fixed, and immediately analyzed by flow cytometry.

Unstained cells, single fluorochrome-stained cells, and cells stained as fluorescence-minus-one control were used to set up the flow cytometer.

MDSC GENERATION FROM BONE MARROW CELLS

Tibias of CD1 mice were surgically removed using sterile techniques, and bone marrow (BM) was flushed. Red blood cells were lysed using LBS. To obtain bone marrow-derived MDSCs (BM-MDSCs), 2.5×10^6 cells were plated into Petri dishes with 50 mm diameter in 5 ml of medium supplemented with GM-CSF (40 ng/ml) (Biolegend, USA) and IL-6 (40 ng/ml) (Life technologies, USA), the cytokines that have previously been defined as promoting MDSC generation from precursors [22]. TNFα (Life technologies, USA) was added to experimental tubes at the final concentration of 40 ng/ml. For both cultures, DMEM-based medium (Sigma-Aldrich, USA), supplemented with 2 mM L-glutamine, 20 μM 2-ME, 150 U/ml streptomycin, 200 U/ml penicillin, and 10% heat-inactivated FBS (Sigma-Aldrich, USA), was used. Cells were maintained at 37 °C in humidified atmosphere containing 5% CO_2 for 4 days. After that, the cells were washed and either used for *in vitro* T cell suppression assay or stained with fluorochrome-conjugated Abs and analyzed by flow cytometry.

In Vitro T Cell Suppression Assay

Mononuclear cells were obtained from spleens of intact mice by gradient centrifugation on Ficoll-Paque (GE Healthcare Bio-Sciences AB, Germany) gradient at 400 g for 30 min at 20 °C, labeled with 5 μM 6-

carboxyfluorescein succinimidyl ester (CFSE) (Sigma-Aldrich, USA) for 6 min at 4 °C, washed twice with cold PBS, and plated at the concentration of 2.5×10^5 cells/ml in 96-well round bottom plates in complete medium. Fifty micrograms per milliliter of concanavalin A (ConA, Sigma-Aldrich, USA) was used for activation of mitogen-induced T cell proliferation. Increased numbers of BM-MDSCs were added at the suppressor:responder ratios of 0.5:1 and 1:1, correspondingly. In 4 days, CFSE dilution in $CD8^+$ T cells was analyzed by flow cytometry.

ELISA

Sera was obtained from all subjects by centrifugation and stored at − 20 °C for determination of cytokines. The levels of TNFα, IL-10, IL-1β, IL-6, SDF-1α, GM-CSF, and S100 in control and experimental mice were analyzed by a quantitative sandwich ELISA in accordance with the manufacturer's recommendations using the kits: Mouse CXCL12/SDF-1α Quantikine ELISA (R&D Systems, USA); GM-SCF, IL-10, TNFα, IL-1β, IL-6 Mouse ELISA Max™ Standard ELISA Kit (BioLegend, USA); and Mouse S100 Calcium Binding Protein (S100) ELISA Kit (Mybiosource, USA). Absorbance was read at 450 nm, and the concentration of the cytokines was determined by extrapolation from a standard curve.

Statistical Analysis

Flow cytometric data were analyzed using BD CellQuest Pro software. Statistical analysis of the obtained data was performed using GraphPad Prism software version 4 (GraphPad Software, USA). Data are presented as scatter plots or column bar graphs with mean ± SD from at least six mice per group. A p value of less than 0.05 was considered statistically significant according to a two-tailed Student's t test and indicated as $*p < 0.05$, $**p < 0.005$, and $***p < 0.0005$ unless otherwise stated.

RESULTS

Chronic Inflammation Promotes Tumor Growth

To explore the impact of ongoing chronic inflammation on tumor progression, in 2 weeks after induction of adjuvant arthritis, the animals were subcutaneously inoculated with Ehrlich carcinoma cells. By the end of the second week after tumor cell inoculation, the ATA group showed considerably faster rate of tumor growth when compared to the TA animals not subjected to inflamed conditions (Fig. 1).

Increased Tumor Growth in Inflamed Mice Is Accompanied by Expansion of Splenic MDSCs

Earlier, we have shown that adjuvant arthritis as an experimental model of chronic inflammation was accompanied by peripheral accumulation of $CD11b^+Ly6G^{high}$ G-MDSCs and $CD11b^+CD49d^+$ M-MDSCs characterized by the ability to inhibit mitogen-activated T cell proliferation [12]. Using the Ly6C/Ly6G-based strategy, we also found that the numbers of splenic G-MDSCs defined as $CD11b^+Ly6G^{high}Ly6C^{low}$ and M-MDSCs defined as $CD11b^+Ly6G^-Ly6C^{high}$ were increased under chronic inflammation. Following the previous research [12], we observed an increase in the proportion of $CD11b^+Ly6G^{high}Ly6C^{low}$ and $CD11b^+Ly6G^-Ly6C^{high}$ cells by the end of the second week after induction of adjuvant arthritis, and the increase in M-MDSCs persisted until the fourth week (Fig. 2a, b). In addition, we examined whether CD49d staining can be used to distinguish $Ly6G^{high}Ly6C^{low}$ and $Ly6G^-Ly6C^{high}$ subsets from each other in our model of adjuvant arthritis. According to obtained results, CD49d was expressed mainly by $Ly6G^-Ly6C^{high}$ subset, but not by $Ly6G^{high}Ly6C^{low}$ (Fig. 2c), suggesting that CD49d marker can be alternatively used for identification of M-MDSCs. Therefore, for further experiments, we

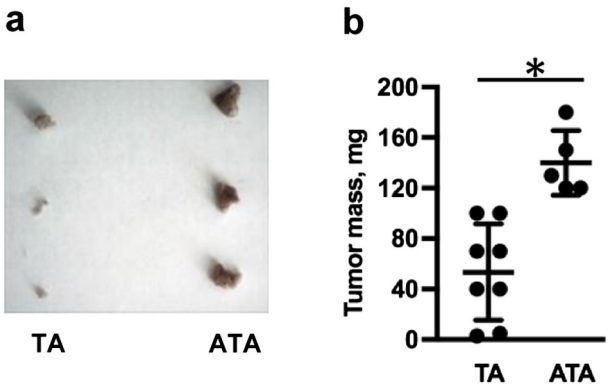

Fig. 1. Chronic inflammation promotes tumor growth**.** In 2 weeks after injection of CFA (ATA group) or PBS (TA group), mice were subcutaneously inoculated with Ehrlich carcinoma cells. By the end of the second week after tumor cell inoculation, mice were sacrificed and tumors were weighted. Representative (**a**) and cumulative (**b**) results for each group are indicated. Student's t test showed that there was a significant difference between the groups, as indicated: $*p = 0.004$.

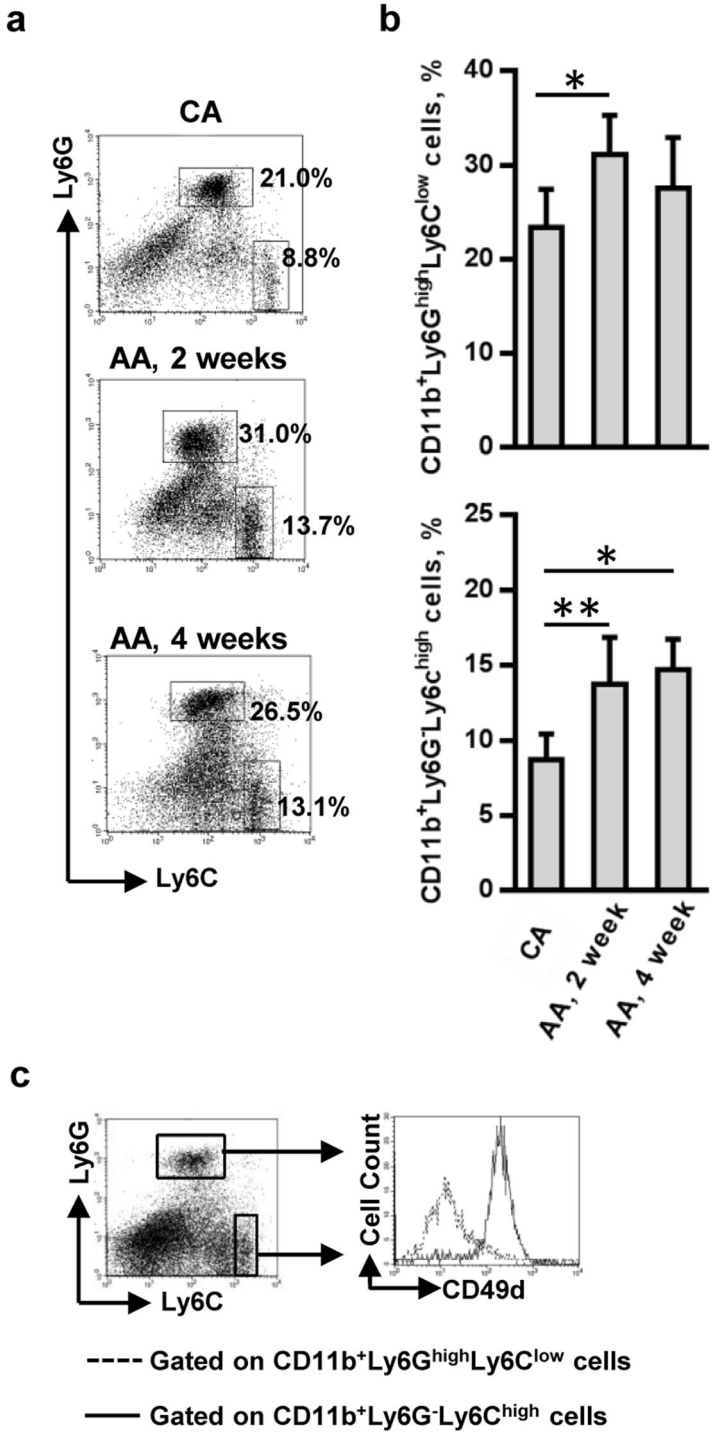

Fig. 2. Prevalence of granulocytic $CD11b^{+}Ly6G^{high}Ly6C^{low}$ and monocytic $CD11b^{+}Ly6G^{-}Ly6C^{high}$ myeloid cells is elevated in the spleen of mice with chronic inflammation. Splenocytes from the CA and AA mice were obtained in 2 and 4 weeks after initiation of adjuvant arthritis; washed and labeled with anti-CD11b, anti-Ly6G, and anti-Ly6C; and the frequency of G-MDSCs and M-MDSCs was analyzed. Each group included at least six mice. Representative flow cytometry (**a**) and cumulative (**b**) results for each group are shown. Significant differences between columns assessed by Student's *t* test are indicated as: $*p < 0.05$, $**p < 0.005$. **c** Freshly isolated splenocytes from the AA group were depleted of erythrocytes and stained with anti-CD11b, anti-Ly6G, anti-Ly6C, and anti-CD49d. CD49d expression was analyzed after gating on $CD11b^{+}Ly6G^{high}Ly6C^{low}$ (dotted) and $CD11b^{+}Ly6G^{-}Ly6C^{high}$ (solid) cells.

used both CD49d and Ly6C antibodies for identification of M-MDSCs.

Earlier, it was demonstrated that adoptive transfer of MDSCs obtained from CFA-treated mice strongly inhibited T cell-mediated tumor rejection [18]. To determine the impact of MDSCs induced by chronic inflammation on tumor growth in our model of inflammation-related tumor progression, splenocytes harvested from the experimental animals were analyzed for G-MDSCs and M-MDSCs. We detected a significant increase in the proportion of both splenic G-MDSCs and M-MDSCs in the AA, TA, and ATA groups when compared to the control group (Fig. 3a). Increased tumor growth in the ATA group was accompanied by a larger subpopulation of splenic M-MDSCs when compared to TA (Fig. 3a). To test the role of inflammation-induced MDSCs as a potential pathogenic factor, involved in tumor promotion, we depleted these cells with gemcitabine. Gemcitabine-mediated inhibition of MDSC accumulation has been well documented [23]. Direct anti-tumor effect of gemcitabine [23] was excluded by administration of gemcitabine 4 days prior tumor inoculation. Administration of gemcitabine in mice with adjuvant arthritis resulted in decreased frequencies of G-MDSCs and M-MDSCs (Fig. 3b) and delayed tumor growth (Fig. 3c) as compared to untreated mice with adjuvant arthritis.

Production of arginase-1 (Arg-1) and release of reactive oxygen species (ROS) by M-MDSCs and G-MDSCs, respectively, are shown to negatively regulate T cell functions [24]. Therefore, we further examined the activity of Arg-1 in M-MDSCs and the level of ROS in G-MDSCs. We observed a significant increase in ROS production by G-MDSCs in the TA and ATA groups as compared to the AA and CA groups (Fig. 4a). No difference in the production of Arg-1 by M-MDSCs between the animal groups was detected (Fig. 4b).

To assess the effect of chronic inflammation on the suppression of immunosurveillance against tumors, we analyzed the levels of $CD8^+$, $CD4^+$, and NK (defined as $CD3^-CD49b^+$) cells in the spleen of experimental mice. We found that the levels of the cell populations were not affected by ongoing chronic inflammation or tumor process when compared to the control group (data not shown).

MDSCs Obtained from the ATA Group Are Characterized by Increased *l*-Selectin Expression

The analysis of the pattern of migratory molecules demonstrated that both G-MDSC and M-MDSC subpopulations obtained from the ATA group were characterized by upregulated expression of CD62L when compared to CA, AA, and TA (Fig. 5a). In contrast, G-MDSCs isolated from the ATA and TA expressed CD184 marker at lower levels when compared to the CA and AA groups (Fig. 5b), while we detected no difference in the expression of CD184 by M-MDSCs among the groups (data not shown).

MyD88-dependent expansion of MDSCs has been demonstrated in tumor [25] and inflammation [26]. In our experiments, we used a chronic inflammation model induced by CFA containing heat-killed *Mycobacterium tuberculosis*. Considering *Mycobacterium tuberculosis* as a source of specific ligands predominantly recognized by TLR2 [27], we assessed expression of TLR2 by MDSCs. We found that both $CD11b^+Ly6G^{high}$ G-MDSC and $CD11b^+CD49d^+$ MDSC subpopulations expressed TLR2 at the same level (data not shown). Further analysis of these MDSC subsets demonstrated decreased TLR2 expression by G-MDSCs in the AA and ATA groups as compared to CA (Fig. 5c). No significant differences between the animal groups were found in the expression of TLR2 by M-MDSCs (data not sown).

ATA Animals Are Characterized by Elevated Serum Levels of S100 and TNFα

IL-6 [28], IL-1β [29], and TNFα [30] are often over-produced during a number of chronic inflammatory diseases. S100 is an important marker of inflammatory process and, together with SDF-1α and IL-10, can sustain and exacerbate tumor progression [31]. GM-CSF plays an important role in differentiation of MDSCs [32]. Therefore, we determined systemic levels of these cytokines and chemokines. The ATA animals demonstrated the highest serum levels of S100 and TNFα among all groups. Concentration of SDF-1α was elevated in the TA and ATA groups compared to the control animals (Fig. 6). We detected no difference in the levels of IL-1β, IL-10, IL-6, and GM-CSF between the animal groups (data not shown).

TNFα Modulates Suppressive Potential and Expression of CD62L by MDSCs *In Vitro*

To examine a possible effect of TNFα on modulating CD62L expression by MDSCs, we used mouse MDSCs, generated from the BM *in vitro*. First, freshly isolated mouse BM cells were treated with GM-CSF + IL-6 with or without TNFα for 4 days to allow MDSC induction. As shown in

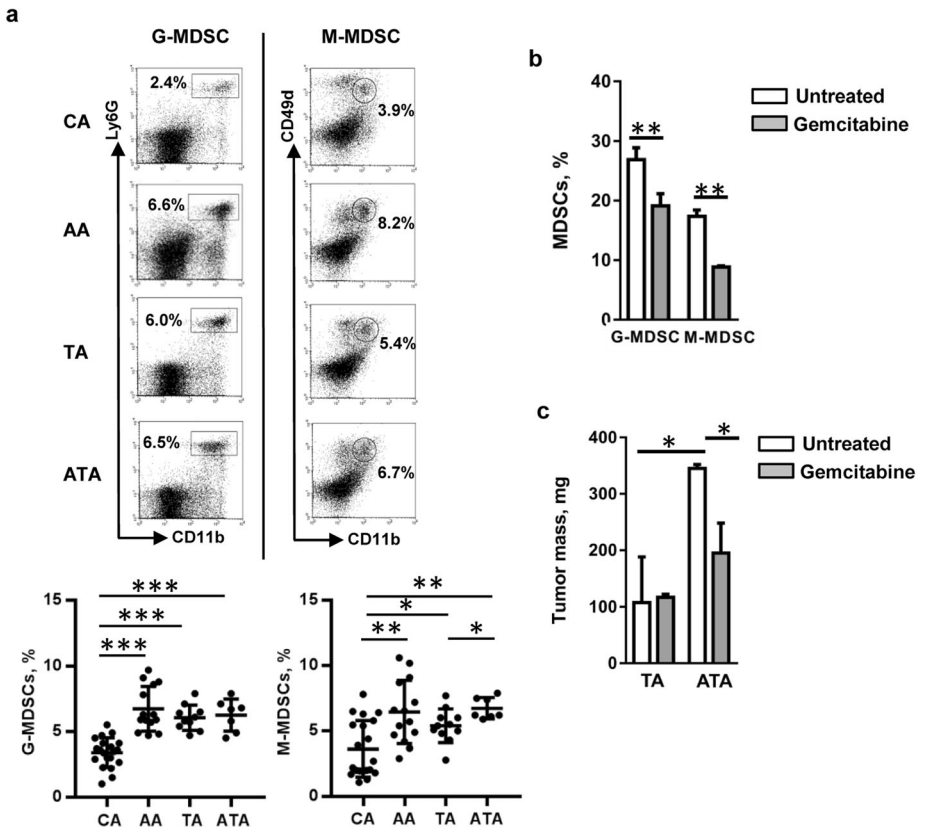

Fig. 3. Inflammation-induced MDSCs exert tumor-promoting functions. **a** Splenocytes received from the CA, AA, TA, and ATA mice were depleted of erythrocytes, washed extensively, and stained with fluorescent-conjugated antibodies. The frequency of G-MDSCs cells and M-MDSCs was analyzed. Representative results of G-MDSCs defined as $CD11b^{+}Ly6G^{high}$ and M-MDSCs defined as $CD11b^{+}CD49d^{+}$ and cumulative results for each group are shown. **b** For depletion of inflammation-induced MDSCs, mice received an intraperitoneal injection of PBS (untreated) or gemcitabine on day 7 and 10 after initiation of adjuvant arthritis. Ehrlich carcinoma cells were inoculated subcutaneously in 4 days after the last injection. Mice were sacrificed in 14 days after tumor inoculation, and the frequency of splenic G-MDSCs defined as $CD11b^{+}Ly6G^{high}Ly6C^{low}$ and M-MDSCs defined as $CD11b^{+}Ly6G^{-}Ly6C^{high}$ was analyzed. **c** TA and ATA received an intraperitoneal injection of PBS (untreated) or gemcitabine 7 and 4 days before tumor inoculation. Mice were sacrificed in 14 days after tumor inoculation and tumors were weighted. Cumulative data for each group ($n = 4$) are shown. Significant differences between columns assessed by Student's t test are indicated as: $^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.0005$.

Fig. 7a, in 4 days of stimulation, we consistently observed a significant increase in the number of $CD11b^{+}$ cells. T cell suppression assay showed that BM-MDSCs strongly suppressed $CD8^{+}$ T cell proliferation in a dose-dependent manner (Fig. 7b). Moreover, the addition of TNFα to BM-MDSC culture resulted in enhanced suppressive potential when compared to BM-MDSCs obtained by cultivation only with GM-CSF + IL-6 combination (Fig. 7b). Finally, we evaluated the expression of CD62L by MDSC subsets. We found that exogenous TNFα induced enhanced expression of CD62L by both G-MDSCs and M-MDSCs when compared to unstimulated cells, while the GM-CSF + IL-6 combination alone did not result in the increased expression of CD62L by both MDSC subsets (Fig. 7c).

DISCUSSION

Chronic inflammatory processes leading to sustained local or systemic immune suppression have been recognized as an important factor underlying tumor initiation and progression. However, the protumerigenic pathways of chronic inflammation remain not fully understood. In this study, we assessed the progression of a transplanted tumor under normal and chronic inflammatory conditions. We

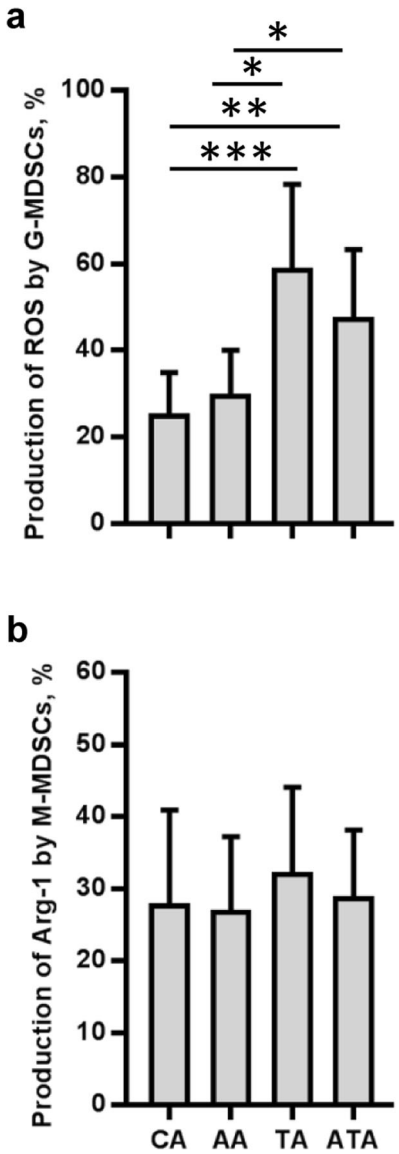

Fig. 4. The activation status of MDSCs obtained from inflamed and tumor mice. **a** Freshly isolated splenocytes from the CA, AA, TA, and ATA mice were depleted of erythrocytes and incubated in serum-free medium with 2 μM DCFDA for 30 min, then washed extensively and labeled with anti-Ly6G and anti-CD11b. The percentage of cells producing ROS was measured in the $CD11b^{+}Ly6G^{high}$ gate by flow cytometry. **b** Splenocytes received from CA, AA, TA, and ATA were stained with anti-CD11b and anti-CD49d or anti-CD11b, anti-Ly6C, anti-Ly6G, and anti-Arg-1, and the frequency of $Arg\text{-}1^{+}$ cells in the $CD11b^{+}CD49d^{+}$ or $CD11b^{+}Ly6G^{-}Ly6C^{high}$ gates was analyzed by flow cytometry. Significant differences between columns assessed by Student's *t* test are indicated as: $*p < 0.05$, $**p < 0.005$, and $***p < 0.0005$.

demonstrated that a sterile local chronic inflammatory process promoted a significantly faster rate of tumor growth, when compared to tumor growth under non-inflammatory conditions, which was accompanied by a systemic accumulation of MDSCs and increased serum levels of proinflammatory factors TNFα and S100. Using gemcitabine for depletion of inflammation-induced MDSCs, we further demonstrate that these cells are directly involved in tumor progression.

It has been well documented that various inflammation-associated mediators including IL-1β, IL-6, MCP-1, MIP-1α, GM-CSF, VEGF, PGE2, S100, SDF-1α, and TNFα are associated with tumor progression and required to drive MDSC accumulation and activation [19, 33]. In our model, enhanced generation of TNFα and S100 and increased tumor growth in inflamed mice were accompanied by an elevation of M-MDSC frequencies and a consistent increase in the expression of CD62L by both G-MDSC and M-MDSC subsets. CD62L, also known as L-selectin, is constitutively expressed by all classes of leukocytes and mediates leukocyte adhesion to blood vessel walls [34], a prerequisite step for cell migration from the circulatory system into peripheral tissues. Expression of CD62L has been shown to guide cells to secondary lymphoid organs, sites of inflammation, and tumor growth *via* high endothelial venules [35]. Earlier, it was shown that *in vitro*-generated MDSCs expressing high levels of CD62L efficiently migrated to secondary lymphoid organs and potently inhibited GVHD rejection limiting proliferation and activation of alloreactive T cells [36]. An ability of MDSCs to accumulate in lymph nodes in tumor-bearing mice has been also demonstrated [19]. This suggests that MDSC adhesion and migration in blood vessels are similar to the migration of leukocytes. Moreover, an extensive phenotype analysis performed by Liechtenstein et al. demonstrated that CD62L expression was a reliable MDSC surface marker: an *ex vivo* model showed that while dendritic cells progressively lost CD62L expression, MDSCs increased its expression [37].

An ability of TNFα to increase CD62L expression by BM progenitor cells has been documented earlier [38]. Given the increased serum amounts of TNFα in the ATA mice, we assessed the effect of TNFα on the expression of CD62L by MDSCs generated from the BM. *In vitro* cultures of BM-derived MDSCs demonstrated that exogenous TNFα increased expression of CD62L by both M-MDSC and G-MDSC subsets. Whether this is the case *in vivo* and whether other mechanisms exist requires further investigation. Nevertheless, the obtained results imply that TNFα

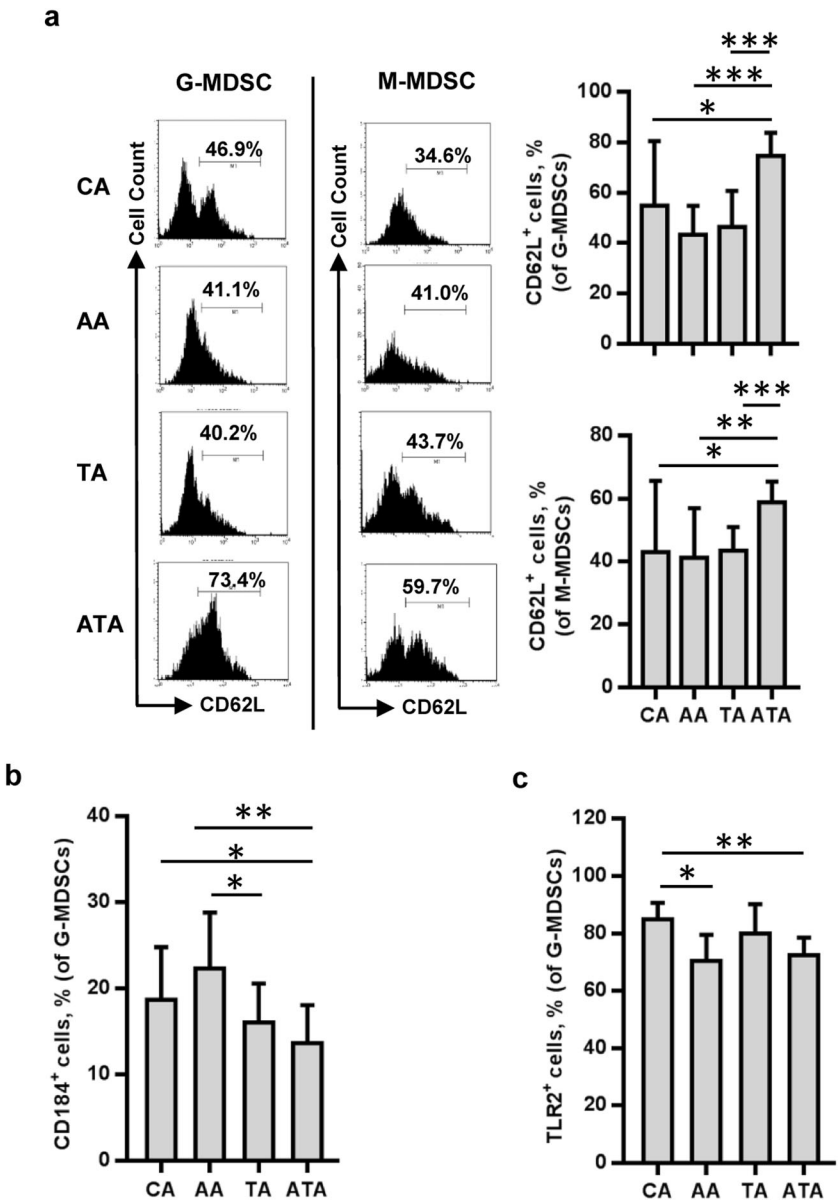

Fig. 5. The migratory potential of MDSCs obtained from inflamed and tumor mice**.** Splenocytes received from the CA, AA, TA, and ATA mice were depleted of erythrocytes, washed extensively, and stained with fluorescent-conjugated antibodies. **a** Representative and cumulative results of CD62L expression by G-MDSCs and M-MDSCs are shown. Cumulative results of CD184 (**b**) or TLR2 (**c**) expression by G-MDSCs defined as $CD11b^{+}Ly6G^{high}$ are demonstrated for each group ($n = 8–13$). Significant differences between columns assessed by Student's t test are indicated as: $*p < 0.05$, $**p < 0.005$, and $***p < 0.0005$.

released during chronic inflammatory processes may promote MDSC expansion *via* induction of CD62L expression. These results are in conjunction with the previous data showing a decreased accumulation of MDSCs in $Tnf^{-/-}$ mice within the tumor and during chronic inflammation [20, 21]. Interestingly, in the absence of TNFα, the production of IFNγ, IL-1, IL-6, and IL-12 was upregulated; nevertheless, the proinflammatory conditions generated in the absence of TNFα could not support MDSC accumulation [21]. Our results showed that the tumor promotion in mice with chronic inflammation was not associated with

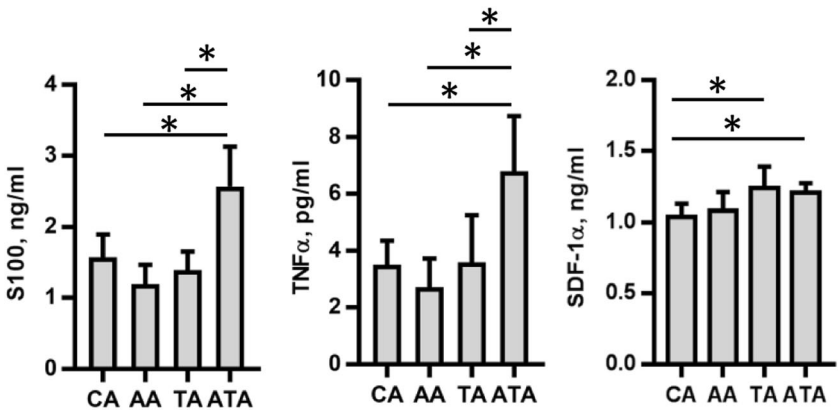

Fig. 6. Levels of sera S100, TNFα, and SDF-1α are increased in the ATA mice. Levels of S100, TNFα, and SDF-1α were examined by ELISA in serum of the CA, AA, TA, and ATA mice. Each group included at least six mice. *p* values calculated using the Student's *t* test and indicated as: $*p < 0.05$.

increased serum levels of IL-1 or IL-6, emphasizing a critical role of TNFα signaling in MDSC accumulation, particularly the M-MDSC subset, upregulated frequencies of which we observed in the circulation of the ATA mice. Moreover, previous studies demonstrated that adjuvant-induced M-MDSCs produced higher levels of TNFα [13]. Therefore, the increased numbers of M-MDSCs, observed in the ATA mice, could also be an additional source of exogenous TNFα, which could operate as an autocrine feedback loop that sustains peripheral expansion of MDSCs *via* induction of CD62L expression.

In addition, it has been earlier shown that TNFα induces increased S100A8 and S100A9 and RAGE production in MDSCs, which is associated with poor MDSC differentiation into mature myeloid cells, conditions that could be reversed by neutralizing TNFα or blocking RAGE and its ligands, the S100A8 and S100A9 proteins [21]. These data may explain the increased concentrations of serum S100 observed by us in the ATA mice, which could be a result of an increased generation of S100 by MDSCs induced by TNFα. Whether this cytokine acts in concert with TNFα to increase CD62L expression by MDSCs is a subject for future studies.

Exogenous TNFα displayed broad effects on *in vitro* generated MDSCs. The addition of TNFα to the IL-6 + GM-CSF combination was able to generate BM-MDSC cultures with augmented suppressive activity against $CD8^+$ T cells. The finding agrees with the previously reported research showing that the suppressive activity of MDSCs from $Tnf^{-/-}$ mice was significantly reduced as compared to that observed in $Tnf^{+/+}$ mice [21]. However, functional analysis of MDSCs obtained from experimental mice demonstrated no difference in the production of Arg-1 and ROS between inflamed and not inflamed mice with tumor. Possibly, other suppressive factors are involved in TNFα-dependent activation of MDSCs.

Further analysis of migratory molecules demonstrated multiple pathways involved in MDSC expansion in the tumor model and the model of chronic inflammation. Our data showed a decrease in CD184 expression by G-MDSCs in both groups of tumor mice (ATA and TA), which was accompanied by increased serum levels of SDF-1α. SDF-1α, also known as C-X-C motif chemokine 12 (CXCL12), is upregulated in many types of tumors and acts mainly *via* its receptor CD184, also known as CXCR4. Recently, it has been demonstrated that CXCR4-SDF-1α axis is involved not only in tumor cell trafficking, but also in the accumulation of suppressive immune cells, particularly MDSCs. Thus, the CXCR4-SDF-1α signaling pathway was reported to be involved in MDSC migration in a breast cancer mouse model [39]. PGE2 was shown to regulate the accumulation of M-MDSCs in ovarian cancer *via* induction of the CXCR4 expression by M-MDSCs and the production of CXCL12 by ovarian cells [40]. CXCR4-SDF-1α exerted a pro-migratory effect on MDSCs by activating p38 protein-kinase in a lung cancer mouse model [41]. In our tumor model of subcutaneous carcinoma, increased serum levels of SDF-1α were accompanied by decreased levels of surface CXCR4 on G-MDSCs, which may be explained by the well documented internalization of the CXCR4 receptor complex triggered by the binding with its ligand SDF-1α [42]. Interestingly enough,

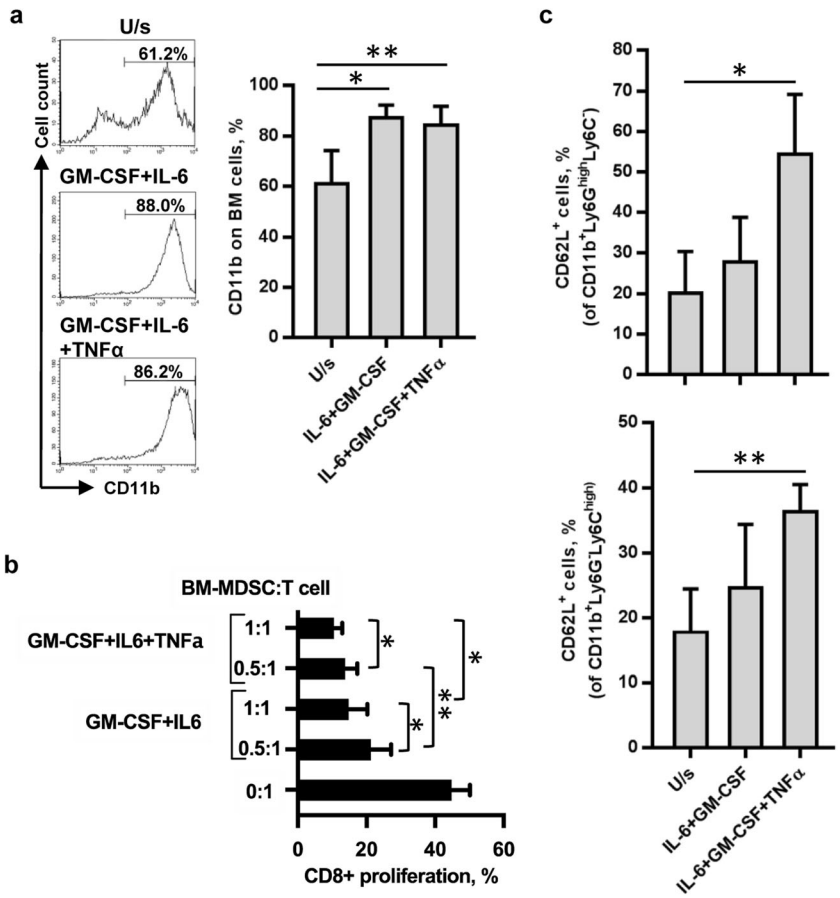

Fig. 7. TNFα-stimulated BM-derived MDSCs exert enhanced suppressive activity and expression of CD62L. **a** Accumulation of $CD11b^+$ cells after 4 days of cultivation of mouse BM cells with IL-6 + GM-CSF or GM-CSF + IL6 + TNFα cytokine combinations is demonstrated as representative histograms and cumulative results of six independent experiments. **b** MDSCs derived from BM in the presence of GM-CSF + IL6 or GM-CSF + IL6 + TNFα were co-cultured with ConA-stimulated PBMCs from intact mice for 72 h, and the level of suppression of $CD8^+$ T cell proliferation was measured using CFSE-test. Five independent experiments were performed for each group; each repeated in triplicate. Cumulative results for each group are shown. **c** Expression of CD62L by G-MDSCs defined as $CD11b^+Ly6G^{high}Ly6C^{low}$ and M-MDSCs defined as $CD11b^+Ly6G^-Ly6C^{high}$ was analyzed after cultivation of BM cells in the presence of GM-CSF + IL6 or GM-CSF + IL6 + TNFα for 4 days. Cumulative results of six independent experiments are represented. Significant differences between columns assessed by Student's *t* test are indicated as: $*p < 0.05$ and $**p < 0.005$.

although CXCR4 was also expressed by M-MDSCs (data not shown), we did not observe any alterations in its expression in tumor mice.

TLR2 was another receptor that participated in activation of G-MDSCs but not M-MDSCs. Recent studies have demonstrated that TLR pathways play an important role in the expansion of MDSCs in cancer and chronic inflammation. TLR2 activation by Pam2CSK4 lipopeptide leads to accumulation of $CD11b^+Gr1^+$ MDSCs in the tumors, as well as peripheral tissues of tumor-bearing mice [43]. Similar to Pam2CSK4, endogenous TLR2 ligand, Hsp72, derived from cancer cells, promoted tumor growth by inducing generation and systemic accumulation of MDSCs [44]. One other study demonstrated that TLR2 ligand administration did not enhance the suppressive activity of G-MDSCs, but did enhance the suppressive activity of M-MDSCs by inducing their differentiation into $iNOS^+$ macrophages that inhibited T cell proliferation through NO production [45]. Our data showed alterations in the TLR2 expression by G-MDSCs in mice with adjuvant-induced arthritis, suggesting that the TLR2 pathway can be specifically involved in the expansion of G-MDSCs in this model of chronic inflammation. Earlier, it was shown that TLR ligands decreased the surface level of the receptor but upregulated the mRNA levels of the protein as a compensatory mechanism to counteract ligand-

induced TLR downregulation [46]. This observation may explain the reduction in the TLR2 cell surface expression by G-MDSCs in mice with chronic inflammation. Thus, the alterations in the expression of CXCR4 and TLR2 by G-MDSCs in our models of chronic inflammation and tumor reflect their high tropism to peripheral tissues. In the ATA mice, such involvement of several migratory pathways could contribute to an enhanced recruitment of G-MDSCs into sites of chronic inflammation and tumor.

In summary, the obtained data further substantiate the link between chronic inflammation and cancer. Our model of inflammation-related tumor progression suggests that chronic inflammation facilitates tumor growth *via* activation of CD62L-expressing MDSCs. Further studies are required to investigate whether elevated expression of CD62L by MDSCs favors increased distribution of MDSCs into tumor sites and secondary lymphoid organs within the vicinity of antigen-specific T cells. Another major finding of this work is the principal role of the proinflammatory mediator TNFα that can potentially upregulate expression of CD62L by MDSCs and increase their suppressive activity against immune effector cells. The results suggest that TNFα blockade can be a useful strategy to dampen MDSC migration and decrease their suppressive potential in clinic.

It should be pointed out that the present study has several limitations. The majority of experimental studies on the role of chronic inflammation in the tumorigenesis are conducted using models of inflammation-associated carcinogenesis; among them, the model of colitis-associated colon cancer is the most popular. These models help investigate the mechanisms of cancer development given the background of inflammation localized in the same tissue, whereas we used the model of adjuvant-induced arthritis, which is not histologically associated with cancer development. Therefore, our experimental model differs in certain aspects from the proposed natural disease process. However, the adjuvant-induced model of inflammation allowed us to examine the systemic effect of a local chronic inflammatory process on the promotion of distant tumor growth. The results obtained by us highlight the importance of chronic inflammation control for prevention of cancer development even in sites remote from the focus of inflammation.

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COMPLIANCE WITH ETHICAL STANDARDS

All experiments were carried out in compliance with the Guide for Care and Use of Laboratory Animals and approved by the Ethical Committee of M.A. Aitkhozhin’s Institute of Molecular Biology and Biochemistry, Almaty, Kazakhstan.

Conflict of Interest. The authors declare that they have no competing interests.

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