The Inflammasome Component Nlrp3 Impairs Antitumor Vaccine by Enhancing the Accumulation of Tumor-Associated Myeloid-Derived Suppressor Cells

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Abstract

The inflammasome is a proteolysis complex that generates the active forms of the proinflammatory cytokines interleukin (IL)-1β and IL-18. Inflammasome activation is mediated by NLR proteins that respond to microbial and nonmicrobial stimuli. Among NLRs, NLRP3 senses the widest array of stimuli and enhances adaptive immunity. However, its role in antitumor immunity is unknown. Therefore, we evaluated the function of the NLRP3 inflammasome in the immune response using dendritic cell vaccination against the poorly immunogenic melanoma cell line B16-F10. Vaccination of Nlrp3⁻/⁻ mice led to a relative 4-fold improvement in survival relative to control animals. Immunity depended on CD8⁺ T cells and exhibited immune specificity and memory. Increased vaccine efficacy in Nlrp3⁻/⁻ hosts did not reflect differences in dendritic cells but rather differences in myeloid-derived suppressor cells (MDSC). Although Nlrp3 was expressed in MDSCs, the absence of Nlrp3 did not alter either their functional capacity to inhibit T cells or their presence in peripheral lymphoid tissues. Instead, the absence of Nlrp3 caused a 5-fold reduction in the number of tumor-associated MDSCs found in host mice. Adoptive transfer experiments also showed that Nlrp3⁻/⁻ MDSCs were less efficient in reaching the tumor site. Depleting MDSCs with an anti–Gr-1 antibody increased the survival of tumor-bearing wild-type mice but not Nlrp3⁻/‒ mice. We concluded that Nlrp3 was critical for accumulation of MDSCs in tumors and for inhibition of antitumor T-cell immunity after dendritic cell vaccination. Our findings establish an unexpected role for Nlrp3 in impeding antitumor immune responses, suggesting novel approaches to improve the response to antitumor vaccines by limiting Nlrp3 signaling. Cancer Res; 70(24); 10161–9. ©2010 AACR.

Introduction

NLR family, pyrin domain containing 3 (Nlrp3) is a member of the nucleotide-binding domain and leucine-rich repeat containing gene family of intracellular sensors. When activated, Nlrp3 forms a protein complex called the inflammasome (1–3). The inflammasome combines Nlrp3 with the adaptor molecule ASC/PYCARD/TMS/CARD5, Cardinal, and pro–caspase-1 (4) to form a multimer. The result is the proteolytic maturation of caspase-1, which cleaves and activates proIL-1β and proIL-18 to generate interleukin (IL)-1β and IL-18 (3).

Nlrp3 senses a variety of microbial and nonmicrobial molecular motifs. The microbial signals are part of the pathogen-associated molecular patterns (PAMP) and include gram-positive and -negative bacteria, RNA and DNA viruses, polynosinic:polycytidylic acid, and lipopolysaccharide (LPS; ref. 1). The nonmicrobial signals include exogenous compounds such as asbestos and endogenous signals such as urate crystals (1). Many of these nonmicrobial stimuli are referred to as damage-associated molecular patterns (DAMP; ref. 5). Activation by these signals not only promotes innate immunity but also enhances Th1, Th2, and Th17 responses (6). Thus, the inflammasome responds to many pathologic insults and participates in multiple immune pathways (7).

In contrast to this literature on PAMPs and DAMPs, there have been few studies characterizing Nlrp3 in the tumor microenvironment, though stimuli for its activation are no doubt present (8). However, studies on IL-1β suggest a potential tumor promoting role in several mouse models (9–10). Conversely, reduction of IL-1β diminished metastases to the lung (11) and eliminating IL-1β prevented progression in a murine melanoma model (12).

Human studies have been less definitive but remain consistent with the murine data. For example, gastric cancer is...
associated with genetic polymorphisms linked to enhanced IL-1β expression (13). Similar studies on IL-18 polymorphisms have shown an increased risk for other epithelial cancers (14). More directly, serum IL-18 concentrations are inversely correlated with survival in hepatocellular cancer (15).

These cytokines can contribute to tumorigenesis in several ways though more recent attention has focused on their role in promoting myeloid-derived suppressor cells (MDSC; refs. 16, 17). MDSCs are a heterogeneous population of immature myeloid cells that are most readily identified in the mouse by their expression of Gr-1 and CD11b (18). These cells suppress T-cell responses directly by a variety of mechanisms (19). MDSCs also contribute to tumorigenesis indirectly by inducing regulatory T cells (20) and a Th2 immune response (21), suppressing natural killer (NK) cells (22), and increasing angiogenesis (23). Clinical studies have documented these cells in several human cancers (24) including head and neck (25), renal cell (26), and hepatocellular (22) cancers. Both murine and human studies have found the number of MDSCs increases with tumor burden (24, 27).

These data suggest that Nlrp3 activation could inhibit the antitumor immune response to a cancer vaccine by enhancing the function of immunosuppressive cells. We were led to test this hypothesis after completing gene array studies that showed an association between Nlrp3 and MDSCs.

Materials and Methods

Mice

Nlrp3−/− mice were generated as described (28). EGFP × Nlrp3−/− transgenic mice were produced by crossing F1 progeny of EGFP transgenic and homozygous Nlrp3−/− mice. All other mice were purchased from The Jackson Laboratory. All experiments were conducted using protocols approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Dendritic cell vaccination model

Tumor lines were purchased from the American Type Culture Collection and were expanded by 6 passages in 7.5% fetal calf serum/Dulbecco’s modified Eagle’s medium (Gibco). B16-F10 cells were authenticated by the presence of melanin. Subcutaneous tumors were formed by injecting either 1 × 10⁶ cells (B16-F10, Lewis Lung) or 5 × 10⁵ cells (EG7-OVA) in the left leg. The leg diameter was measured 3 times a week. Mice were euthanized when this diameter was greater than 6 mm and the survival time was interpolated from the last 2 measurements. A tumor was designated as “nonpalpable” if the left leg diameter was within 0.2 mm of the right leg and the tumor was visible on gross dissection.

Dendritic cells (DC) were generated from bone marrow cultures treated with granulocyte macrophage colony stimulating factor and IL-4 (Peprotech Inc.; ref. 29). DCs were pulsed at an 8:1 ratio with B16-F10 lysates formed by irradiation (16,000 rad), 5 rounds of freeze/thaw, and shearing by a 30-gauge needle. After antigen pulsing, DCs were matured with 100 ng/mL of LPS for 24 hours. A total of 1 × 10⁶ cells were subcutaneously injected on days 3 and 10 after tumor injection.

Cell depletion was accomplished by the intraperitoneal injection of 200 μg of PK136 (NK cell) and RB6-8C5 (MDSC) and 500 μg of 53.6.72 (CD8 T cell) and GK1.5 (CD4 T cell) mAb (BioExpress). The injection schedule for lymphoid cell depletion was day −1, day 0, and then biweekly. MDSC depletion injections were biweekly starting on day 6.

Flow cytometry

Flow cytometry was performed as previously described (30). For the migration assays, MDSCs were harvested from the spleens of EGFP transgenic C57BL/6 or Nlrp3−/− mice by immunomagnetic bead separation (Miltenyi) 2 weeks after intravenous tumor injection. These cells were then cryopreserved. Nlrp3−/− mice were injected with 25,000 B16-F10 cells whereas wild-type (WT) mice received 1 × 10⁶. When the tumor was palpable, the mice received an intravenous injection of 5 × 10⁵ EGFP MDSCs. Three days after this injection, the tumor was harvested, digested, and processed for flow cytometry.

MDSC assay

MDSCs for the in vitro suppression assays were harvested by FACsorting from the lungs of WT and Nlrp3−/− mice 2 weeks after intravenous injection with 1 × 10⁶ B16-F10 cells. Immunosuppression was evaluated by adding these cells to a mixed lymphocyte reaction (MLR). Stimulator cells were taken from the adherent fraction of BALB/cj splenocytes after 2 hours of culture; responder cells were harvested from the nonadherent fraction of C57BL/6j splenocytes. Stimulators and CD11b+ Gr-1+ cells were treated with mitomycin C and responders were labeled with carboxyfluorescein diacetate, succinimidyl ester.

Real-time PCR

Total RNA was isolated from sorted cells using RNeasy columns (Qiagen) and converted to cDNA with random hexamers and MMLV Reverse Transcriptase (Invitrogen). PCR amplification was performed using Nlrp3 specific primers and probes 5′-CTCCCGCATCTCATTGTTG-3′, 5′-GCCGTAGCGACTTGTGA-3′, and FAM-CCACACTCACC-TAGACCGC-TAMRA with TaqMan PCR reagents and 7900HT Thermocycler (Applied Biosystems). Expression values were normalized to cell number.

Statistics

Data are reported as a mean ± SEM. Results were considered significant if P ≤ 0.05 as determined by the Mann-Whitney test. Comparisons in survival were done by the Cox proportional hazard regression method.

Results

The Nlrp3 is expressed in MDSCs

We have previously shown that peripheral tolerance mechanisms are a major impediment to successful antitumor vaccines in the FVB/neu mouse (31). To delineate mechanisms by which MDSCs function in this model, we utilized
Figure 1. Nlrp3 is expressed by tumor-associated myeloid cells. A, Expression of Nlrp3 by real-time PCR in (1) Gr-1⁺, CD11b⁺; (2) Gr-1⁻, CD11b⁻; and (3) Gr-1⁺, CD11b⁻ sorted cells. On the left are representative dot plots of sorted cells from a subcutaneous B16-F10 tumor in a vaccinated WT (A) and Nlrp3⁻/⁻ mouse (B). On the right are bar graphs of Nlrp3 transcripts normalized to cell number.

Figure 2. DC vaccination improves survival in Nlrp3⁻/⁻ mice. A, survival curves of WT and Nlrp3⁻/⁻ mice after receiving a subcutaneous injection of $1 \times 10^5$ B16-F10 melanoma cells. B, survival of Nlrp3⁻/⁻ mice was significantly improved after receiving $1 \times 10^5$ tumor lysate-pulsed DCs (DC Vx) on days 3 and 10 after tumor injection. No improvement was seen in WT mice. C, Nlrp3⁻/⁻ mice show improved survival after receiving vaccines from either Nlrp3⁻/⁻ or WT mice. D, WT mice show no improvement in survival after receiving vaccines from WT or Nlrp3⁻/⁻ mice.

16 genes, Nlrp3 and IL-1β were substantially increased in expression (Nlrp3, 11.9 ± 3.2-fold; IL-1β, 3.85 ± 0.7-fold) in the tumor environment. These results suggested activation of the Nlrp3 inflammasome occurs in MDSCs present in the tumor microenvironment.

We tested the role of the Nlrp3 inflammasome more directly using the B16-F10 tumor and B6 mice whose background would allow us to compare WT with Nlrp3⁻/⁻ mice. First, we verified the expression of Nlrp3 in the tumor-associated Gr-1⁺, CD11b⁺ cells, which had been isolated by cell sorting. Both Gr-1⁺, CD11b⁺ and Gr-1⁻, CD11b⁻ cells expressed Nlrp3 at transcript numbers significantly greater than found in CD11b⁻ cells (4.30 ± 0.13, 4.69 ± 0.94 versus 0.00 ± 0.0, $P = 0.033$; Fig. 1A). Nlrp3 was not expressed in Nlrp3⁻/⁻ mice (Fig. 1B).

DC vaccination improves survival in Nlrp3⁻/⁻ mice

We then compared the survival of WT and Nlrp3⁻/⁻ mice that received a subcutaneous injection of $1 \times 10^5$ B16-F10 melanoma cells. In this model, none of the WT or Nlrp3⁻/⁻ mice survived (Fig. 2A). The median survival was also not significantly different (15.3 versus 16.6 days, $P > 0.05$).

The survival of Nlrp3⁻/⁻ mice could be improved by administering a DC vaccine on days 3 and 10 after tumor injection. In these experiments, mice received inoculations of $1 \times 10^5$ WT DCs pulsed with B16-F10 tumor cell lysate. Although only 9.1% of the WT mice showed long-term survival, survival in the Nlrp3⁻/⁻ mice increased to 35%. The hazard ratio favoring survival in the Nlrp3⁻/⁻ mice was 2.4 ($1.2$–$4.8; $P = 0.017$; Fig. 2B).

The survival benefit was not limited to the B16-F10 model. WT and Nlrp3⁻/⁻ mice were also subcutaneously injected with $5 \times 10^5$ E.G7-OVA tumor cells and then treated with peptide
pulsed DCs using the same schedule. As before, Nlrp3\(^{-/-}\) mice had a substantial improvement in survival compared with WT mice (33.3% versus 62.5%). The hazard ratio favoring survival in the Nlrp3\(^{-/-}\) mice was 2.1 (1.6–3.1; \(P = 0.023\); Supplementary Fig. S1).

**Nlrp3 expression by the host limits the effectiveness of the DC vaccine**

Because this survival advantage may have been dependent on the expression of Nlrp3 by the DCs, we compared survival in Nlrp3\(^{-/-}\) mice treated with DC vaccines from WT or Nlrp3\(^{-/-}\) mice. Thirty-three percent of the Nlrp3\(^{-/-}\) mice injected with Nlrp3\(^{-/-}\) DC vaccine survived compared with 40% injected with the WT vaccine (\(P \geq 0.05\); Fig. 2C). Thus, the Nlrp3\(^{-/-}\) DC vaccine produced survival that was comparable with WT vaccines in Nlrp3\(^{-/-}\) mice.

Subsequently, we evaluated the effectiveness of vaccination using Nlrp3\(^{-/-}\) and WT DCs in WT mice. None of the WT mice survived. Those receiving the WT vaccine had a median survival of 21.8 days versus 16.1 days with the Nlrp3\(^{-/-}\) vaccine (\(P \geq 0.05\); Fig. 2D). From these data, we concluded the poor outcome in WT mice was due to Nlrp3 expression by host cells and not by the cells given with the vaccine.

**The benefit of the DC vaccine in Nlrp3\(^{-/-}\) mice is CD8 dependent**

The immunologic memory of the vaccinated Nlrp3\(^{-/-}\) mice was tested by rechallenging these mice 3 months after their initial tumor exposure. Of the 11 rechallenged Nlrp3\(^{-/-}\) mice, 10 survived a second tumor injection (90.9%; Fig. 3A). Two of the surviving WT mice were also rechallenged and survived (50%). Because of the low number of surviving WT mice, we were unable to establish statistical significance. Nevertheless, these experiments showed an antitumor memory response in the Nlrp3\(^{-/-}\) mice and implied the improved outcome was due to an enhanced immune response to the vaccine.

The specificity of this tumor protection was examined by challenging surviving Nlrp3\(^{-/-}\) mice with an unrelated tumor. Eight Nlrp3\(^{-/-}\) mice received \(1 \times 10^6\) Lewis lung carcinoma cells (LLCa) and 2 mice survived (25%; Fig. 3A). This result was significantly less than those rechallenged with B16-F10 cells (\(P = 0.003\)) but not different from naïve Nlrp3\(^{-/-}\) mice injected with LLCa cells (survival = 20%). Therefore, the immunologic memory generated after vaccination in Nlrp3\(^{-/-}\) mice was specific only for the tumor used in the vaccine.

The efficacy of the adaptive immune response was further tested by measuring survival after antibody depletion of CD4\(^+\), CD8\(^+\), or NK cells (Supplementary Fig. S2). The effect of cell depletion was measured by calculating a hazard ratio using Cox regression analysis. The survival of all 3 cohorts of cell-depleted Nlrp3\(^{-/-}\) mice was decreased compared to the control Nlrp3\(^{-/-}\) mice (Fig. 3B and C). However, this finding was only statistically significant for Nlrp3\(^{-/-}\) mice after the depletion of CD8\(^+\) T cells (hazard ratio = 2.06, \(P = 0.028\)). Therefore, the enhanced activity of tumor vaccination in the Nlrp3\(^{-/-}\) mice required CD8\(^+\) T cells.
This conclusion was supported by determining the number of CD8 effector T cells from the lymph nodes of DC-vaccinated mice. Tumor-draining nodes were harvested on day 14 following DC vaccinations on days 3 and 10. The number of CD62L+CD69+ effector cells was measured by flow cytometry. There were no differences in the number of CD8 effector cells between WT and Nlrp3−/− mice either with or without tumor injection. However, differences became apparent when the tumor-injected mice were further subdivided into groups with palpable or nonpalpable tumors. Using this analysis, Nlrp3−/− mice with no palpable tumor had significantly more CD8 effector cells than WT mice (18.1 ± 0.4 versus 3.5 ± 0.5 x10^4 cells, P = 0.002; Fig. 3D). There were no differences in the number of CD44 effector or in the number of CD8+ cells in mice with palpable tumors.

**Nlrp3−/− MDSCs are morphologically and functionally equivalent to WT MDSCs in vitro**

Given this result, we compared the microscopic appearance and the functional activity of MDSCs from Nlrp3−/− and WT tumor-bearing mice. Tumor-associated MDSCs from both types of mice could be further divided into 2 subpopulations based on their expression of Gr-1 and CD11b. The Gr-1Hi, CD11b+ cells had a neutrophil morphology and the Gr-1Int, CD11b+ cells had a monocytic morphology. These subpopulations corresponded to the granulocytic and monocytic MDSCs described by Youn and colleagues (32). However, there were no microscopic differences between Nlrp3−/− and WT MDSCs within each subpopulation (Fig. 4A).

Because the number of MDSCs within the tumors was inadequate for *in vitro* suppression assays, we isolated these cells from the lungs of WT and Nlrp3−/− mice with B16-F10 metastasis. Once again, 2 subpopulations of MDSCs could be identified by CD11b and Gr-1 expression. The monocytic MDSCs displayed a greater suppressive capacity compared with the granulocytic MDSCs. However, we did not find differences in T-cell suppression between WT and Nlrp3−/− MDSCs from either subpopulation (Fig. 4B). There were also no differences in the suppressive ability of MDSCs isolated from the spleens of WT and Nlrp3−/− mice with metastatic melanoma after DC vaccination (Supplementary Fig. S3).

Regardless of the differences among Gr-1Hi and Gr-1Int MDSCs, the central observation from these experiments was that no differences were detected when comparing cells from WT and Nlrp3−/− mice. These data indicate the disparity in the vaccine response between the WT and Nlrp3−/− mice was not due to differences in the suppressive capacity of their respective MDSCs.

**Nlrp3−/− mice have fewer MDSCs at the tumor site**

Because the survival advantage of the Nlrp3−/− mouse could not be explained by functional differences in MDSCs, we hypothesized this advantage was due to a reduction in the number of MDSCs. We found no significant differences in the number of Gr-1+, CD11b+ cells isolated from tumor-draining lymph nodes (TDLN) 14 days after tumor injection (Fig. 5A). There was a trend to a lower percentage of splenic MDSCs in the Nlrp3−/− mice but this finding was not statistically significant (1.61 ± 0.24% versus 2.67 ± 0.40%, P = 0.11; Fig. 5A).

We next evaluated the number of MDSCs within the tumor by immunofluorescent microscopy. Mice were included in this analysis if their tumor was visible by light microscopy. This approach revealed a 5-fold increase in the number of CD11b+ Gr-1+ cells in the tumor and surrounding stroma of WT mice compared with Nlrp3−/− mice [18.6 ± 3.0/low-power field (LPF) versus 3.5 ± 0.5/LPF, P = 0.02; Fig. 5B; Supplementary Fig. S4].

Because immunohistochemistry cannot distinguish between Gr-1Hi and Gr-1Int cells, the percentage of these 2 populations was measured using flow cytometry. Tumors

![Figure 4](https://www.aacrjournals.org)
were harvested when palpable; mice without tumor were excluded. Gating on the Gr-1<sup>+</sup>, CD11b<sup>+</sup> cells revealed a 4.7-fold increase of the monocytic MDSCs in WT mice compared with Nlrp3<sup>−/−</sup> mice (2.1 ± 0.5% versus 0.45 ± 0.24%, P = 0.003), whereas a 6-fold increase was noted in the granulocytic MDSCs (0.54 ± 0.18% versus 0.09%, P = 0.003; Fig. 5C). Thus, both flow cytometry and immunohistochemistry showed a significant reduction in the number of tumor-associated MDSCs in Nlrp3<sup>−/−</sup> compared with WT mice.

**Depletion of MDSCs improves survival in vaccinated WT but not Nlrp3<sup>−/−</sup> mice**

We established that the decrease in MDSCs accounted for the improved survival in Nlrp3<sup>−/−</sup> mice by measuring survival following MDSC depletion. Anti–Gr-1 antibody was injected twice a week beginning on day 6. This treatment produced a 2.6-fold decrease in MDSCs (Supplementary Fig. S5). Depletion of MDSCs eliminated the survival advantage of the vaccinated Nlrp3<sup>−/−</sup> mice over WT mice (hazard ratio = 1.15, P = 0.75; Fig. 5D). This change was exclusively due to an improvement in overall survival in the WT mice from 9.1% to 38.9% (hazard ratio = 2.06, P < 0.05). There was no difference in overall survival of the vaccinated Nlrp3<sup>−/−</sup> mice after MDSC depletion (40.0% versus 35.0%, P = 0.82). As a control, we measured survival in unvaccinated WT and Nlrp3<sup>−/−</sup> mice following MDSC depletion. Survival was not significantly different from unvaccinated mice without MDSC depletion (Supplementary Fig. S6). Together, these results strongly suggested the decreased number of tumor-associated MDSCs accounted for the increased efficacy of the DC vaccine in Nlrp3<sup>−/−</sup> mice.

**Nlrp3 expression promotes migration of MDSCs into the tumor**

We assessed the effect of Nlrp3 on migration by isolating splenic MDSCs from WT and Nlrp3<sup>−/−</sup> mice and then transferring those cells to Nlrp3<sup>−/−</sup> mice with subcutaneous tumors. A population of MDSCs with greater than 90% purity could be effectively isolated with proper titration of the Gr-1 antibody and immunomagnetic bead selection (Supplementary Fig. S7). Furthermore, there were no differences in percentages of the subpopulations between WT and Nlrp3<sup>−/−</sup> cells (Fig. 5E).

This approach revealed that significantly fewer Nlrp3<sup>−/−</sup> MDSCs migrated into the tumor compared with WT MDSCs (178.1 ± 91.0 cells/tumor versus 448.0 ± 36.1 cells/tumor,
These results were not biased by tumor size because no difference in tumor size was detected at the time of injection (4.1 ± 0.06 versus 4.2 ± 0.25 mm; \( P \geq 0.05 \)).

**Discussion**

DC vaccines represent a promising therapy for cancer; however, their efficacy is frequently suboptimal. For example, a recent prostate cancer vaccine trial found that treatment resulted in a superior outcome, but the improvement in median survival was only 4.1 months (33). The very modest efficacy of DC vaccination was substantiated in our model by the vaccine’s inability to improve survival in WT mice. However, we found the efficacy of DC vaccination could be markedly improved in the absence of Nlrp3.

The effect of Nlrp3 in the tumor environment was unanticipated because the established signals for inflammasome priming and activation are not present. Our results and those of others suggest this concept is changing. Li and colleagues (35) have shown that the Nlrp3 inflammasome can be activated in a sterile setting by necrotic cancer cells. Priming is accomplished by an excess of extracellular matrix components and activation is completed by ATP released from dying cancer cells (36). These observations required either the administration of \( 1 \times 10^7 \) pressurized cells (37) or chemotherapy (36). Our work extends these ideas to the activation of Nlrp3 in the DC vaccine setting.

Further analysis implicated differences in MDSC number as the reason for the survival advantage in vaccinated Nlrp3−/− mice. Nlrp3 does not appear to affect the ability of MDSCs to suppress T cells. Instead, the reduction in quantity of MDSCs resulted in an inhibition of cytotoxic CD8⁺ T cells and prompted a more effective antitumor response.

Our interpretation also explains why the administration of anti–Gr-1 antibodies restored the efficacy of the vaccine in WT mice to a degree comparable with Nlrp3−/− mice. Antibody depletion of Gr-1⁺ cells is well established in the literature (35, 38–41) though the effect of this depletion has had mixed results in cancer models (43). Improvement in antitumor immunity has been documented in models using UV light–induced tumors (42), liver carcinoma (35), and 15-12RM sarcoma cells (39). Our study also shows improvement in WT mice and is the first to do so using a DC vaccine. On the contrary, injection with the Gr-1 antibody inhibited the immune response to a colon cancer cell line (38) and an NK-sensitive lymphoma (40). These differences reflect the functional heterogeneity of MDSCs that is in part due to the tumor model used (43).

Though WT MDSCs were 2.5 times more efficient in migrating to the tumor, we did not find differences in the number of MDSCs in the TDLN. This finding suggests MDSCs are mobilized to the lymph node independently of Nlrp3. We believe that mobilization to the spleen is also Nlrp3 independent because lower number of splenic Nlrp3−/− MDSCs correlated with tumor size. This observation suggests a critically important role for Nlrp3 in the recruitment of MDSCs solely to the tumor microenvironment.

It is conceivable that the differences in the number of MDSCs present at the site of tumor growth are due to changes in proliferation or survival. We believe that our explanation of a role for Nlrp3 in MDSC migration is more consistent with the literature. Nlrp3 is more likely to induce cell death than cell survival. This effect has been documented in monocytes (47). Furthermore, Nlrp3−/− myeloid cells are resistant to cell death induced by bacterial pathogens (28). Wild-type MDSCs are also not likely to have a proliferative advantage over Nlrp3−/− cells. MDSC expansion appears to take place in niches distant from the tumor in the spleen, bone marrow, and liver (48, 49). The modest difference in the number of MDSCs in the spleen is not compatible with the significantly different numbers of MDSCs at the tumor site and strongly argues against proliferation as the sole reason for differences in tumor-associated MDSC number.

Though the improvement in the response to the DC vaccine is encouraging, a majority of tumors continue to grow even in Nlrp3−/−-vaccinated mice. This finding is not particularly surprising because there are no vaccine-only strategies that have consistently eliminated established B16 F10 tumors (50). Though other forms of immune escape may also be at work, we are particularly intrigued by the significant contribution of the Nlrp3 inflammasome, given the presence of Nlrp3 independent inflammasomes. For example, inflammasomes containing absent in melanoma 2 (51) and ice protease-activating factor contribute to IL-1β production independent of Nlrp3. An even greater antitumor response might be seen with inhibition of apoptosis-associated speck-like protein containing a CARD (ASC) because ASC is common to multiple inflammasomes. Those studies are underway in our laboratory.

At first glance, our result stands in contrast to recent findings that Nlrp3 might enhance adaptive immunity. For example, Ghiringhelli and colleagues (36) showed that the Nlrp3 inflammasome was critically important in the P2×7R-dependent activation of DCs to generate IFN-γ-producing CD8⁺ T cells. As previously mentioned, this was mediated by the release of ATP from dying tumor cells in the presence of the chemotherapeutic drugs. Besides the inclusion of chemotherapy, 1 significant difference in this study was the addition of IL-12, which bypasses the requirement for the Nlrp3 inflammasome. We have found that IL-12 is significantly generated by the DCs used by our group. Therefore, our findings suggest that in the presence of IL-12, activation of the Nlrp3 inflammasome enhances the accumulation of MDSCs and suppresses immune responses.

In summary, the expression of Nlrp3 in the tumor microenvironment diminishes antitumor immunity and vaccine efficacy by facilitating the migration of MDSCs to the site of the tumor. Because MDSCs express Nlrp3, their influx becomes part of a positive feedback loop leading to further expansion of these cells. These findings suggest novel means of increasing the effectiveness of DC vaccines by targeting the Nlrp3 inflammasome.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
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