Blockade of Myd88 Signaling by Novel MyD88 Inhibitor Prevents Colitis-associated Colorectal Cancer From Development by Impairing Myeloid-Derived Suppressor Cells

Lu Wang

Huazhong University of Science and Technology

Dan Hu

Renmin Hospital of Wuhan University

Bin Xie

Huazhong University of Science and Technology

Lin Xie (✉ xielintj@126.com)

Tongji Hospital of Tongji Medical College of Huazhong University of Science and Technology

Research Article

Keywords: MyD88 signaling, myeloid-derived suppressor cells, colitis-associated colorectal cancer, MyD88 inhibitor

Posted Date: November 1st, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-997649/v1>

License: © ⓘ This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Version of Record: A version of this preprint was published at Investigational New Drugs on January 28th, 2022. See the published version at <https://doi.org/10.1007/s10637-022-01218-6>.

Abstract

Background: Myeloid-derived suppressor cells (MDSCs) are currently confirmed to be due to escaping the host immune system of tumor by developing a highly suppressive environment. MDSC elimination provides an ideal target for tumor immunotherapy. However, little is still known about the exact molecular mechanism behind MDSC-mediated tumor immune evasion.

Methods: We used a novel myeloid differentiation factor 88 (MyD88) inhibitor TJ-M2010-5 to block the MyD88 signaling and prevent colitis-associated colorectal cancer (CAC) development in mice.

Results: We showed that CAC growth inhibition was involved in diminished MDSC generation, expansion, and suppressive function, and that the MDSC-mediated immune escape was dependent on the MyD88 signaling pathway activation. The MyD88 inhibitor treatment decreased accumulation of CD11b⁺Gr1⁺ MDSC in mice with CAC, thereby reducing cytokine (GM-CSF, G-CSF, IL-1 β , IL-6 and TGF- β) secretion associated with MDSC accumulation, and reducing expression of molecules (iNOS, Arg-1 and IDO) associated with the suppressive capacity of MDSC. In addition, the MyD88 inhibitor treatment reduced the differentiation of MDSC from myeloid cells and the suppressive capacity of MDSC on proliferation of activated CD4⁺ T cell in vitro.

Conclusion: Therefore, MDSC is a primary cellular target of the novel MyD88 inhibitor during CAC development. Our findings prove that MyD88 signaling is involved in the regulation of the immunosuppressive functions of MDSC. Our novel MyD88 inhibitor, TJ-M2010-5, is a new and effective agent that modulates MyD88 signaling to overcome MDSC suppressive functions and to enable the development of a successful antitumor immunotherapy.

Introduction

As tumor may escape the host immune system by developing a highly suppressive environment, nowadays more studies have focused on tumor immune evasion[1]. Tumor immune evasion is mediated by factors released from the tumor or by immunosuppressive cells infiltrating the tumor microenvironment, such as myeloid-derived suppressor cells (MDSCs), natural killer (NK) cells, macrophages, dendritic cells (DC), eosinophils and regulatory T cells (Treg)[2]. Tilting the balance from an immunosuppressive environment to an immune-active environment may be effective in tumor therapy[3, 4].

An increased count of MDSCs has been found in the peripheral blood (PB), bone marrow (BM), lymph nodes, and tumor sites of patients and experimental animals with tumors. MDSCs have been confirmed to suppress host antitumor immunity and continue tumor progression[5-7]. Therefore, eliminating MDSCs may be the ideal target for tumor immunotherapy[8, 9]. Nowadays, several treatment approaches have been suggested to overcome MDSC-induced immunosuppression[10]. The key point of these strategies is to either decrease the number of expanding MDSC or to attenuate their immunosuppressive activity in the tumor-bearing host.

Although the exact mechanism behind MDSC generation, expansion, and suppressive function is not completely known, recent studies have suggested that MDSC accumulation is mediated by tumor-associated mediators, including granulocyte macrophage-colony stimulating factor (GM-CSF)[11], interleukin (IL)-1 β [12], IL-6[13], vascular endothelial growth factor (VEGF)[14] and transforming growth factor- β (TGF- β)[15]. Furthermore, the main immunosuppressive mechanism driven by MSDCs involves inhibition of T cell responses[16], suppression of NKT-mediated lysis[17], polarizing macrophage differentiation towards type 2[18], limiting the availability of mature DC[19] and promoting Treg expansion[6] in the tumor-bearing host.

Sustained toll-like receptor (TLR) activation is known to be associated with persistent inflammatory cytokine production and tissue damage. Moreover, TLR signaling elicited in the inflammatory or tumor microenvironment has the potential to induce MDSCs in different organs. TLR2/6 heterodimers expand and recruit MDSCs to the skin to suppress T cells expansion[20, 21]. Lipopolysaccharide (LPS) promotes MDSC development in a TLR4-dependent manner to suppress T cell function in the lungs[22]. TLR9 blockage inhibits the suppressive activity of MDSC on T cell proliferation in tumor-bearing mice[23]. Therefore, activation of the TLR signal pathway may play a critical role in MDSC-mediated tumor immune evasion.

Myeloid differentiation factor 88 (MyD88) is a key adaptor-signaling molecule for all TLRs except TLR3[24]. MDSCs have been proven to inhibit inflammation in the lungs as they are a TLR4/MyD88-induced cell type that can influence T cell responses[22, 25]. MyD88^{-/-} MDSC fails to suppress tumor antigen specific T cells' immune response[26]. Moreover, our previous study proves that the administration of our novel MyD88 inhibitor TJ-M2010-5 may prevent colitis-associated colorectal cancer (CAC) from developing in mice by controlling inflammation and carcinogenesis[27]. Therefore, we investigate the impact of the novel MyD88 inhibitor on the number, phenotype, and function of MDSCs in mice with CAC. We focus on the underlying molecular mechanisms of the MyD88 inhibitor targeting MDSC and provide novel insights on therapeutic strategies targeting MDSC in tumor-bearing hosts and cancer patients.

Methods

Animals

Female BalB/c mice (6 weeks old) were obtained from the Weitonglihua Company (Beijing, China). All animals were housed in a specific pathogen free facility. The experimental protocol was approved by the Animal Care and Research Committee of Huazhong University of Science and Technology.

Colitis-associated colorectal cancer model

CAC in mice was induced as previously described. To induce CAC, each mouse was injected intraperitoneally (i.p.) with 10mg/Kg azoxymethane (AOM, Sigma-Aldrich Chemical, Germany). Seven days later, they began to receive three cycles of 2.5% dextran sodium sulfate (DSS, MP Biomedicals, USA)

in drinking water for one week and two weeks of regular drinking water throughout a 10-week observation period. A group of age- and gender-matched healthy control mice received vehicle (sterile water) injection and plain drinking water. Samples (colon, spleen, PB, BM) were harvested at weeks 5,7, 8 and 10 (W5, W7, W8 and W10) of the protocol.

MyD88 inhibitor treatment

All mice were randomly divided into the normal control (NC), CAC model (CAC), and MyD88 inhibitor-treated groups (I, n=4-5 per group). The mice in the MyD88 inhibitor group were treated with 50mg/kg TJ-M2010-5 (i.p.) daily beginning two days before the first DSS administration throughout the 10-week observation period. The mice in the control group were injected with the same volume of sterile water.

Evaluation of colorectal tumors

The entire colons of each mouse was opened longitudinally, and the numbers of tumors in each colon was counted. The paraffin-embedded colon tissue sections (4 μ m) were stained with hematoxylin-eosin (HE) staining.

Preparation of single cell suspensions

Lamina propria mononuclear cells (LPMCs)

LPMCs were isolated from colonic tissues using the Lamina Propria (LP) Dissociation Kit, according to the manufacturer's instructions (Miltenyi Biotec, Germany, in Supplementary Methods).

BM cells

Femurs of mice were aseptically removed and debrided of surrounding muscle tissue. BM cells were flushed from the femur using phosphate buffer saline (PBS).

Splenocytes

The spleen of the mice were excised and sliced into small pieces. The excised pieces were pressed through a strainer, and the harvested cells were washed using PBS.

PBMCs

PBMCs were isolated from freshly obtained whole blood from mice using density gradient separation (TBD sciences, China, in Supplementary Methods).

Antibodies (Abs)

The Abs used in this study are shown in Supplementary Methods.

Flow cytometry analysis

Quantitative flow cytometric analyses were performed using standard procedures. For analysis of intracellular cytokine production, cells were permeabilized for 20 min at 4 °C. Data acquisition was performed using FACS Celesta flow cytometer and analyzed by the FlowJo software (Version 10.0).

IHC and IF

The paraffin-embedded colon tissue sections (4 µm) were incubated with Abs. The bound Abs were detected sequentially with biotin-conjugated secondary antibody and streptavidin-HRP and visualized using a DAB Kit (Beyotime Biotech, China) for IHC. For IF, Alexa Flour 488 anti-rabbit or Cy3 anti-rat Abs (Servicebio, China) were used. The slides were mounted with DAPI (Servicebio, China) and examined under fluorescence microscope.

Magnetic beads sorting cells

CD3⁻ splenocytes, CD11b⁻ BM cells, CD11b⁺Gr-1⁺ MDSC and CD4⁺ splenic T cells were purified by magnetic bead negative/positive selection according to the manufacturers' instructions (Miltenyi Biotec., Germany).

Western blot

Colon tissue samples were homogenized and separated on SDS-PAGE for Western blot analysis. Cell lysates from sorted CD11b⁺Gr-1⁺ splenocytes were prepared and diluted using a sample preparation kit (Protein Simple) for the automated capillary western blot system, WES System (Protein Simple, in Supplementary Methods).

RAW264.7 cell culture and stimulation

RAW 264.7 cell line was obtained from the China Center for Type Culture Collection (CCTCC #GDC143, Wuhan, Hubei). Frozen aliquots were used in the experiments within six months of culture period, after the first thawing of the cells. The cell line has been tested and authenticated. Cells were cultured in a RPMI medium 1640 (Gibco, USA). After stimulation with 100 ng/mL of LPS (Sigma-Aldrich Co., Germany) for four hours with 40 µM TJ-M2010-5 one-hour pretreatment; subsequently, cells and the supernatant in individual wells were harvested for mRNA extraction and ELISA, respectively.

Quantitative real-time polymerase chain reaction (RT-qPCR)

The total RNA was extracted from mouse colon tissues or RAW264.7 cells as described using the Trizol reagent (Invitrogen, USA). cDNA was synthesized using the ThermoFisher Kit (USA). RT-qPCR was performed using the Hieff qPCR SYBR Green Master Mix (Yeasten Biotech., China) and a StepOne System (Life Technologies, USA). Relative fold changes were determined using the $\Delta\Delta CT$ calculation method. Values were normalized to internal control β -actin. The sequences of the primers were given in Supplementary Methods.

ELISA

The concentrations of GM-CSF, IFN- γ , IL-1 β , IL-6, and TGF- β 1 in the supernatant were analyzed using ELISA, according to the manufacturers' instructions (eBioscience, USA).

In vitro MDSC differentiation from immature myeloid cells

CD11b⁻ BM cells flushed from the femur using PBS grown in RPMI medium supplemented with 10% heat-inactivated FBS (Yeasen Biotech., China), 1 mM sodium-pyruvate (Meilunbio, China), and 50 μ M β -mercaptoethanol (Solarbio, China). Cultures were supplemented with 10 ng/mL of GM-CSF (Peprotech, USA) and 1 μ g/mL LPS, and were incubated at 37 °C for 8 days. At the end of the culture period, the population of CD11b⁺Gr-1⁺ MDSC that differentiated from immature myeloid cells was detected by flow cytometry.

In vitro suppressive activity of MDSC on CD4⁺ T cell proliferation

CD11b⁺Gr-1⁺ MDSCs were obtained from splenocytes of mice with CAC with or without the MyD88-inhibitor treatment. For evaluation of MDSC suppressive activity, 2×10^5 CD11b⁺Gr-1⁺ MDSC and 2×10^5 CD4⁺ splenic T cells from naive BalB/c mice were cultured in flat-bottom 96-well plates in complete RPMI medium. The proliferation assay of CD4⁺ T cells were performed using a carboxy-fluorescein diacetate succinimidyl ester (CFSE, Invitrogen, USA) assay, according to the manufacturer's protocol. T cells were stimulated by the addition of anti-CD3/anti-CD28-coated microbeads (Invitrogen, USA) at a bead-to-cell ratio of 1:75 for 72 hours.

Statistics

Data are expressed as the mean $\pm$ standard deviation (SD). Comparisons between groups were analyzed using Student's t-test or the log rank test. Individual differences versus various controls were assessed using one-way ANOVA. All statistical analyses were performed using the SPSS software package (version 17.0; IBM Corp, Armonk, NY, USA). Statistical significance was set to $P < 0.05$.

Results

MDSC expansion during chronic colitis and CAC in mice

Our previous report has proved that mice with AOM/DSS-induced CAC developed multiple tumor lesions in their colons over a 10-week period. Severe colitis and an increased frequency of tumor growth were noted their colons[27]. Furthermore, we investigated the presence of different populations of myeloid cells. These mice had a significantly higher proportion (1.6-4.7 times the control) of CD11b⁺Gr-1⁺MDSC in their spleen, PB, and BM five weeks postinduction, and also a higher presence (>5 times the control) of these cells in the LP eight weeks postinduction (Figure 1A). These results indicated that MDSC accumulation in the CAC model aggravated both the colitis and tumor development.

Lessened accumulation of MDSCs in mice with AOM/DSS-induced CAC in the absence of MyD88 signaling through MyD88 inhibitor (TJ-M2010-5) treatment

We have previously shown that the MyD88 signaling blockade with MyD88 inhibitor treatment in mice with AOM/DSS-induced CAC leads to reduced colitis and complete suppression of CAC development. We assessed the effects of the MyD88 inhibitor on MDSC accumulation during CAC development. Flow cytometry data have shown that the populations of CD11b⁺Gr-1⁺ MDSC in the LP, spleen, PB, and BM in mice with CAC using MyD88 inhibitor treatment were significantly decreased to 40% (W8); 33% (W5) and 29% (W8); 21% (W5) and 28% (W8); 34% (W5) and 61% (W8), respectively, compared to those in mice with CAC (Figure 1B). Consistent with these data, we noted that Ly6G⁺ cells were significantly reduced by about 75%-90% in the colon (Figure 1C), and fewer CD11b⁺Ly6G⁺ cells in the spleen (Figure 1D) were noted five weeks postinduction, compared with those in mice with CAC not receiving MyD88 inhibitor treatment by IHC or IF. Thus, blocking MyD88 signaling using MyD88 inhibitor treatment resulted in the reduced accumulation of MDSC during CAC development.

MyD88 signaling blockade reduces factors associated with MDSC accumulation in mice with AOM/DSS-induced CAC

A MDSC arises from a common myeloid progenitor. Their development is supported by several growth factors (GM-CSF and G-CSF) and other factors, including IL-1 β , IL-6, IL-11, IFN- γ , and tumor-derived exosomes through TLR2 and TGF- β , which are implicated in the activation of signal transducer and activator of transcription (STAT)1, STAT3, and nuclear factor kappa-B (NF- κ B), and the down-regulation of IFN regulatory factor 8 (IRF8) in MDSC; these are also responsible for MDSC expansion[28]. Under pathological conditions of tumors or chronic inflammation, myeloid cells are unable to effectively differentiate into mature myeloid cells, caused by the persistent stimulation of these factors, and the conversion of immature myeloid cells to MDSC, which thereby lead to potent immune-suppressive potential[16]. Thus, we checked whether the levels of these factors supporting MDSC expansion in the absence of MyD88 signaling were decreased in mice with CAC after MyD88 inhibitor treatment. As shown in Figure 2A, treatment with the MyD88 inhibitor significantly decreased the levels of G-CSF (47.3% of CAC group), IL-6 (69.0% of CAC group), and TGF- β (50.2% of CAC group)-producing CD3⁻ splenocytes in mice with AOM/DSS-induced CAC at eight weeks postinduction, which was consistent with our previous findings. Furthermore, a similar pattern in GM-CSF, IL-1 β , IL-6, and TGF- β mRNA expression was detected in mice colon tissue (Figure 2B). As a result, MyD88 signaling blockade may disrupt tumor/inflammation-derived factors secretion associated with MDSC accumulation.

Impact of MyD88 signaling blockade on the expression of molecules involved in MDSC-mediated immune suppression in mice with AOM/DSS-induced CAC

MDSCs utilize a number of mechanisms to mediate immune suppression, including upregulation of iNOS to produce reactive oxygen species (ROS), upregulating Arg-1 expression of TGF- β and IL-10, promoting the immunosuppressive molecule IDO expression, and directly affect CD4⁺ and CD8⁺ T cells[29]. To

further clarify the impact of MyD88 signaling blockade on MDSC, we detected the expression of iNOS, Arg-1 and IDO in the colon tissue of mice with AOM/DSS-induced CAC under MyD88 inhibitor treatment. As shown in Figure 3A, the expression of iNOS, Arg-1 and IDO were significantly reduced compared to mice with CAC after assessing with Western blot, with a decrease of 55-75% noted seven weeks postinduction. IHC staining in Figure 3B also showed fewer iNOS-, Arg-1- and IDO-staining positive cells infiltrating into the LP of the colon, compared to mice with CAC. We further detected the expression of these proteins in CD11b⁺Gr-1⁺ MDSC from the spleens of the mice. Expression of iNOS, Arg-1 and IDO were also significantly decreased in the mice treated with MyD88 inhibitor, compared to mice with CAC by WES, with a decrease of 45-70% seen at seven weeks postinduction (Figure 3C). Thus, the MyD88 signaling blockade did not only reduce the population of MDSC, but also caused the down-regulation expression of molecules involved in the suppressive capacity of MDSC in mice with CAC.

MyD88 signaling blockade suppressed the differentiation of MDSCs from myeloid cells in vitro

Macrophages are an important source of cytokines involved in MDSC expansion. To determine that MyD88 signaling was responsible for the generation of MDSC in vitro, the cytokine secretion profile of RAW 264.7 cells in the presence of LPS with or without the MyD88 inhibitor treatment were shown. As shown in Figure 4A, significantly elevated GM-CSF ($p = 0.004$), IFN- γ ($p = 0.0008$), IL-1 β ($p = 0.003$), IL-6 ($p = 0.0002$), and TGF- β 1 ($p = 0.003$) levels were confirmed for RAW 264.7 cells after stimulation with LPS, compared to the non-stimulated cells. However, the supernatant concentration of these cytokines was statistically reduced almost to the levels of non-stimulated cells after treatment with the MyD88 inhibitor. A similar pattern in these cytokines' mRNA expression was also observed (Figure 4B). The MyD88 inhibitor treatment greatly reduced GM-CSF, IFN- γ , IL-1 β , IL-6, and TGF- β 1 mRNA expression of RAW 264.7 cells in the presence of LPS. In addition, CD11b⁺Gr-1⁺ MDSC was generated in the presence of GM-CSF and LPS from CD11b⁻ BM progenitor cells from BalB/c mice in vitro, with an increased population of 9.83% vs. 0.54% at baseline, while this induction was diminished (1.06%) by the MyD88 inhibitor treatment (Figure 4C). Therefore, the blockade initiated by the MyD88 inhibitor not only caused inhibited MDSC expansion by decreasing the secretion of related cytokines, but also through direct suppression of MDSC differentiation.

Loss of MDSC suppression on CD4⁺ T cells proliferation after MyD88 inhibitor administration

Next, we examined the effect of the MyD88 inhibitor therapy on the suppressive function of MDSC in mice with AOM/DSS-induced CAC. As shown in Figure 5, the proliferation of activated CD4⁺ T cells by CD3/CD28 antibodies declined from 91.7% to 53.1%, with the culture of MDSC in normal control mice in vitro. The proliferation rate of CD4⁺ T cells further decreased to 30.9% with MDSC in mice with CAC. MDSCs, especially from tumor-bearing hosts, effectively suppressed CD4⁺ T cells proliferation. However, MDSCs derived from the spleen of the MyD88 inhibitor-treated mice were significantly less suppressive on the proliferation of activated CD4⁺ T cells, with a reversal proliferation rate of 87.5%. Thus, these results clearly suggested that MyD88 signaling may be crucial to the suppressive capacity of MDSCs.

Discussion

Colorectal cancer is the third most common malignancy in humans; furthermore, it is the second leading cause of cancer-related death in most countries. Several studies have confirmed that colitis is one of the primary drivers of colon tumorigenesis[30]. During the development of inflammation and carcinogenesis in colon, the innate immune system plays a critical role in protecting against CAC by triggering antimicrobial responses, maintaining intestinal homeostasis, and inducing healing; on the other hand, it promotes chronic inflammation in the tumor microenvironment and evades antitumor effector immune responses[31]. Thus, understanding the dual role of the innate immune system in cancer immunology is vital to designing effective therapeutic strategies against CAC.

Chronic inflammation and tumors may actively recruit and condition a complex network of cells belonging to the innate immune system, such as tumor-associated macrophages (TAMs), NKTs, and MDSCs. Among these cells, MDSCs are a heterogeneous population of immune cells that can impair anti-tumor response both locally, in the tumor site, and systemically, in lymphoid organs. A growing number of studies have demonstrated that targeting MDSC improved the effect of anti-cancer therapies in breast, colorectal, prostate, hepatocellular and melanoma cancer[32-36]. In this study, we also proved the contribution of MDSC to the development of CAC driven by AOM/DSS administration in mice. We observed the accumulation of cells with typical MDSC phenotype (CD11b⁺Gr-1⁺) in the spleen, PB, and BM of mice at five weeks postinduction, when the colitis was severe, and in LP at eight weeks postinduction, when the colons were full of neoplastic lesions. These data indicated that MDSC accumulation in this model was a result of both chronic colitis and tumor formation. In addition, MDSC accumulation due to inflammation occurred earlier in systemically lymphoid organs than in the local tumor site, as a consequence of cancer development.

Since MDSCs are important to inflammation and consequently, carcinogenesis, it is essential to understand the molecular mechanisms behind expanding and activating MDSC as potential target for cancer immunotherapy. It has been proven that the signaling through the MyD88 adaptor molecule is critical for the suppressive function of the MDSCs. Blockage of MyD88 signaling by the transgenic method successes to inhibit tumor growth in lung and ovarian in mouse model, which is involved in suppressing MDSC[26, 37]. Despite the inhibition of MyD88 signaling in MDSC proving to be a promising treatment for cancer, administration of these agents has not yet been accomplished. Our previous study has proven that the novel synthetic MyD88 inhibitor (TJ-M2010-5) is an effective MyD88 homodimerization inhibitor, and its administration successfully prevents development of CAC in mice[27]. In this subsequent study, we have shown that the accumulation and differentiation of MDSC were dependent on both the MyD88 signaling activation and the TJ-M2010-5 inhibitor administration. These are shown in Figure 1B and 4C, which reveals the decline of innate immune cells (e.g. macrophages) or cancer-derived factors both in vivo (Figure 2) and in vitro (Figure 4A&B). We have proven that MDSC is generated, proliferates and migrates in response to these cytokines and chemokines; furthermore, MyD88 signaling is essential for inflammatory and tumor cells secretion[38]. Thus, TJ-M2020-5, a MyD88

inhibitor, has the potential to be a clinically available agent that acts on MDSC to prevent CAC development.

Immunosuppressive MDSC has been implied to be involved in tumor immune evasion through the following mechanisms[39-41]: (i) stimulating production of ROS that decreases T cell receptor functionality through NADPH oxidase and iNOS; (ii) producing high levels of Arg-1 that depletes T cells of L-arginine and induces cell cycle arrest; (iii) synthesizing IDO to protect tumor from attack from specific tumor T cell by inducing tolerance through tryptophan catabolism to inhibit T cell proliferation and induce Treg cells, among others. In this study, we have shown that these immunosuppressive functions of MDSCs are also dependent on MyD88 signaling activation. We found that the MyD88 inhibitor administration reduced iNOS, Arg-1 and IDO expression to weaken tumor-immune evasion mediated by MDSC (Figure 3). Thus, the immunosuppressive capabilities of MDSC are also dependent on MyD88 signaling activation. On the other hand, it has been demonstrated that intratumor MDSCs mainly hamper CD8⁺ T cell activation, which thereby causes immune suppression for tumor escape[42, 43]; however, the ability of MDSCs to elicit CD4⁺ T cell tolerance is still controversial and unclear. Our results (Figure 5) showed that MDSC derived from mice with CAC has the significant ability to inhibit CD4⁺ T cell proliferation in vitro and the MyD88 signaling blockade by the MyD88 inhibitor, which reduced the inhibitory effect of MDSC on CD4⁺ T cell proliferation. Another study has also proven that MDSC from mice with chronic infection suppressed OVA-specific CD4⁺ T cell proliferation via a nitric oxide-dependent mechanism[44]. Therefore, these findings indicate that CAC development induces MDSC that suppress CD4⁺ T cell proliferation and promotes inflammation associated with carcinogenesis.

Indeed, the regulation of MDSC immunosuppressive functions plays a critical role in successful immunotherapy against cancer; moreover, Myd88 signaling is involved in the regulation of the immunosuppressive functions of MDSC. Our novel MyD88 inhibitor, TJ-M2010-5, is a new and effective agent to modulate MyD88 signaling, which overcomes MDSCs' suppressive functions, thus making it a successful antitumor therapy.

Declarations

Ethical Considerations

This study was carried out in accordance with the recommendations and guidelines issued by the Ethics Committee of Huazhong University of Science and Technology.

Consent for publication

Not applicable.

Availability of data and material

All data generated or analysed during this study are included in this article.

Competing interests

The authors declare no conflict of interest.

Funding

This work is supported by the Funding: National Natural Science Foundations for Young Scientists of China (Grant # 81702817, #81401051 and #81202604); the Non-Profit Central Research Institute Fund of Chinese Academy of Medical Sciences (2019PT320014).

Authorship Contributions:

Participated in research design: Lin Xie and Lu Wang.

Conducted experiments: Lu Wang, Dan Hu, Bin Xie, and Lin Xie.

Performed data analysis: Lu Wang, Dan Hu, and Lin Xie.

Wrote or contributed to the writing of the manuscript: Lin Xie.

Acknowledgements

We would like to thank Dr. Ping Zhou and Dr. Fengchao Jiang (Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) for providing the MyD88 inhibitor, TJ-M2010-5, used in this study. We would also like to show our gratitude to the Wuhan Regional Center of Life Science, Chinese Academy of Sciences for sharing the WES System (Protein Simple).

References

1. Talmadge, J.E. and D.I. Gabrilovich, History of myeloid-derived suppressor cells. **Nat Rev Cancer**, 2013. **13**(10): p. 739-52.
2. Kerkar, S.P. and N.P. Restifo, Cellular constituents of immune escape within the tumor microenvironment. **Cancer Res**, 2012. **72**(13): p. 3125-30.
3. Kirkwood, J.M., et al., *Next generation of immunotherapy for melanoma*. **J Clin Oncol**, 2008. **26**(20): p. 3445-55.
4. Motz, G.T. and G. Coukos, Deciphering and reversing tumor immune suppression. **Immunity**, 2013. **39**(1): p. 61-73.
5. Filipazzi, P., V. Huber, and L. Rivoltini, Phenotype, function and clinical implications of myeloid-derived suppressor cells in cancer patients. **Cancer Immunol Immunother**, 2012. **61**(2): p. 255-263.
6. Gabrilovich, D.I. and S. Nagaraj, Myeloid-derived suppressor cells as regulators of the immune system. **Nat Rev Immunol**, 2009. **9**(3): p. 162-74.

7. **Ostrand-Rosenberg, S.**, Myeloid-derived suppressor cells: more mechanisms for inhibiting antitumor immunity. **Cancer Immunol Immunother**, 2010. **59(10)**: p. **1593-600**.
8. Khaled, Y.S., B.J. Ammori, **and** E. Elkord, Myeloid-derived suppressor cells in cancer: recent progress and prospects. **Immunol Cell Biol**, 2013. **91(8)**: p. **493-502**.
9. Alizadeh, D. **and** N. Larmonier, Chemotherapeutic targeting of cancer-induced immunosuppressive cells. **Cancer Res**, 2014. **74(10)**: p. **2663-8**.
10. **Umansky, V. and A. Sevko**, Melanoma-induced immunosuppression and its neutralization. **Semin Cancer Biol**, 2012. **22(4)**: p. **319-26**.
11. Dolcetti, L., **et al.**, Hierarchy of immunosuppressive strength among myeloid-derived suppressor cell subsets is determined by GM-CSF. **Eur J Immunol**, 2010. **40(1)**: p. **22-35**.
12. Tu, S., **et al.**, Overexpression of interleukin-1beta induces gastric inflammation and cancer and mobilizes myeloid-derived suppressor cells in mice. **Cancer Cell**, 2008. **14(5)**: p. **408-19**.
13. Bunt, S.K., **et al.**, Reduced inflammation in the tumor microenvironment delays the accumulation of myeloid-derived suppressor cells and limits tumor progression. **Cancer Res**, 2007. **67(20)**: p. **10019-26**.
14. Melani, C., **et al.**, Amino-biphosphonate-mediated MMP-9 inhibition breaks the tumor-bone marrow axis responsible for myeloid-derived suppressor cell expansion and macrophage infiltration in tumor stroma. **Cancer Res**, 2007. **67(23)**: p. **11438-46**.
15. Takaku, S., **et al.**, Blockade of TGF-beta enhances tumor vaccine efficacy mediated by CD8(+) T cells. **Int J Cancer**, 2010. **126(7)**: p. **1666-74**.
16. Kumar, V., **et al.**, The Nature of Myeloid-Derived Suppressor Cells in the Tumor Microenvironment. **Trends Immunol**, 2016. **37(3)**: p. **208-220**.
17. Li, H., **et al.**, Cancer-expanded myeloid-derived suppressor cells induce anergy of NK cells through membrane-bound TGF-beta 1. **J Immunol**, 2009. **182(1)**: p. **240-9**.
18. Sinha, P., **et al.**, Cross-talk between myeloid-derived suppressor cells and macrophages subverts tumor immunity toward a type 2 response. **J Immunol**, 2007. **179(2)**: p. **977-83**.
19. Egelston, C., **et al.**, Suppression of dendritic cell maturation and T cell proliferation by synovial fluid myeloid cells from mice with autoimmune arthritis. **Arthritis Rheum**, 2012. **64(10)**: p. **3179-88**.
20. Sumpter, T.L. and L.D. Falo, Jr., "Toll"-erance in the skin. **Immunity**, 2014. **41(5)**: p. **677-9**.
21. Skabytska, Y., **et al.**, Cutaneous innate immune sensing of Toll-like receptor 2-6 ligands suppresses T cell immunity by inducing myeloid-derived suppressor cells. **Immunity**, 2014. **41(5)**: p. **762-75**.
22. Arora, M., **et al.**, TLR4/MyD88-induced CD11b+Gr-1 int F4/80+ non-migratory myeloid cells suppress Th2 effector function in the lung. **Mucosal Immunol**, 2010. **3(6)**: p. **578-93**.
23. Zoglmeier, C., **et al.**, CpG blocks immunosuppression by myeloid-derived suppressor cells in tumor-bearing mice. **Clin Cancer Res**, 2011. **17(7)**: p. **1765-75**.
24. Siednienko, J., **et al.**, Absence of MyD88 results in enhanced TLR3-dependent phosphorylation of IRF3 and increased IFN-beta and RANTES production. **J Immunol**, 2011. **186(4)**: p. **2514-22**.

25. Ray, A., K. Chakraborty, **and** P. Ray, Immunosuppressive MDSCs induced by TLR signaling during infection and role in resolution of inflammation. **Front Cell Infect Microbiol**, 2013. **3**: p. **52**.
26. Hong, E.H., **et al.**, Blockade of Myd88 signaling induces antitumor effects by skewing the immunosuppressive function of myeloid-derived suppressor cells. **Int J Cancer**, 2013. **132**(12): p. **2839-48**.
27. Xie, L., **et al.**, Targeting of MyD88 Homodimerization by Novel Synthetic Inhibitor TJ-M2010-5 in Preventing Colitis-Associated Colorectal Cancer. **J Natl Cancer Inst**, 2016. **108**(4).
28. Condamine, T., J. Mastio, **and** D.I. Gabrilovich, Transcriptional regulation of myeloid-derived suppressor cells. **J Leukoc Biol**, 2015. **98**(6): p. **913-22**.
29. Condamine, T. and D.I. Gabrilovich, Molecular mechanisms regulating myeloid-derived suppressor cell differentiation and function. **Trends Immunol**, 2011. **32**(1): p. **19-25**.
30. Saleh, M. **and** G. Trinchieri, Innate immune mechanisms of colitis and colitis-associated colorectal cancer. **Nat Rev Immunol**, 2011. **11**(1): p. **9-20**.
31. Berraondo, P., **et al.**, Innate immune mediators in cancer: between defense and resistance. **Immunol Rev**, 2016. **274**(1): p. **290-306**.
32. De Cicco, P., G. Ercolano, **and** A. Ianaro, The New Era of Cancer Immunotherapy: Targeting Myeloid-Derived Suppressor Cells to Overcome Immune Evasion. **Front Immunol**, 2020. **11**: p. **1680**.
33. Katoh, H., **et al.**, CXCR2-expressing myeloid-derived suppressor cells are essential to promote colitis-associated tumorigenesis. **Cancer Cell**, 2013. **24**(5): p. **631-44**.
34. Chun, E., **et al.**, CCL2 Promotes Colorectal Carcinogenesis by Enhancing Polymorphonuclear Myeloid-Derived Suppressor Cell Population and Function. **Cell Rep**, 2015. **12**(2): p. **244-57**.
35. Holmgaard, R.B., **et al.**, Targeting myeloid-derived suppressor cells with colony stimulating factor-1 receptor blockade can reverse immune resistance to immunotherapy in indoleamine 2,3-dioxygenase-expressing tumors. **EBioMedicine**, 2016. **6**: p. **50-58**.
36. Mao, Y., **et al.**, Targeting Suppressive Myeloid Cells Potentiates Checkpoint Inhibitors to Control Spontaneous Neuroblastoma. **Clin Cancer Res**, 2016. **22**(15): p. **3849-59**.
37. Baert, T., **et al.**, Myeloid Derived Suppressor Cells: Key Drivers of Immunosuppression in Ovarian Cancer. **Front Immunol**, 2019. **10**: p. **1273**.
38. Di Mitri, D., A. Toso, **and** A. Alimonti, Molecular Pathways: Targeting Tumor-Infiltrating Myeloid-Derived Suppressor Cells for Cancer Therapy. **Clin Cancer Res**, 2015. **21**(14): p. **3108-12**.
39. Kulsantiwong, P., **et al.**, Pam2CSK4 and Pam3CSK4 induce iNOS expression via TBK1 and MyD88 molecules in mouse macrophage cell line RAW264.7. **Inflamm Res**, 2017. **66**(10): p. **843-853**.
40. Liu, J.T., **et al.**, Inhibition of MyD88 Signaling Skews Microglia/Macrophage Polarization and Attenuates Neuronal Apoptosis in the Hippocampus After Status Epilepticus in Mice. **Neurotherapeutics**, 2018. **15**(4): p. **1093-1111**.
41. Liu, H., **et al.**, Atractylenolide I modulates ovarian cancer cell-mediated immunosuppression by blocking MD-2/TLR4 complex-mediated MyD88/NF-kappaB signaling in vitro. **J Transl Med**, 2016.

14(1): p. 104.

42. Nagaraj, S., **et al.**, Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. **Nat Med**, 2007. **13**(7): p. 828-35.
43. Schouppe, E., **et al.**, Tumor-induced myeloid-derived suppressor cell subsets exert either inhibitory or stimulatory effects on distinct CD8+ T-cell activation events. **Eur J Immunol**, 2013. **43**(11): p. 2930-42.
44. Valanparambil, R.M., **et al.**, Primary Heligmosomoides polygyrus bakeri infection induces myeloid-derived suppressor cells that suppress CD4(+) Th2 responses and promote chronic infection. **Mucosal Immunol**, 2017. **10**(1): p. 238-249.

Figures

Figure 1

A&B) Population of CD11b+Gr-1+ MDSC in the spleen, PB, BM, and LP by flow cytometry at five, seven and eight weeks postinduction (W5, W7 and W8) in (A) the CAC group and the normal control group (NC), and (B) the MyD88 inhibitor-treated group (I). N=5 per group. C&D) IHC staining for Ly6G+ cells of colon sections (C) and IF staining for CD11b+ (green) Ly6G+ (red) cells of spleen sections (D) in the CAC group and the I group at five, seven and eight weeks postinduction (W5, W7 and W8). Scar bar = 50 μ M. Histogram at the bottom (C) shows the percentage of positive Ly6G+ cells in total cells. N=4 per group. Data are expressed as the mean $\pm$ SD of each group. ***P < 0.001.

Figure 2

The MyD88 inhibitor treatment reduced factors associated with MDSC accumulation in mice with CAC. A) Population of G-CSF, IL-6 and TGF- β -producing CD3- splenocytes by flow cytometry at eight weeks postinduction. B) Relative levels of GM-CSF, IL-1 β , IL-6 and TGF- β mRNA transcripts in mouse colons at eight weeks postinduction examined by RT-qPCR. Data are expressed as the mean $\pm$ SD of each group. All data of RT-qPCR were normalized to that in normal control mice. **P < 0.01; ***P < 0.001. N=5 per group. Groups: the normal control (NC), CAC and MyD88 inhibitor-treated (I) groups.

Figure 3

Impact of the MyD88 inhibitor on the expression of molecules involved in MDSC-mediated immune suppression in mice with CAC. A) Protein extracts from colon tissue were analyzed by western blot for iNOS, Arg-1 and IDO. α -tubulin was used as a control. Data are representative images or expressed as the mean $\pm$ SD of each group. Densitometry analysis was performed with Image J software for quantification. $**P < 0.01$. B) Representative IHC stains for iNOS, Arg-1 and IDO in colonic samples at seven weeks postinduction. Scale bar = 100 μ M. N = 4 per group. Groups: the normal control (NC), CAC and MyD88 inhibitor-treated (I) groups. C) Protein extracts of cell lysates from sorted CD11b+Gr-1+ splenocytes were analyzed by WES for iNOS, Arg-1 and IDO. α -tubulin was used as a control. Data are representative images or expressed as the mean $\pm$ SD of each group. Quantitative analysis was performed using Compass software (Protein Simple). $**P < 0.01$; $***P < 0.001$.

Figure 4

The MyD88 inhibitor administration suppressed the differentiation of MDSC from myeloid cells in vitro. Supernatant concentrations by ELISA (A) and relative levels of mRNA transcripts by RT-qPCR (B) of GM-CSF, IFN- γ , IL-1 β , IL-6 and TGF- β 1 in RAW 264.7 cells are shown. Data are expressed as the mean $\pm$ SD of each group from three independent experiments. Data from the RT-qPCR were normalized to non-stimulated cells. *P < 0.05; **P < 0.01; ***P < 0.001. C) BM cells were cultured with GM-CSF and LPS for eight days and a phenotypic analysis on induced CD11b+Gr-1+ MDSC was performed using flow cytometry. Groups: the non-stimulated, LPS or LPS/GM-CSF-stimulated and MyD88 inhibitor-treated (I) cells groups.

Figure 5

Suppressive ability of CD11b+Gr-1+ MDSC on activated CD4+ T cells proliferation was reserved by MyD88 inhibitor administration. MDSC from the normal control, CAC and MyD88 inhibitor-treated (I) mice were cultured with CFSE-labeled autologous CD4+ T cells in proportion (1:1) with the stimulation of anti-CD3/anti-CD28-coated microbeads, and T-cell proliferation, which were all analyzed by flow cytometry. Data were from three independent experiments.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryMethods.doc](#)