Group 1 Innate Lymphocyte-Derived IFN-γ Regulates Macrophage Alternative Activation In Colon Cancer

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Group 1 innate lymphocyte-derived IFN-γ regulates macrophage alternative activation in colon cancer

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Abstract

Background

Tumor-associated macrophage (TAM) is an important innate immune cell-subset in tumor microenvironment, and that is also a pivotal orchestrator of tumor-promoting inflammation and tumor progression. Evidence proved that TAMs are up-regulated in a great number of cancers, and most of them are alternative activated M2 phenotype, which greatly promote the progress of cancer diseases. Group 1 innate lymphocytes including conventional NK cells and type 1 innate lymphocytes (ILC1s), are abundant in intestinal tissue, and characterized by expressing transcription factor T-bet and secreting interferon (IFN)-γ, which can promote the macrophage to classically activated anti-tumor M1 phenotype. However, the relationship between these two cell subsets remains unclear in colon cancer.

Methods

Flow cytometry was used to detect the percentage of M1 phenotype macrophage, M2 phenotype macrophage and group 1 innate lymphocytes in colon cancer tissue and paracancer healthy colon tissue of AOM/DSS-induced colon cancer mice model. In vitro isolating group 1 innate lymphocytes and inducing bone marrow-derived macrophage to detect the cross-talk when co-cultured. Adoptively transfer or blocking group 1 innate lymphocytes in vivo to investigate the role of group 1 innate lymphocytes on tumor-infiltrating macrophage and the tumor growth.

Results

We found that M1 phenotype macrophage and group 1 innate lymphocytes were down-regulated in colon cancer tissue, and they were positively correlated. Group 1 innate lymphocytes promoted macrophage to classically activated M1 phenotype *in vitro*, and that could be blocked by anti-IFN-γ. *In vivo* results showed that the administration of group 1 innate lymphocytes-blocking antibody anti-NK1.1 could decrease the number of M1 phenotype macrophage in tumor tissue of MC38 tumor-bearing mice and promote the tumor growth, while adoptively transferring

group 1 innate lymphocytes led to tumor-inhibiting and level of M1 phenotype macrophage up-regulating in MC38 tumor-bearing mice.

Conclusions

Our studies preliminarily prove that group 1 innate lymphocytes promote the alternative activation of M1 macrophage by secreting IFN-γ to inhibit the progress of colon cancer for the first time, which may provide an insight in the immunotherapy of colon cancer.

Key words: Tumor-associated macrophage; M1 macrophage; Group 1 innate lymphocytes; Colon cancer; IFN-γ

Background

Colon cancer is a malignant and lethal gastrointestinal cancer disease, which is the second leading reason of tumor-related death all of the world[1]. Currently, surgery combined with radiotherapy and chemotherapy is the most common treatment to colon cancer, but the therapeutic effect is not satisfactory[2]. To date, immunotherapy, as a promising therapeutic strategy, has been applied in a variety of tumor diseases[3, 4]. Understanding the immune regulatory mechanism in the occurrence and development of colon cancer is a key step in developing immunotherapy strategies to treat colon cancer.

Innate lymphocytes (ILCs), as a subset of innate immune cells, participate in the regulation of multiple tumor diseases[5]. Studies have shown that ILCs are abundant in colon cancer tissue and play an important role in the development of colon cancer[6], but the regulatory mechanism is not fully clear. According to the transcription factors and secreted cytokines, ILCs are divided into three subsets, group 1 innate lymphocytes, group 2 innate lymphocytes, and group 3 innate lymphocytes[7]. Among of them, group 1 innate lymphocytes, which consist of conventional NK cells and ILC1s, are characterized by secreting interferon (IFN)-γ and tumor necrosis factor

(TNF)-α, exerting a powerful anti-tumor function in various tumors[8]. TNF-α was proved to have a potent anti-tumor effect. It can not only kill tumors directly, but also recruit and stimulates anti-tumor macrophages and dendritic cells (DCs) to promote the anti-tumor effect. Besides, it is also required for the induction and activation of cytotoxic T cells (CTL), which is the most effective tumor-killing cells[9, 10]. IFN-γ, as a main effector factor of group 1 innate lymphocytes, is proved to have the ability to inhibit the proliferation and angiogenesis of tumor cells, and that can also enhance the cytotoxic activity of CTL to promote the anti-tumor effect[11, 12]. It is worth noting that IFN-γ can promote the induction of classically activated M1 phenotype macrophage, which can further promote the tumor-inhibiting effect[13, 14]. But it remains unclear if group 1 innate lymphocytes could promote the M1 phenotype macrophage polarization in colon cancer.

Tumor-associated macrophages (TAMs) are the main myeloid cell-subset infiltrated in solid tumors, which play a key role in coordinating tumor-promoted inflammation[15]. It is reported that TAMs are mainly tumor-promoting M2 phenotype macrophage in tumor microenvironment, and under certain stimuli, TAM also could polarize into anti-tumor M1 phenotype macrophage. It has been proved this functional heterogeneity of TAMs is common in a variety of tumors[16, 17]. It is widely believed that IFN-γ and Lipopolysaccharide (LPS) can promote macrophage polarizing to classically activated anti-tumor M1 phenotype, while IL-4 and IL-13 result in the activation of M2 phenotype macrophage, and that could be used as the immunotherapy target in the macrophage-based tumor immunotherapy[18-20]. Studies have shown that M2 macrophage, which is characterized by the expression of CD206 and Arg-1, is up-regulated in colon cancer microenvironment while M1 macrophage, which is featured by the expression of CD86 and iNOS, decreases in colon cancer microenvironment, which leads to the immunosuppressive and tumor-promoting effect in colon cancer[21, 22]. How to reverse the M1/M2 axis becomes a key in the immunotherapy of colon cancer[23]. However, the mechanism of M1 down-regulation in the colon cancer microenvironment is still not fully understood.

In this study, we found that M1 phenotype macrophage and group 1 innate lymphocytes were both down-regulated in colon cancer microenvironment, and the percentage of M1 phenotype macrophage is positively correlated with the percentage of group 1 innate lymphocytes. Furthermore, *in vitro* studies showed that group 1 innate lymphocytes can promote macrophage polarized to M1 phenotype macrophage when co-cultured and this effect was reversed by anti-IFN-γ. *In vivo* results demonstrated that adoptively transferred group 1 innate lymphocytes to MC38 tumor-bearing mice increased the percentage of M1 phenotype macrophage in tumor microenvironment and inhibited the tumor growth while administration of ILC1s-blocking antibody exhibited an opposite effect.

Materials and methods

2.1 Mice

Male and Female C57BL/6 (8 weeks, 20 g) were purchased from the Animal Center of Jilin University (Jilin, China). All the mice were bred and maintained in a specific pathogen-free animal facility with a temperature of 23°C ± 2°C and relative humidity of 55% ± 10%. All animal experiments have complied with the protocol of Jilin University Medical School Animal Ethics and Experimentation Committee.

2.2 Cell line and cell culture

Mice colon cancer cell line, MC38 and macrophage cell line, RAW264.7 were purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Mice bone marrow-derived macrophage (BMDM) was induction from C57BL/6 mice bone marrow, and mice group 1 innate lymphocytes were isolated from the colon cancer tissue of C57BL/6 mice. Cells were cultured in the DMEM medium supplemented with 10% FBS. All the cells were cultured in a humidified incubator with 5% CO_2 and a 37 °C temperature.

2.3 Isolation of group 1 innate lymphocytes

Group 1 innate lymphocytes were isolated from the colon cancer tissue of C57BL/6 mice, with a method of combining magnetic beads and flow sorting. Collagenase II and DNase I were used to digesting the tumor tissue for 1 hour, and then go through the 70 mesh screen to get the single cell suspension. Per-coll (BD, New Jersey, USA) was used to get the immune cells from digested tumor tissue single cell suspension[24]. Obtained cells were incubated with biotin-binding (anti-CD3ε, anti-CD45R, anti-Gr-1, anti-CD11c, anti-CD11b, anti-Ter119, anti-TCR-αβ, and anti-FCεRI) (Miltenyi, Belgish, Germany) to enrich Lineage-negative cells following the Miltenyi magnetic beads cell isolation protocol. Enriched lineage-negative single cell suspension stained with anti-mouse Lineage cocktail (Biolegend, 145-2C11, RB6-8C5, RA3-6B2, Ter-119, M1/70), anti-mouse CD127 (Biolegend, A7R34), anti-mouse NK1.1 (Biolegend, PK136), and anti-mouse NKp46 (Biolegend, 29A1.4) for flow cytometry sorting the $Lineage^{-}$ $CD127^{+}$ $NK1.1^{+}$ $NKp46^{+}$ cells, as group 1 innate lymphocytes.

2.4 Induction of bone marrow-derived macrophage

Obtaining the leg of of C57BL/6 mice (6 weeks, female), and using a syringe to get bone marrow cells to 10 cm cell-culture plate in the ultra-clean desk. Washing the bone marrow cells for three time and making cells through the 70 μm cell strainer to a 15 ml cell collecting tube. 1000 rpm centrifuge for 5 minutes, adding 5 ml ACK to break red cells, and then 1000 rpm centrifuge for 5 minutes again. Culturing the bone marrow cells in 6-well plate with 4 ml culture medium (10% FBS and 20ng/ml M-CSF) and a 1×10^{6} cell density. On the third day, changing half of the culture medium and adding the corresponding stimulate factor M-CSF. On the fifth day, changing all the culture medium and adding the corresponding stimulate factor M-CSF. On the seventh day, obtaining the bone marrow-derived macrophage (BMDM) for research, and he purity of BMDM is detect by flow cytometry **(Figure S1B)**.

2.5 Building mice tumor model

For AOM/DSS-induced colitis-associated colon cancer, 8 weeks mice were intraperitoneal injected of azoxymethane (AOM, 10 mg/kg body weight) (Ray Biotech, Georgia, USA). 4 days later, the mice were treated with 1.5% dextran sodium sulfate (DSS) in drinking water for six days. Replacing the DSS water with common water for two weeks, then treated with 1.5% DSS water for another 6 days. After 3 cycle of DSS treated, evaluating the tumor formation. For MC38 tumor bearing mice model, collecting the cultured MC38 cells, and then the mice were subcutaneously injected in the right flank with 1×10^6 tumor cells. Tumor length (L), diameter and width (W) were measured with a caliper.

2.6 Flow cytometry analysis

Tumor tissues from MC38 tumor-bearing mice and colon cancer mice samples were isolated and cut into small pieces. Paracancer tissue as a control and digested as the tumor tissue. The tumor tissue pieces and paracancer tissue pieces were digested with digestion solution containing 5% FBS, collagenase II (1 mg/mL), DNase I (200 μg/mL) and hyaluronidase (4U/mL) (Sigma, St. Louis, USA) in 37°C water bath for 40 minutes. 70 mesh screen to collect digested single cells, centrifuge, and then obtain the single tumor tissue cell suspension. Anti-mouse CD45 (30-F11), anti-mouse F4/80 (BM8) were used to analyze macrophage, anti-mouse CD86 (A17199A) for M1 phenotype macrophage, anti-mouse CD206 (15-2) for M2 phenotype macrophage. anti-mouse Lineage (145-2C11), anti-mouse CD127 (A7R34), and anti-mouse T-bet (4B10) were used to detect group 1 innate lymphocytes. For surface staining, antibody mixed with single cell suspension (100 ul/10^6 cells) for 20 minutes at 4 degree. For intracellular staining, cells were cultured in complete 1640 medium with phorbol 12-myristate (PMA, 1ug/ml), ionomycin (1 ug/ml) and brefeldin A (10 μg/ml) (eBioscience, San Diego, USA) for 4-6 hours. Collecting the cells, and then surface-stained, fixed, permeabilized. Using the Fixation and Permeabilization Buffer Kit (Thermo Fisher Scientific, Massachusetts, USA) to stain the intracellular factors

following the manufacturer's recommendations, and the data was analyzed using flowj7.6.

2.7 RNA isolation and quantitative of Real-time PCR

Total RNA was extracted by TRIZOL reagent (Thermo Scientific, Massachusetts, USA) according the manufacturer' s recommendations. cDNA was reverse transcribed by total RNA using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). Real-time PCR was performed in duplicate using Bio-Rad SYBR Green Super Mix (TAKARA, Tokyo, Japan)according to the manufacturer's instructions. The Primers were designed using Premier 5.0 software and synthesized by Shanghai Invitrogen. The mouse IFN-γ and β-actin primer sequences were as follows: IFN-γ, Forward (5′_3′), TGGCGGTGCTGAGCTACTGG; Reverse (5′_3′), TGTACCAGGAGTGTCAAGGCTCTC. β-actin, Forward (5′_3′), TGGAATCCTGTGGCATCCATGAAA;Reverse (5′_3′), TAAAACGCAGCTCAGTAACAGTCCG.

2.8 Western Blot Analysis:

Cells were lysed with radio immunoprecipitation assay (RIPA) buffer containing a complete protease inhibiting tablet, and then centrifuge to get the total protein. The concentration of extracted protein was detected by CBA kit. The equal total protein was loaded into gel and separated by 12% SDS-PAGE, and the transferred onto polyvinylidene fluoride (PVDF) transfer membrane (Bio - Rad, CA, USA). 5% BSA was used to block the non-specific binding at a room temperature for 2 hours. Washing the membrane with PBS for 1 time and then the membranes were incubator with iNOS and Arg-1 primary antibody and β-actin (Abcam, Shanghai, China) for one night. Washing the membranes with TBS supplemented 5% Tween 20 (TBST) for 3 times, each time for 10 minutes. Then incubate the second antibody at a room temperature for 1 hours. Luminescent Image Analyzer (Image Quant LAS4000mini, GE Healthcare, China) was used to detect the gene expression and relevant blots were quantified by ImageJ.

2.9 Statistical analysis

The Error bars represent the mean ± SEM, which was analyzed by GraphPad Prism Version 7 software (GraphPad Software, CA, USA).Each in vitro experiment was performed in triplicate and each in vitro experiment uses 3–6 mice per treatment group. Statistical significance was determined by a t-test and one-way ANOVA and statistical significance was defined as a p-value < 0.05.

Results

3.1 Macrophage is up-regulated in colon cancer microenvironment and is mainly M2 phenotype

Tumor-infiltrating macrophage has always been considered as one of the important factor regulating tumor development and progression, especially the ratio of anti-tumor M1 phenotype macrophage and tumor-promoting M2 phenotype macrophage[25, 26]. To examine the role of macrophage in colon cancer, we made the AOM/DSS-induced colon cancer mice model, and detected the percentage of tumor-infiltrating macrophage ($CD45^+$ $CD11b^+$ $F4/80^+$), M1 phenotype macrophage ($CD45^+$ $F4/80^+$ $CD86^+$), and M2 phenotype macrophage ($CD45^+$ $F4/80^+$ $CD206^+$) in mouse colon cancer tissue and paracancer healthy colon tissue. Fc-receptor-blocking antibody was used before staining the macrophage for flow cytometry analysis, and flow cytometry results showed that tumor-infiltrating macrophage was up-regulated in the mouse colon cancer tissue compared to the healthy tissue **(Figure 1A, B)**, which is consistent with previous studies that macrophage is increased in colon cancer microenvironment when tumor occurs[27]. Flow cytometry result also proved that M1 phenotype macrophage was decreased while M2 phenotype macrophage was increased in colon cancer microenvironment **(Figure 1C, D, E, F, S1A)**, which demonstrated that macrophage could be polarized to tumor-promoting M2 phenotype in colon cancer microenvironment, then accelerated the cancer progression. But the mechanism of M1 phenotype macrophage decreasing and M2 phenotype macrophage increasing in colon cancer is not fully understood.

3.2 Group 1 innate lymphocytes is decreased in colon cancer microenvironment

and is positive correlation with M1 phenotype macrophage

Previously studies have shown that ILCs are abundant in intestinal tissue, and ILC2s-derived IL-4 can promote tumorigenesis by polarizing macrophage towards a pro-tumorigenic M2 phenotype[28-30]. Furthermore, group 1 innate lymphocytes are characterized by secreting IFN-γ, which can polarize macrophage towards M1 phenotype[31, 32]. On basis of this, we guessed that whether the decreased M1 phenotype macrophage is due to the down-regulated IFN-γ levels in cancer microenvironment. We detected the percentage of group 1 innate lymphocytes ($CD45^+$ $Lineage^-$ $CD127^+$ $T\text{-}bet^+$) in AOM/DSS-induced mice colon cancer tissue and paracancer healthy colon tissue. Flow cytometry result showed that group 1 innate lymphocytes is decreased in mouse colon cancer tissue compared to healthy tissue **(Figure 2A, B, S1C)**. RT-PCR result showed that the expression of IFN-γ was also decreased in mouse colon cancer tissue compared to healthy tissue **(Figure 2C)**. Besides, the percentage of M1 phenotype macrophage was positive correlation with the percentage of group 1 innate lymphocytes in mouse colon cancer tissue, while M2 phenotype macrophage was negative correlation.**(Figure 2D, E)**. Whether the decrease of M1 phenotype macrophage attributes to the decrease of group 1 innate lymphocytes in mouse colon cancer tissue remains unclear.

3.3 Group 1 innate lymphocytes promotes M1 phenotype macrophage polarizing in vitro

To prove whether group 1 innate lymphocytes could promote macrophage polarizing towards a M1 phenotype, we sorted group 1 innate lymphocytes from mouse colon cancer tissue and co-cultured with macrophage cell line RAW264.7 or bone marrow-derived macrophage (BMDM). Our results showed that CD86 was significantly up-regulated on RAW264.7 and BMDM when co-cultured with group 1 innate lymphocytes while CD206 expression was decreased **(Figure 3A, B)**. To determine whether group 1 innate lymphocyte-derived IFN-γ promotes the M1 phenotype polarization, we chose to add the neutralizing antibody of group 1 innate lymphocytes secreting factors, IFN-γ and TNF-α into co-cultured system. Flow cytometry result showed that anti-IFN-γ decreased the expression of CD86 on RAW264.7 and BMDM while anti-TNF-α dialed to alter the CD86 expression **(Figure 3C, D)**. Western blotting result of iNOS (M1 phenotype macrophage protein)

and Arg-1 (M2 phenotype macrophage protein) also proved this result **(Figure 3E, F)**. Together, our results showed that group 1 innate lymphocytes could promote macrophage polarize towards a M1 phenotype by secreting IFN-γ *in vitro*.

3.4 Adoptively transferred group 1 innate lymphocyte significantly decrease the tumor growth of MC38 tumor-bearing mice

To determine whether group 1 innate lymphocytes could regulate the polarization of tumor-infiltrated macrophage to participate in the progression of colon cancer, we isolated group 1 innate lymphocytes from mouse spleen and adoptively transferred $1*10^6$ group 1 innate lymphocytes to mice after implanting MC38 cells for 3 days. At the endpoint of this research, we found that the tumor size and weight of group 1 innate lymphocyte-transferred mice significantly decreased compared to control mice **(Figure 4A, B, C)**. To prove if the distinguish of tumor growth in control and group 1 innate lymphocyte-transferred MC38 tumor-bearing mice is attributed to the phenotype change of tumor-infiltrated macrophage, we then tested the percentage of M1 and M2 phenotype macrophage in the tumor tissues of different groups. Flow cytometry results showed that the percentage of M2 macrophage was significantly decreased after adoptively transferred group 1 innate lymphocytes, while M1 macrophage increased simultaneously **(Figure 4D, E, F)**. Due to the anti-tumor effect of M1 phenotype macrophage, these results preliminarily explained that the tumor-growth inhibit effect in group 1 innate lymphocyte-transferred mice. Western blotting result also proved this phenomenon **(Figure 4G)**. The above *in vitro* experiments proved that the group 1 innate lymphocytes could promote macrophage to polarizing towards an anti-tumor M1 phenotype by secreting IFN-γ. So we wondered that whether the phenotype change of macrophage is due to the up-regulated group 1 innate lymphocytes and its-derived cytokine IFN-γ. Flow cytometry was used to detect the percentage of group 1 innate lymphocytes and IFN-γ in tumor tissue. Results showed that the percentage of group 1 innate lymphocytes in tumor microenvironment was significantly increased in group 1 innate lymphocyte-transferred mice, and that further proved that adoptively transferred group 1 innate lymphocytes could arrived in tumor microenvironment **(Figure 4H, I, S1D)**. Flow cytometry result also proved that the level of IFN-γ was up-regulated in tumor of group 1 innate lymphocyte-transferred mice **(Figure 4J, K)**. Together, we preliminary proved that the group 1 innate lymphocytes could promote macrophage

polarize towards a M1 phenotype to exert anti-tumor effect through secreting IFN-γ *in vivo*.

3.5 Blocking group 1 innate lymphocyte promotes the tumor growth of MC38 tumor-bearing mice by down-regulating M1 phenotype macrophage

To further proved that group 1 innate lymphocytes could exert anti-tumor function by reverse macrophage to anti-tumorigenesis M1 phenotype. We operated the group 1 innate lymphocyte-blocking assay with previous reported group 1 innate lymphocyte-neutralizing antibody, anti-NK1.1, in MC38 tumor-bearing mice[33]. Our results demonstrated that blocking group 1 innate lymphocyte significantly promoted the tumor growth of MC38 tumor-bearing mice compared to control mice **(Figure 5A, B, C)**, and that directly proved the anti-tumor role of group 1 innate lymphocyte in MC38 tumor-baring mice.

To prove whether this effect rely on tumor-infiltrated macrophage, we detected the percentage of M1 and M2 phenotype macrophage in tumors of different groups. Flow cytometry results showed that blocking group 1 innate lymphocytes increased the levels of tumorigenesis M2 phenotype macrophage, meanwhile decreased the levels of anti-tumorigenesis M1 phenotype macrophage **(Figure 5D, E, F)**. Western blotting result also proved this phenomenon **(Figure 5G)**. These results further proved that the anti-tumor role of group 1 innate lymphocytes might partially rely on the phenotype change of tumor-associated macrophage. To determine reason of the phenotype change of macrophage, we detected the percentage of group 1 innate lymphocytes and IFN-γ expression level in tumors of different groups. Flow cytometry result showed that the percentage of group 1 innate lymphocytes and the level of IFN-γ significantly decreased in the tumors of group 1 innate lymphocyte-blocking mice **(Figure 5H, J, I, K)**. Together, these part results further proved the anti-tumorigenesis role group 1 innate lymphocytes, and preliminary demonstrated that group 1 innate lymphocytes might promote the macrophage phenotype change through secreting IFN-γ to exert the anti-tumorigenesis effect *in vivo*.

Discussion

In this study, we explored the potential relationship between tumor-infiltrated group 1 innate lymphocytes and TAM. We found that the percentage of group 1 innate lymphocytes and anti-tumorigenesis M1 phenotype macrophage were decreased in colon cancer microenvironment, and the decreased rate is positively correlated. *In vitro* results proved that group 1 innate lymphocytes could promote macrophage to polarizing to classical activated M1 phenotype, and this effect was reversed by anti-IFN-γ. This preliminarily demonstrated the potential relationship of group 1 innate lymphocytes and tumor-infiltrated macrophage, and partially explained the positive correlation of down-regulated rate of group 1 innate lymphocytes and M1 phenotype macrophage. *In vivo* studies uncovered that group 1 innate lymphocytes indeed could inhibit the tumor growth and up-regulate the level of M1 phenotype macrophage while group 1 innate lymphocyte-blocking antibody promoted the tumor growth of MC38 tumor-bearing mice and down-regulating the M1 phenotype macrophage level. These results further proved the endogenously regulated approach of group 1 innate lymphocytes to tumor-infiltrated macrophage, and enriched our knowledge to the anti-tumor role of group 1 innate lymphocytes. Altogether, our study preliminarily proved that group 1 innate lymphocytes could promote the polarization of anti-tumor M1 phenotype macrophage through secreting IFN-γ in colon cancer microenvironment, thus inhibiting the tumor growth. It is worth noting that anti-NK1.1 does not only deplete ILC1s, but also deplete the NK cells, so we can only draw the conclusion that group 1 innate lymphocytes mediate the effect. However, group 1 innate lymphocytes consist of conventional NK cells and ILC1s, and it seems that ILC1s mainly mediate the effect. So more research should be done to confirm the exact cell subset, which responsible for the effect we found. In our next project we will solve this problem.

ILCs, as a special lymphoid cell-subset, were widely studied in recent years, and that do not express specific antigen receptor, but anticipate in a variety of diseases, such as allergic diseases, autoimmune diseases and tumor diseases[34, 35]. Compared to $CD4^+$ T

cells, ILCs are divided into three subset: Group 1 innate lymphocytes expressing transcription factor T-bet, characterized by secreting IFN-γ and TNF-α[36, 37]; group 2 innate lymphocytes expressing transcription factor RORα and GATA3, characterized by secreting type II cytokines IL-4, IL-5, IL-9 and IL-13[38, 39]; and group 3 innate lymphocyte expressing transcription factor RORγt, characterized by secreting IL-17 and IL-22[40]. Group 1 innate lymphocytes, like Th1 cells, have been reported to have a significant role in the intestine inflammatory diseases[41, 42]. In recent years, a great number of studies focused on the regulatory function of group 1 innate lymphocyte in tumor diseases[43]. A great number of the studies have shown the tumor-inhibiting effect of group 1 innate lymphocyte by secreting tumor-killing factors IFN-γ and TNF-α[44, 45]. Herein, we found a new way that group 1 innate lymphocyte exerted their anti-tumorigenesis role in colon cancer. We found that group 1 innate lymphocyte could promote the macrophage alternative activation to an anti-tumor M1 phenotype through secreting IFN-γ and then inhibiting the growth of tumors, which highlight a new anti-tumor role by group 1 innate lymphocyte. But our study also has some limitations that we only clearly proved that group 1 innate lymphocyte could promote the induction of M1 phenotype macrophage by secreting IFN-γ *in vitro,* and our *in vivo* results does not show the directly effect of group 1 innate lymphocyte on tumor-infiltrated macrophage by secreting IFN-γ to exert anti-tumor role in the tumor microenvironment. We only preliminary proved this phenomenon, more evidences should be explored to further clarify these findings.

Tumor microenvironment (TME) is an abstract space that tumor grows and invades, and that is composed of tumor cells, interstitial cells, immune cells, capillaries, intercellular substance, tissue fluid and biomolecules[46]. Mostly TME is a highly immunosuppressive environment, and this is the reason why the tumor can evade the host's immune surveillance, and grow out of control[47, 48]. Studies have proved that the incompetent and exhausted effector T cells[49], myeloid-derived suppressor cells (MDSCs)[50], TAM[51], regulatory T cells[52] and a variety of immunosuppressive factors are the main actors to form the immunosuppressive microenvironment of TME[53].

Among them, TAM especially plays a critical role, due to that they are the main immune cells infiltrated in the tumor microenvironment[54]. They can recognize and eliminate tumor cells, but as tumors occurring and developing, they are polarized to M2 phenotype, and play a key role in tumor growth, invasion and metastasis[55]. TAM plays a "double-edged sword" role in the occurrence and development of tumors. Finding out the reason that TAM has two function effects, one is anti-tumor M1 phenotype, and the other is tumor-promoting M2 phenotype, is a key step in macrophage-based tumor immunotherapy. Studies have proved that immunosuppressive TME always promote macrophage polarized to a M2 phenotype, which greatly further push the tumor invasion and growth. Here we found that M2 phenotype macrophage was greatly up-regulated in colon tumor tissue compared to healthy tissue, and that promoted the tumor growth. Reversing the M1 and M2 phenotype by adoptively transferred ILC1s, greatly increased the levels of M1 phenotype and inhibited the tumor growth, which directly proved that the ratio of M1/M2 phenotype macrophage is important for the development of tumors. How to increase the M1 phenotype macrophage and decrease the M2 phenotype macrophage level is an important task for tumor immunotherapy.

Conclusions

Our studies found that decreased group 1 innate lymphocytes level might contribute to the increase of tumor-promoting M2 phenotype macrophage. Group 1 innate lymphocytes could promote the polarization of macrophage to M1 phenotype through secreting IFN-γ. *In vivo* studies demonstrated that adoptively transferred group 1 innate lymphocytes increased the M1 phenotype macrophage and inhibited the tumor growth, while blocking group 1 innate lymphocytes reached the opposite. Our study may provide an insight in the immunotherapy of colon cancer.

Abbreviations

TAM: Tumor-associated macrophage; ILC1s: Type 1 innate lymphocytes; IFN-γ: Interferon-γ; ILCs: Innate lymphocytes; TNF-α: Tumor necrosis factor-α; DCs: Dendritic cells; CTL: Cytotoxic T cells; BMDM: Bone marrow-derived macrophage; TME: Tumor microenvironment; MDSCs: Myeloid-derived suppressor cells

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The data used to support the findings of this study are available in the supplemental data.

Declaration

The authors declare that they have no competing interests.

Authors' contributions

Yandong Zhang did the research and wrote the manuscript. Shu Ma, Tie Li and Yu Tian modified the language. Huangao Zhou and Hongsheng Wang revised the study. Lan Huang designed the study, drafted, and revised the manuscript. All authors read and approved the final manuscript.

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Supplementary description

Supplemental material includes the original data supporting our results.

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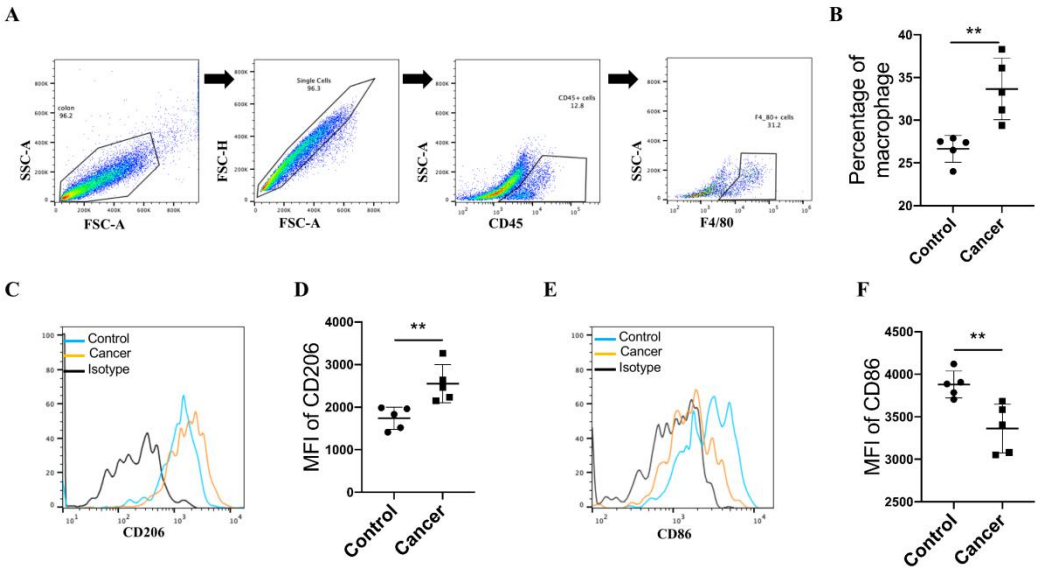
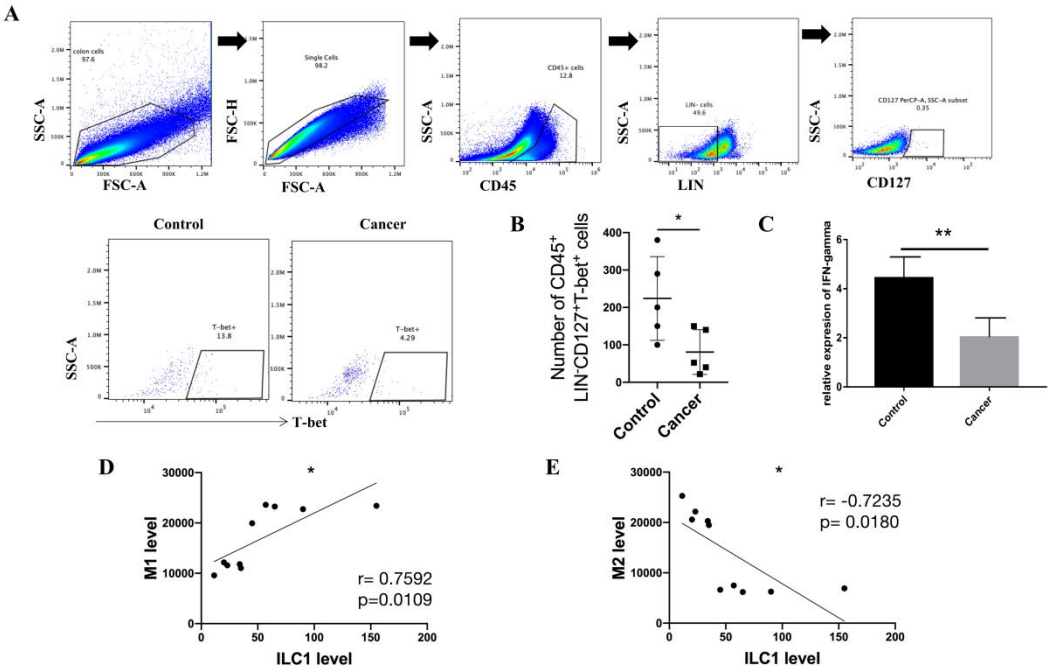
Figure legends



1. M2 phenotype macrophage up-regulated in colon cancer tissue

A: representative flow cytometry graphs of getting the colon cancer tissue and

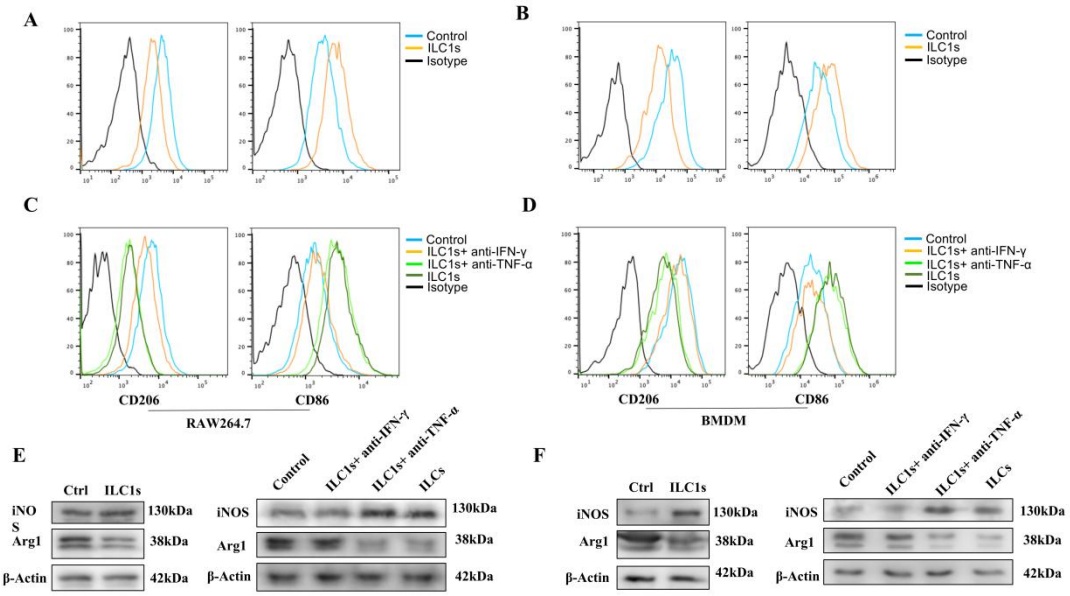
paracancer control tissue macrophage from AOM/DSS-induced mice colon cancer model, Fc-receptor-blocking antibody was used before staining the macrophage for flow cytometry analysis. **B:** percentage of macrophage ($CD45^+F4/80^+$) in tumor tissue and control, n=5. **C and D:** percentage of M2 phenotype macrophage ($CD45^+F4/80^+CD206^+$) was detected by flow cytometry, n=5. **E and F:** percentage of M1 phenotype macrophage ($CD45^+F4/80^+CD86^+$) was detected by flow cytometry, n=5. All the experiments repeated for 3 times. Data are shown as mean ± SD (n = 5 per group, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).



2. Percentage of group 1 innate lymphocyte is positive correlation with the level of M1 phenotype macrophage

A: representative flow cytometry graphs of getting the colon cancer tissue and paracancer control tissue group 1 innate lymphocytes from AOM/DSS-induced mice colon cancer model. **B:** percentage of group 1 innate lymphocytes

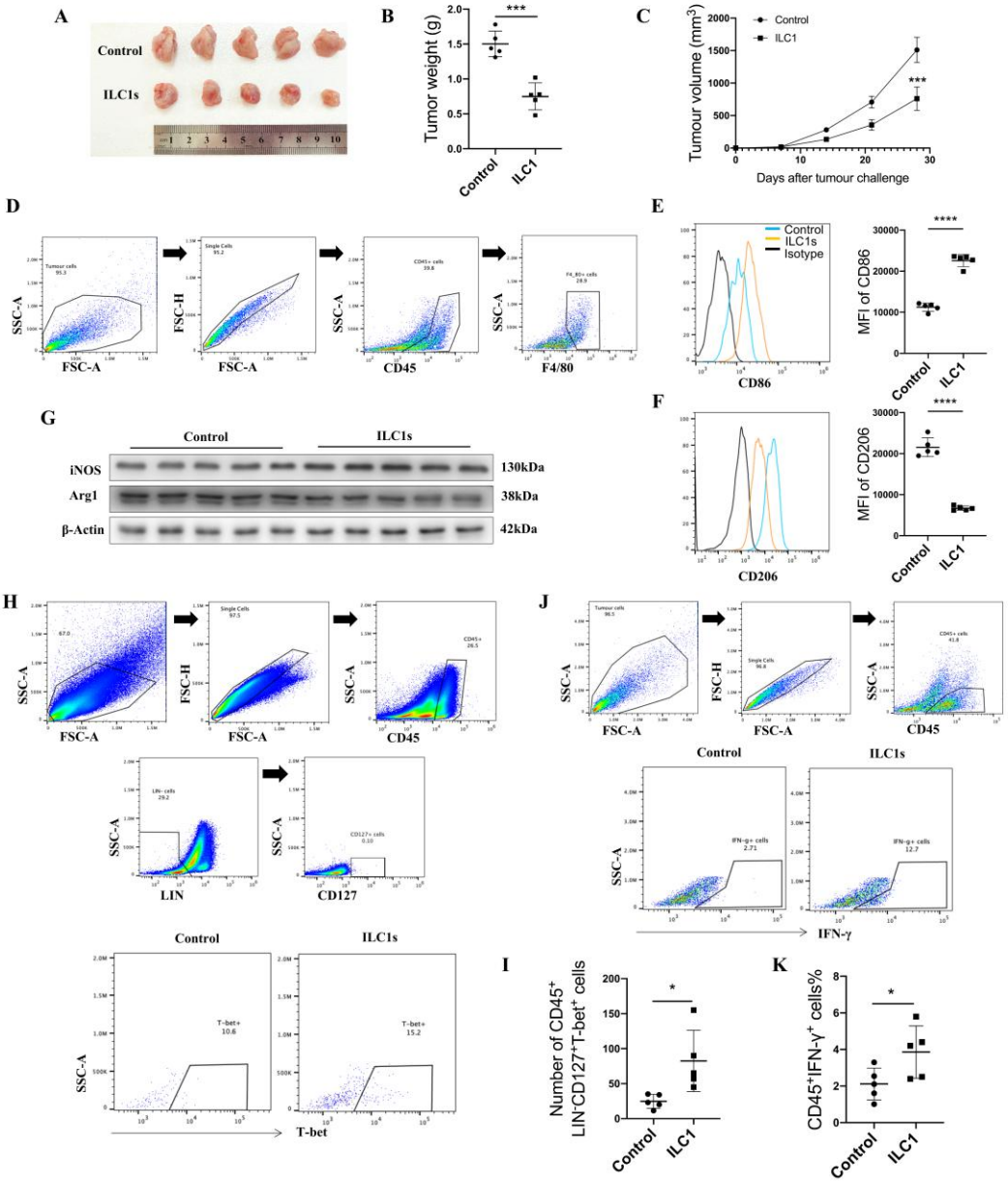
($CD45^{+}Lineage^{-}CD127^{+}T\text{-}bet^{+}$) in tumor tissue and control, n=5. **C:** relative expression of IFN-γ in cancer tissue and control was detected by RT-PCR. **D and E:** the levels of tumor-infiltrated group 1 innate lymphocytes compared to tumor-infiltrated M1 and M2 phenotype macrophage was made the regression analysis. All the experiments repeated for 3 times. Data are shown as mean ± SD (n = 5 per group, *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001).



3. Group 1 innate lymphocytes promote M1 phenotype macrophage polarizing in vitro

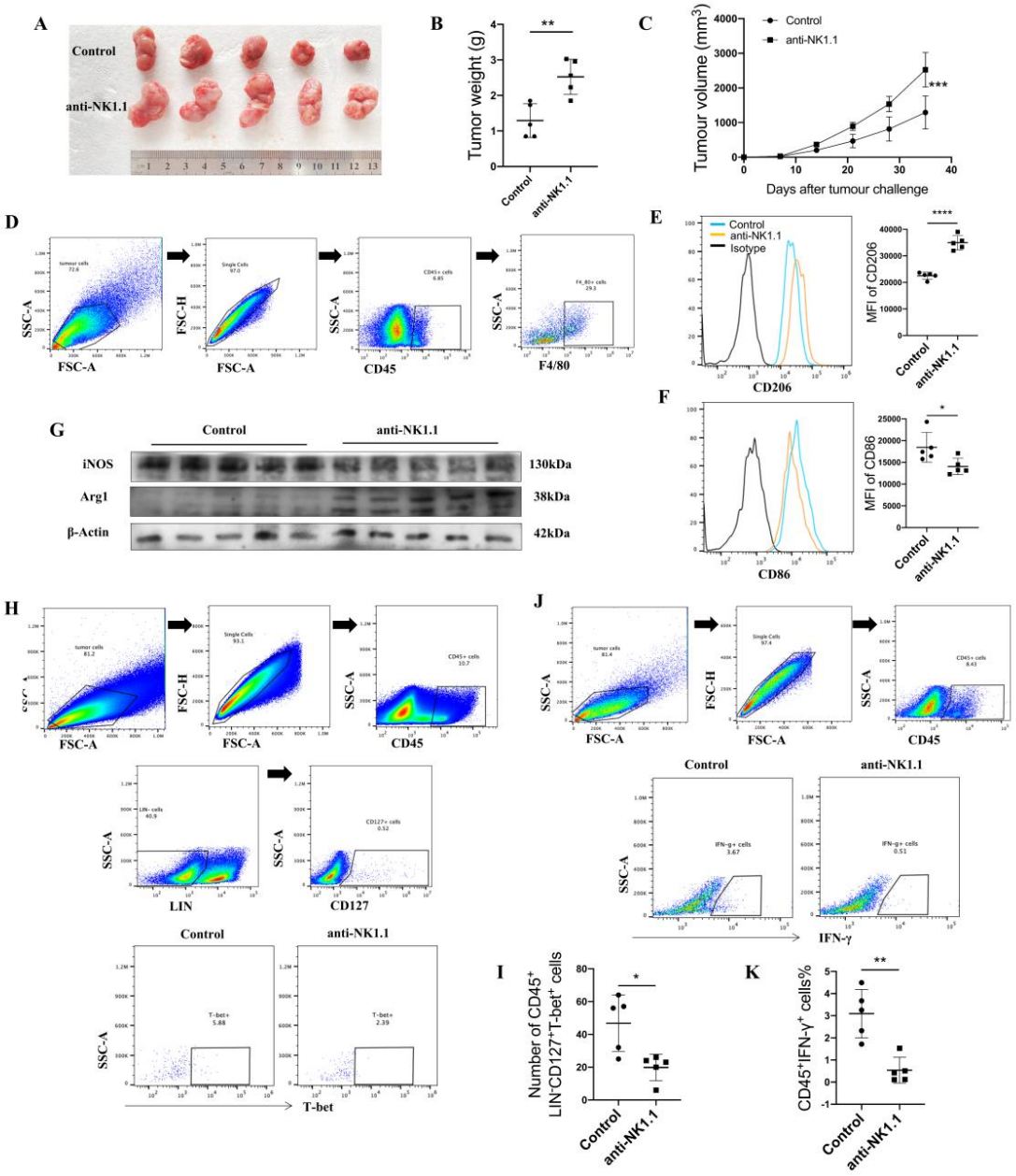
Sorted group 1 innate lymphocytes ($2*10^5$) from mouse colon cancer tissue co-cultured with macrophage cell line RAW264.7 ($1*10^5$) and bone marrow-derived macrophage (BMDM) for 24 hours. **A:** representative flow cytometry graphs of the expression of CD206 and CD86 in group 1 innate lymphocytes co-cultured RAW264.7 and control RAW264.7. **B:** representative flow cytometry graphs of the expression of CD206 and CD86 in group 1 innate lymphocytes co-cultured BMDM

and control BMDM. **C and D:** 10 ug/ml anti-IFN-γ and TNF-α were added to co-culture system, flow cytometry was used to detected the expression of CD206 and CD86 in RAW264.7 and BMDM of different treated groups. **E:** western blotting was used to detect the expression of iNOS and Arg-1 in group 1 innate lymphocytes co-cultured, anti-IFN-γ and TNF-α treated co-culture system, and control RAW264.7. **F:** western blotting was used to detect the expression of iNOS and Arg-1 in group 1 innate lymphocytes co-cultured, anti-IFN-γ and TNF-α treated co-culture system, and control BMDM. All the experiments repeated for 3 times.



4. Adoptively transferred group 1 innate lymphocytes inhibit the tumor growth of MC38 tumor-bearing mice by promoting the M1 phenotype macrophage polarization

A-C: the tumor graph, tumor weight, and tumor volume in $1*10^6$ group 1 innate lymphocytes adoptively transferred mice and control mice. **D:** representative flow cytometry graphs of getting the tumor tissue macrophage. **E:** percentage of M1 phenotype macrophage ($CD45^+F4/80^+CD86^+$) in the tumor of group 1 innate lymphocytes adoptively transferred mice and control mice was detected by flow cytometry. **F:** percentage of M2 phenotype macrophage ($CD45^+F4/80^+CD206^+$) in the tumor of group 1 innate lymphocytes adoptively transferred mice and control mice was detected by flow cytometry. **G:** western blotting was used to detect the expression of iNOS and Arg-1 in the tumor of group 1 innate lymphocytes adoptively transferred mice and control mice. **H:** representative flow cytometry graphs of getting the tumor tissue group 1 innate lymphocytes in different groups. **I:** percentage of group 1 innate lymphocytes in different groups. **J:** representative flow cytometry graphs of getting the tumor tissue IFN-γ in different groups. **K:** the expression level of IFN-γ in the tumor of group 1 innate lymphocytes adoptively transferred mice and control mice. All the experiments repeated for 3 times, n=5. Data are shown as mean ± SD (n = 5 per group, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$).



5. Blocking group 1 innate lymphocytes promoting the tumor growth of MC38 tumor-bearing mice by inhibiting the M1 phenotype macrophage polarization

A-C: the tumor graph, tumor weight, and tumor volume in group 1 innate lymphocyte-blocking mice and control mice, n=5. **D:** representative flow cytometry graphs of getting the tumor tissue macrophage. **E:** percentage of M1 phenotype macrophage ($CD45^+F4/80^+CD86^+$) in the tumor of ILC1s-blocking mice and control mice was detected by flow cytometry, n=5. **F:** percentage of M2 phenotype macrophage ($CD45^+F4/80^+CD206^+$) in the tumor of group 1 innate

lymphocyte-blocking mice and control mice was detected by flow cytometry, n=5. **G:** western blotting was used to detect the expression of iNOS and Arg-1 in the tumor of group 1 innate lymphocyte-blocking mice and control mice. **H:** representative flow cytometry graphs of getting the tumor tissue ILC1s in different groups. **I:** percentage of group 1 innate lymphocytes in different groups, n=5. **J:** representative flow cytometry graphs of getting the tumor tissue IFN-γ in different groups. **K:** the expression level of IFN-γ in the tumor of group 1 innate lymphocyte-blocking mice and control mice, n=5. All the experiments repeated for 3 times. Data are shown as mean ± SD (n = 5 per group, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$).

Supplementary Files

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