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Activated NK cells reprogram MDSCs via NKG2D-NKG2DL and IFN-γ to modulate antitumor T-cell response after cryo-thermal therapy

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ABSTRACT

Background Myeloid-derived suppressor cells (MDSCs) can potently inhibit T-cell activity, promote growth and metastasis of tumor and contribute to resistance to immunotherapy. Targeting MDSCs to alleviate their protumor functions and immunosuppressive activities is intimately associated with cancer immunotherapy. Natural killer (NK) cells can engage in crosstalk with multiple myeloid cells to alter adaptive immune responses, triggering T-cell immunity. However, whether the NK-cell-MDSC interaction can modulate the T-cell immune response requires further study. Cryo-thermal therapy could induce the maturation of MDSCs by creating an acute inflammatory environment to elicit a $CD4^+$ Th1-dominant immune response, but the mechanism regulating this process remains unclear.

Methods NK cells were depleted and NKG2D was blocked with monoclonal antibodies in vivo. MDSCs, NK cells and T cells were assessed by flow cytometry and isolated by magnetic-activated cell sorting (MACS). MDSCs and NK cells were cocultured with T cells to determine their immunological function. The transcriptional profiles of MDSCs were measured by qRT-PCR and RNA-sequencing. Isolated NK cells and MDSCs by MACS were cocultured to study the viability and maturation of MDSCs regulated by NK cells. TIMER was used to comprehensively examine the immunological, clinical, and genomic features of tumors.

Results NK-cell activation after cryo-thermal therapy decreased MDSC accumulation and reprogrammed immunosuppressive MDSCs toward a mature phenotype to promote T cell antitumor immunity. Furthermore, we discovered that NK cells could kill MDSCs via the NKG2D-NKG2DL axis and promote MDSC maturation by interferon gamma (IFN-γ) in response to NKG2D. In addition, $CD4^+$ Th1-dominant antitumor immune response was dependent on NKG2D, which promoted the major histocompatibility complex II pathway of MDSCs. High activated NK-cell infiltration and NKG2D level in tumors were positively correlated with better clinical outcomes.

Conclusions Cryo-thermal therapy induces effective $CD4^+$ Th1-dominant antitumor immunity by activating NK cells to reprogram MDSCs, providing a promising therapeutic strategy for cancer immunotherapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Immunotherapies based on T cells are challenged with immunosuppression mediated by myeloid-derived suppressor cells (MDSCs) and targeting MDSCs is a major clinical challenge as they are a heterogeneous myeloid cell population.

⇒ Natural killer (NK) cells can crosstalk with multiple innate immune cells, including dendritic cells, monocytes and macrophages to promote T cell immunity, but whether NK cells can regulate MDSCs remains unknown.

WHAT THIS STUDY ADDS

⇒ Cryo-thermal therapy on local tumor can systematically activate NK cells to reprogram MDSCs, promoting antitumor T-cell immunity.

⇒ Activated NK cells could kill MDSCs via the NKG2D-NKG2DL axis and promote MDSC maturation by IFN-γ to induce $CD4^+$ Th1-dominant antitumor immunity.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Immunotherapies based on NK cells can target not only their cytotoxicity against tumor cells, but also their powerful immunological regulation of innate immunity.

⇒ The NKG2D-NKG2DL axis is a potential target and biomarker for cancer immunotherapy beyond tumor type.

INTRODUCTION

In recent years, immunotherapies harnessing T-cell immunity to fight cancer have been developed, such as immune checkpoint inhibitors and adoptive T cell transfer.[1] However, the efficacy of immunotherapies against solid tumors is unsatisfactory because of the challenging tumor microenvironment, which can dampen T-cell activation and cytotoxicity.[2] Myeloid-derived suppressor cells (MDSCs) can suppress the proliferation and cytokine production of circulating and

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tumor-infiltrating T cells through multiple pathways.[3 4] On the other hand, as a mixture of immature myeloid cells, MDSCs have the potential to support adaptive immunity through differentiation into mature myeloid cells.[5 6] Thus, reprogramming MDSCs from immunosuppression toward a mature phenotype would be a promising strategy to achieve the optimum therapeutic outcome.

Natural killer (NK) cells are specialized lymphocytes that are activated without prior sensitization but through the balance between activating and inhibitory signals from cell surface receptors, protecting the host against viral infection and cancer.[7–9] NK cells have potent cytotoxic and immunoregulatory activity and can also produce several cytokines, including interferon gamma (IFN-γ), GM-CSF, and tumor necrosis factor-alpha (TNF-α), regulating recruitment and activity of numerous immune cell types.[10 11] However, NK cells are often dysfunctional in advanced cancer.[12] Therefore, understanding how to effectively enhance NK cells will be helpful to improve tumor treatment in the future.

NK cells play a significant role in interacting with myeloid cells to aid antigen presenting cells (APCs), especially dendritic cell (DC) maturation, enhancing innate immunity against tumors and shaping the adaptive immune system.[13] NK-cell-DC interactions result in NK-cell activation and the maturation and/or apoptosis of DCs through direct contact and soluble cytokines, skewing T-cell differentiation toward the Th1 phenotype.[14] In addition, M0 and M2 macrophages can be eliminated by activated NK cells, but M1 macrophages are resistant to lysis because they upregulated major histocompatibility complex (MHC) molecules.[15] Moreover, NKG2D-MICA interaction mediates direct communication between NK cells and monocytes during innate immune responses.[16] However, although MDSCs are often the largest population of myeloid cells in cancer patients, the role of NK cells in MDSC regulation is poorly understood.

We developed a thermal physical therapy for treating solid tumors with a combination of precooling and subsequent radiofrequency ablation called cryo-thermal therapy. Cryo-thermal therapy increased the release of HSP70, participating in the remolding of MDSCs and promoting DC and macrophage differentiation.[17] Furthermore, cryo-thermal-induced M1 macrophages directly regulate $CD4^+$ Th1 differentiation, which result in the long-term protection against tumors.[18 19] Although the activation of innate immunity could promote $CD4^+$ Th1 differentiation,[18 20–22] the mechanism by which cryo-thermal therapy induces $CD4^+$ Th1-dependent antitumor immunity requires further study. We hypothesized that, the interaction of NK cells and MDSCs led to reprogram of MDSCs before the differentiation of MDSCs into mature APCs after cryo-thermal therapy, which could regulate $CD4^+$ Th subsets differentiation.

This study determined the role of NK cells in antitumor immunity in the B16F10 melanoma model and focused on the regulatory role of NK cells in the differentiation and functions of MDSCs. Our results showed that NK cells were markedly activated and decreased the accumulation of MDSCs after cryo-thermal therapy, reprogrammed immunosuppressive MDSCs toward a mature phenotype and alleviated MDSC-mediated suppression. RNA-seq and in vitro studies confirmed that activated NK cells reprogrammed MDSCs toward a mature phenotype, resulting in the promotion of a $CD4^+$ Th1-dominant immune response. Furthermore, we demonstrated that activated NK cells could kill MDSCs to reduce their accumulation through the NKG2D-NKG2DL axis and promote their maturation via IFN-γ in response to NKG2D. We clarified a novel mechanism by which NK cells regulate MDSC maturation and function after cryo-thermal therapy to trigger optimal systemic antitumor $CD4^+$ Th1-dominant immunity.

METHODS

Animal model and cell culture

The culture of B16F10 cell line and management of C57BL/6 mice are performed as previously described.[23] To prepare the tumor-bearing model, female mice at the age of 6–8 weeks were injected subcutaneously with 5×10^5 B16F10 tumor cells into the right flank.

Cryo-thermal therapy

The system and program of cryo-thermal therapy was used as previously described.[23] Cryo-thermal therapy was performed on B16F10 tumors 12 days postinoculation, the tumor volume reaching approximately 0.25 cm^3. All procedures were performed aseptically.

Single-cell suspension preparation and flow cytometry

Blood and spleens were harvested from sacrificed mice and single-cell suspension of samples was prepared as previously described.[23] Cell surface staining, transcription factor and intracellular cytokine staining were performed as previously described.[23] The absolute number of immune cells in peripheral blood was obtained by Precision Count Beads (Biolegend) following manufacturer's instructions. Flow data were recorded by the BD FACS Aria II cytometer and analyzed with FlowJo software.

Tumor rechallenge

Survivors were infused with 1×10^5 B16F10 tumor cells intravenously 14 days after treatment. Nodules of lung tumors were enumerated 21 days after rechallenging.

Depletion of NK cells and blockade of NKG2D in vivo

Mice were injected with 250 µg anti-NK1.1 antibodies (Biolegend) i.p. on day −1 and 4 post cryo-thermal therapy or tumor rechallenge to deplete NK cells. NK-cell depletion was confirmed in vivo previously. To block NKG2D, mice were injected i.p. with 200 µg anti-NKG2D antibodies (Biolegend) on day −1 and 4 post cryo-thermal therapy.

Cell isolation

MDSCs, NK cells and T cells were isolated from spleen with EasySep system according to the manufacturer's

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instructions and were purified to >80%. For isolation of MDSCs, cells were first labeled with Gr-1-PE, and then, PE^+ cells were selection by positive selection kit. All the kits used were listed in online supplemental material 1.

T cell proliferation assay

T cells isolated from naïve mice were labeled with CFSE (carboxyfluorescein diacetate succinimidyl ester) (5 µM/mL) and cocultured with MDSCs with/without NK cells at a ratio of 2:1, in the presence of anti-CD3 activation for 72 hours. T-cell proliferation was then measured by flow cytometry. The reduction of CFSE was regarded as cell proliferation and the percentage of proliferated T cells was calculated.

mRNA sequencing of MDSCs

MDSCs were isolated as described above, and the total RNA was extracted by Nucleospin RNA II kits. Enrichment of mRNA, fragmentation, reverse transcription, library construction, HiSeq X Ten and data analysis were performed by Genergy Biotechnology (Shanghai, China).

Statistical analysis

All data are presented as the mean±SD. Significance was determined using a two-sided Student's T-test. GraphPad Prism V.9.0 (La Jolla, California, USA) was used for all statistical analysis.

RESULTS

Cryo-thermal-activated NK cells reprogram MDSCs toward a mature phenotype

The crosstalk between myeloid cells and NK cells has been reported in many studies. NK cells can be activated by DCs, monocytes and macrophages; conversely, activated NK cells can promote maturation of APCs, thereby promoting T-cell immunity.[16] Our previous study showed that MDSCs, which are the major population of myeloid cells, could differentiate into mature DCs after cryo-thermal therapy, but whether interaction between NK cells and MDSCs happened, thereby affecting the T-cell response, remains unclear. Therefore, the changes of NK cells and MDSCs in blood and spleen were analyzed at different times in the B16F10 model post cryo-thermal therapy (online supplemental figure S1A). The percentage of NK cells was increased in blood at the early stage (1 d) after cryo-thermal therapy, as well as in blood and spleen at the late stage (7–14 dys) (figure 1A). Moreover, the proportion of MDSCs in blood after cryo-thermal therapy was lower at the late stage (10–14 days) and remained at a low level in the spleen (figure 1B). Thus, cryo-thermal therapy increased NK-cell expansion but inhibited MDSC accumulation. Thus, we investigated whether NK cells could regulate MDSC function and accumulation after cryo-thermal therapy by depleting NK cells in vivo (figure 1C). As shown in figure 1D and online supplemental figure S1B, the percentage of MDSCs in spleen and the absolute number in blood in the anti-NK1.1 group was much higher than that in the isotype group. Moreover, the level of MHC II and CD86 on MDSCs in the anti-NK1.1 group was significantly decreased (figure 1E). Furthermore, MDSC subsets (M-MDSCs and G-MDSCs) showed the same trend (online supplemental figure S1C, D). These results revealed that NK cells reduced the accumulation of MDSCs and promote MDSC maturation. To determine the role of NK cells in antitumor immunity, tumor rechallenge was performed with NK-cell depletion, and the tumor growth in lung was detected (figure 1F). As shown in figure 1G, NK cells depletion resulted in numerous tumor nodules growth in lung, which meant that NK cells were critical for cryo-thermal-induced antitumor immunity. To assess the immunoregulatory factor profile of MDSCs, MDSCs were isolated from the spleen by magnetic-activated cell sorting (MACS), and their gene expression was measured by qRT-PCR. MDSCs from the isotype group showed higher levels of immunostimulatory cytokines (TNF-α, IL-6, IL-1β, IL-7, IL-12, and IL-15) and lower levels of immunosuppressive factors (VEGF, Arg1, iNOS, and IL-10) than those from the untreated group (figure 1H). However, MDSCs in the anti-NK1.1 group showed a completely different gene profile from those in the isotype group, which was characterized by the upregulation of immunosuppressive factors (VEGF, Arg1, iNOS, and IL-10) and the downregulation of immunostimulatory cytokines (TNF-α, IL-6, IL-1β, IL-7, IL-12, and IL-15) (figure 1H). These results demonstrated that NK cells mediated MDSCs reprogramming after cryo-thermal therapy, reducing their accumulation, promoting their maturation with less immunosuppressive function.

In addition, the subsets of newly formed NK cells in the anti-NK1.1 group were analyzed. The proportion of NK cells in spleen and absolute number in blood in the anti-NK1.1 group was significantly lower than that in the isotype group (figure 1I and online supplemental figure S1E). The percentage of activated NK cells ($CD11b^+$ $CD27^+$) and mature NK cells ($CD11b^+$ CD27) and their expression of IFN-γ, granzyme B, perforin, and NKG2D in the isotype group were much higher than those in the untreated group, but these indices were significantly decreased in the anti-NK1.1 group (figure 1J and K). Moreover, the level of PD-1 in NK cells of anti-NK1.1 group was markedly upregulated compared with that in the isotype group (figure 1K). These data revealed that on NK-cell depletion, MDSCs inhibited the antitumor effects of newly formed NK cells. In summary, these results showed that NK cells were markedly activated post cryo-thermal therapy and could reprogram immunosuppressive MDSCs toward a mature phenotype and relieve MDSC-mediated suppression.

Activated NK cells regulate MDSCs to enhance the T-cell immune response

NK cells bridge innate and adaptive immune responses, inducing the subsequent development of tumor-specific T-cell responses.[10] Previously, we demonstrated that Th1 mediated long-term antitumor immune memory of cryo-thermal therapy.[24] Therefore, changes in T-cell subsets after cryo-thermal therapy with NK-cell depletion were analyzed. Compared with those in the untreated group,

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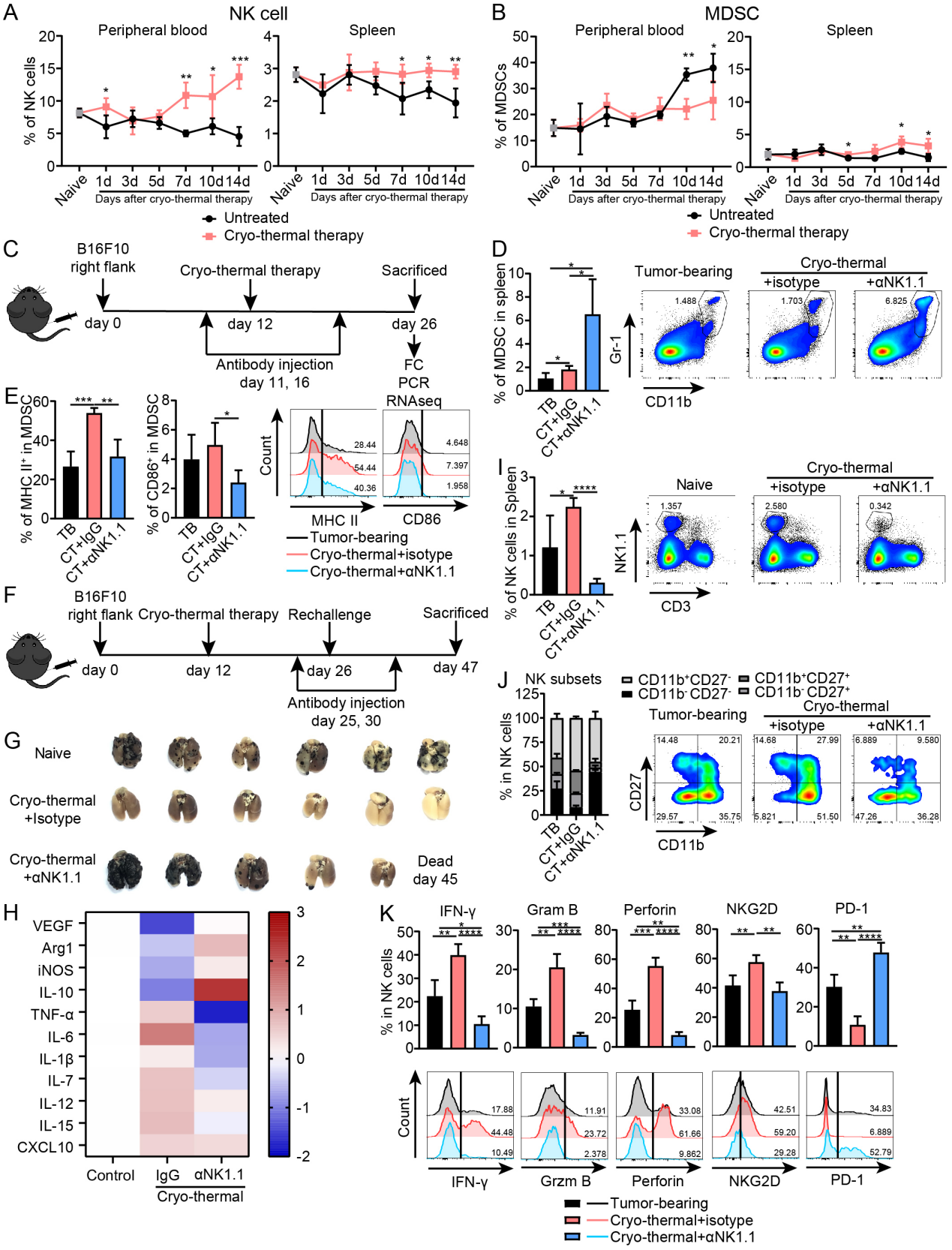

Figure 1 Phenotype of NK cells and MDSCs and NK-cell depletion. (A, B) percentage of NK cells (A) and MDSCs (B) after cryo-thermal therapy. (C) Scheme of the NK-cell depletion model. (D) Frequency of MDSCs. (E) Maturation of MDSCs. (F) Scheme of tumor rechallenge model. (G) Picture of tumor nodules in the lung after tumor rechallenge. n=6 per group. (H) Gene expression of MACS-isolated MDSCs. (I) frequency of NK cells. (J) Activation and maturation of NK cells. (K) Cytokine and receptor expression in NK cells. All data are shown as the mean ±SD n=4 for each group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. MACS, magnetic-activated cell sorting; MDSCs, myeloid-derived suppressor cells; NK, natural killer.

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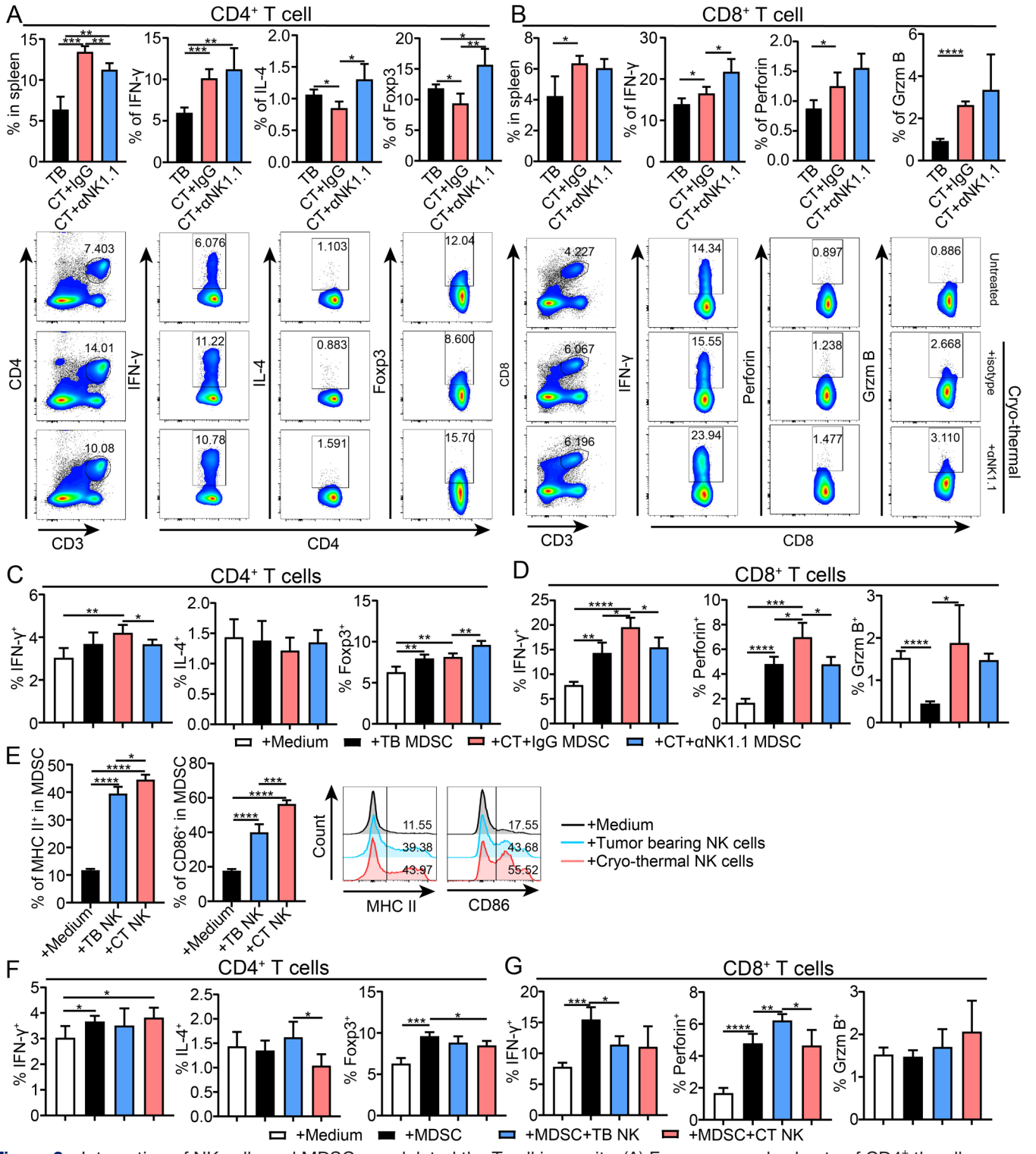

Figure 2 Interaction of NK cells and MDSCs modulated the T-cell immunity. (A) Frequency and subsets of CD4⁺ th cells. (B) Frequency and cytokines of CD8⁺ CTL cells. (C, D) T cells were cocultured with medium or MDSCs from different groups for 24 hours, and the th subsets (C) and CD8⁺ CTLs (D) were analyzed. (E) isolated MDSCs were cocultured with NK cells for 24 hours, and maturation was assessed by MHC II and CD86. (F, G) T cells were cocultured with medium or MDSCs and NK cells from different groups for 24 hours, and the th subsets (F) and CD8⁺ CTLs (G) were analyzed. All data are shown as the mean ±SD n=4 for each group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. CTL, cytotoxic T lymphocyte; MDSCs, myeloid-derived suppressor cell; MHC, major histocompatibility complex; NK, natural killer.

the proportion of CD4⁺ T cells and CD8⁺ T cells in spleen and absolute number in blood in the isotype group were significantly increased, indicating cryo-thermal therapy promoted T-cell proliferation (figure 2A,B and online supplemental figure S1F, H). However, the proportion of CD4⁺ T cells in spleen and absolute number of both CD4⁺

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T cells and $CD8^+$ T cells in blood was obviously decreased in the anti-NK1.1 group compared with the isotype group (figure 2A and online supplemental figure S1F, H). Moreover, the abundance of $CD4^+$ Th1 cells was obviously increased in the isotype group compared with the untreated group, which was accompanied by decreased levels of Th2 and regulatory T cells (Tregs), but on NK-cell depletion, the levels of Th2 and Tregs were notably increased (figure 2A). The expression of IL-17, Bcl6, Thpok, perforin, and granzyme B in $CD4^+$ T cells were not obviously changed in the anti-NK1.1 group or isotype group (online supplemental figure S1G). In addition, PD-1 on $CD4^+$ T cells was much higher in the anti-NK1.1 group than in the isotype group (online supplemental figure S1G). Meanwhile, the level of Tim-3 and Lag-3 on $CD4^+$ T cells was not significantly changed (online supplemental figure S1G). These results showed that NK cells promoted $CD4^+$ Th1 differentiation, prevented Th2 and Treg differentiation and inhibited $CD4^+$ T-cell exhaustion. Moreover, $CD8^+$ T cells were activated and had increased levels of IFN-γ, perforin, and granzyme B in the isotype group compared with the untreated group (figure 2B). Surprisingly, in the anti-NK1.1 group, $CD8^+$ T cells showed further increased expression of IFN-γ and exhibited similar expression profiles of the cytotoxic genes perforin and granzyme B compared with those in the isotype group (figure 2A and B). In addition, the level of PD-1, Tim-3 and Lag-3 on $CD8^+$ T cells was not significantly changed (online supplemental figure S1I). These results indicated that the activation of $CD8^+$ T cells was regulated by NK cells. Overall, these data indicated that cryo-thermal-activated NK cells enhanced $CD4^+$ Th1-dominant immunity and decreased $CD4^+$ T-cell exhaustion but limited $CD8^+$ T-cell activation.

To further investigate whether NK cells modulate the T-cell immune response by reprogramming MDSCs, MDSCs from the untreated, isotype and anti-NK1.1 groups were isolated by MACS and cocultured with naïve T cells to examine T-cell proliferation, Th differentiation and cytotoxic T lymphocyte (CTL) activation (figure 2C,D and online supplemental S2A–C). As shown in online supplemental figure S2C, MDSCs in the isotype group promoted the proliferation of $CD4^+$ T cells but inhibited the proliferation of $CD8^+$ T cells compared with MDSCs in the untreated group. However, MDSCs in the anti-NK1.1 group significantly inhibited $CD4^+$ T-cell proliferation of but promoted $CD8^+$ T-cell proliferation compared with those in the isotype group (online supplemental figure S2C). These data indicated that MDSCs were reprogrammed by NK cells and could promote $CD4^+$ T-cell proliferation but inhibit $CD8^+$ T-cell proliferation. In addition, MDSCs in the isotype group enhanced Th1 differentiation but inhibited Treg differentiation compared with those in the anti-NK1.1 group, corresponding with in vivo results (figure 2C). However, the level of IFN-γ and perforin in $CD8^+$ T cells that were cocultured with MDSCs from the isotype group were significantly higher than those from the anti-NK1.1 group (figure 2D). These results revealed that MDSC maturation was promoted by activated NK cells and promoted $CD4^+$ Th1-cell dominance but regulated $CD8^+$ T-cell activation.

To examine whether NK cells could directly rescue the immunosuppressive effects of MDSCs after therapy, NK cells from untreated and cryo-thermal treated mice were cocultured with MDSCs from the anti-NK1.1 group (online supplemental figure S2B). The viability of MDSCs was measured 6 hour after coculture, and MDSC phenotype was analyzed after 24 hours of coculture. The viability of MDSCs after being cocultured with NK cells from the cryo-thermal group was higher than that in the untreated group (online supplemental figure S2D). Moreover, NK cells from the cryo-thermal group significantly increased the levels of MHC II and CD86 on MDSCs compared with those in the untreated group and the control group (figure 2E). These data indicated that NK cells could both directly eliminate MDSCs and promote the maturation of MDSCs.

Furthermore, to assess the effect of MDSCs on T-cell proliferation and differentiation, naïve T cells were added to the coculture system containing NK cells and MDSCs, and Th differentiation and CTL activation were analyzed 24 hours after coculture. The proliferation of $CD4^+$ T cells was significantly increased when cocultured with NK cells from the cryo-thermal group and MDSCs, but the proliferation of $CD8^+$ T cells was inhibited compared with NK cells from the untreated group and MDSCs (online supplemental figure S2E). In addition, MDSCs cocultured with NK cells from the cryo-thermal group markedly reduced Th2 and Treg differentiation compared with MDSCs cocultured with NK cells from the untreated group (figure 2F). However, the expression of perforin in $CD8^+$ T cells was decreased when MDSCs were cocultured with NK cells from the cryo-thermal group compared with those from the untreated group (figure 2G). These data showed that the immunosuppressive effects of MDSCs could be inhibited by activated NK cells after therapy, leading to $CD4^+$ Th1-cell response and impairment of the $CD8^+$ T-cell response. In summary, our in vivo and in vitro results indicated that NK cells activated by cryo-thermal therapy could reprogram immunosuppressive MDSCs toward a mature phenotype, resulting in a $CD4^+$ T Th1-dominant immune response while impairing $CD8^+$ T-cell response.

MDSC maturation in response to activated NK cells depends on IFN-γ

To determine the mechanism NK cells regulating accumulation and function of MDSCs, 3′ RNA sequencing of MACS-isolated MDSCs from the spleens of untreated and cryo-thermal treated mice was conducted. MDSCs from cryo-thermal treated mice underwent more robust transcriptional changes than those from untreated mice (figure 3A,B). KEGG and GO term analysis revealed that MDSCs from cryo-thermal treated mice had marked changes in immune system pathways and the cell cycle, as shown in the Top 20 GO terms (figure 3C). Hallmark gene set enrichment analysis (GSEA) of MDSCs from

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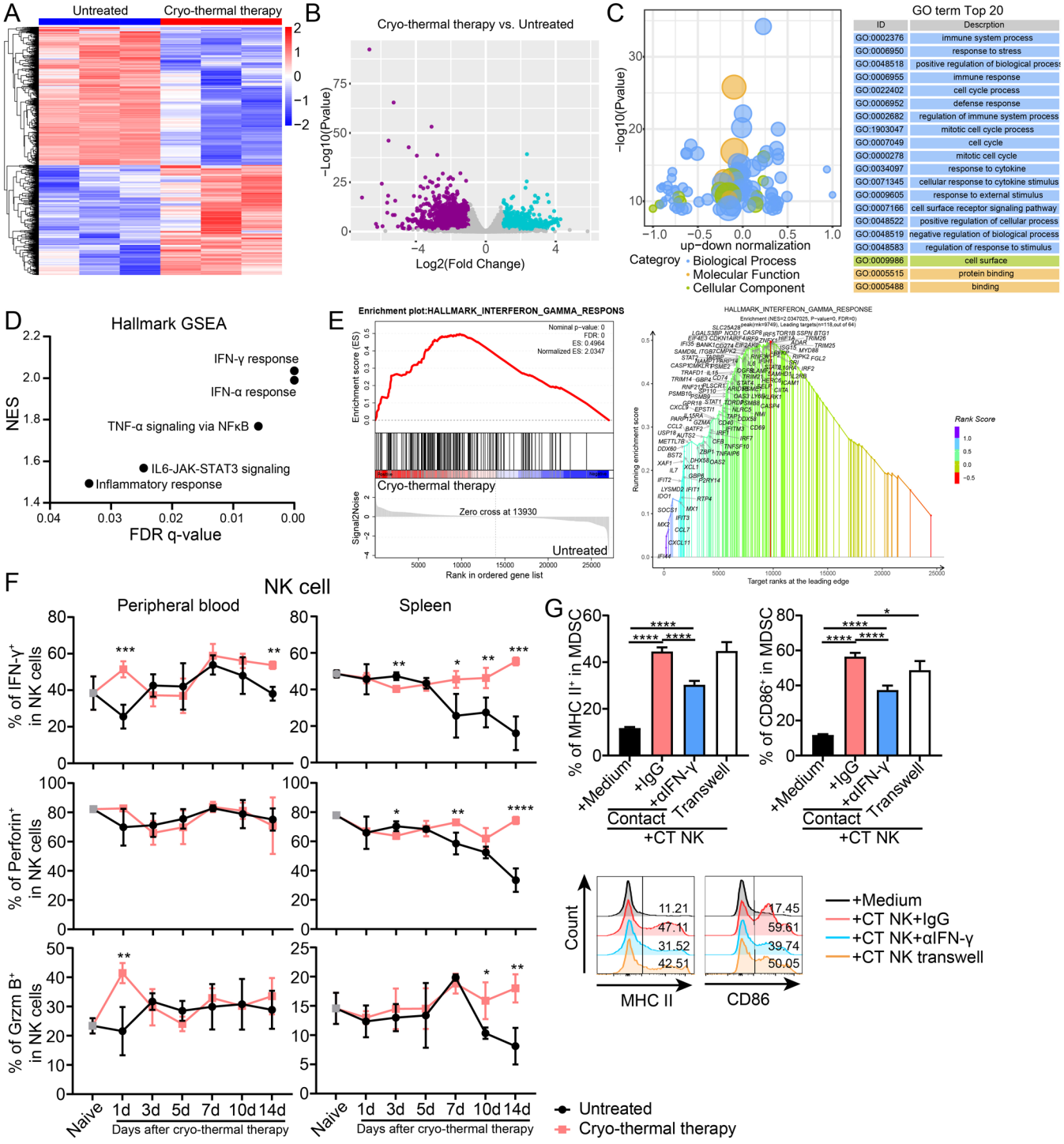

Figure 3 MDSCs regulated by activated NK cells. MDSCs were isolated by MACS and analyzed by mRNA-seq. (A) Heatmap of differentially expressed genes, encompassing sample and gene clustering. (B) Volcano plot of gene expression change of MDSCs from cryo-thermal treated mice over untreated mice, encompassing p value and fold change. (C) Z score bubble map of GO term top 20 of MDSCs. (D) GSEA showing significantly enriched gene sets of MDSCs from cryo-thermal treated mice. (E) Individual GSEA enrichment plot for the hallmark IFN-γ response gene set (left), GSEA dot plot (right). (F) Cytokine expression of NK cells. (G) Isolated MDSCs were cocultured with NK cells for 24 hours in the presence of anti-IFN-γ or isotype or in transwells, and maturation was assessed by MHC II and CD86. All data are shown as the mean ±SD n=4 for each group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. FDR, false detection rate; GSEA, gene set enrichment analysis; MDSC, myeloid-derived suppressor cell; MHC, major histocompatibility complex; NES, normalized enrichment score; NK, natural killer.

cryo-thermal treated mice revealed that the response to IFN-γ was the top pathway among the enriched pathways (figure 3D), which was confirmed by individual IFN-γ target genes (figure 3E). Moreover, the comprehensive down-regulation of the cell cycle and upregulation of antigen

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processing and presentation were revealed by KEGG pathway analysis (online supplemental figures S4, 5). Notably, both the MHC I and MHC II pathways in MDSCs were activated after cryo-thermal therapy (Online supplemental figure S5). Overall, RNA sequencing further verified that after cryo-thermal therapy, MDSCs underwent a process in response to IFN-γ and antigen processing and presentation, as well as decreased accumulation. Moreover, NK cells, which produce IFN-γ, regulated the proportion and maturation of MDSCs in vivo (figure 1B and E), and we hypothesized that IFN-γ produced by NK cells could regulate the accumulation and function of MDSCs. Compared with those in the untreated group, the levels of IFN-γ and granzyme B of NK cells were significantly increased in PBMCs at the early stage (1 day) (figure 3F). Furthermore, the levels of IFN-γ, perforin and granzyme B were increased in the spleen in the late stage (10–14 days) after cryo-thermal therapy (figure 3F). These results indicated that cryo-thermal-activated NK cells could secrete high level of IFN-γ and exert strong cytotoxicity. To verify whether the regulation of MDSCs by NK cells mediated via IFN-γ, NK cells and MDSCs were cocultured in vitro with anti-IFN-γ antibodies or isotype. Transwell assays were used to assess NK-cell regulation on MDSCs. As shown in online supplemental figure S3, the viability of MDSCs was decreased in the anti-IFN-γ group, suggesting that IFN-γ increased the viability of MDSCs. However, the viability of MDSCs separated from NK cells by the transwell was much higher (online supplemental figure S3), suggesting that NK-cell-mediated killing of MDSCs was dependent on direct contact. In addition, the level of MHC II and CD86 on MDSCs was decreased in the anti-IFN-γ group, and the expression of CD86 on MDSCs that were separated from NK cells by the transwell was decreased (figure 3G), indicating that the effect of IFN-γ secreted by NK cells on MDSC maturation was stronger than that of NK-cell-MDSC contact. These data suggested that NK-cell-mediated killing of MDSCs was contact-dependent and that NK-cell-mediated MDSC maturation was mainly mediated by IFN-γ.

The activation of NK cells is dependent on NKG2D

We showed that the activation of NK cells played a critical role in reducing the accumulation of MDSCs in a contact-dependent manner and promoting MDSC maturation via IFN-γ. However, how NK cells are activated after cryo-thermal therapy is not clear. NKG2D is a dominant activation receptor of NK cells that can directly bind to a diverse family of ligands and induce cytotoxicity and IFN-γ production.[25] As shown in figure 4A, the level of NKG2D on NK cells was significantly increased after cryo-thermal therapy. Other receptors and associated molecules (DAP10, DNAM-1, CD96, TIGIT, and NKG2A) on NK cells were also analyzed by qRT-PCR on day 14 after cryo-thermal therapy. The expression of DNAM-1, CD96, TIGIT, and NKG2A on NK cells were not obviously changed compared with that in untreated mice, but the expression of DAP10, which is the intracellular adaptor of NKG2D, was markedly upregulated (figure 4B), which was consistent with the high level of NKG2D, further confirming the activation of NK cells through NKG2D after cryo-thermal therapy. Meanwhile, almost all the ligands of NK-cell receptors (RAE1a-e, MULT1, CD155, CD122) on MDSCs were decreased on day 14 after cryo-thermal therapy (figure 4C). However, the level of NKG2D on NK cells was increased at multiple time points (figure 4A), thus the dynamic changes in its ligand (pan-RAE1) on MDSCs were further measured by flow cytometry from 1 day to 14 days after cryo-thermal therapy. Interestingly, the expression of RAE1 on MDSCs was much higher than that in the untreated group in the early stage (1 day) after therapy, which was consistent with NK-cell activation 1 day after therapy (figure 4D). Because the level of NKG2D on NK cells and the level of the NK cell receptor ligand RAE1 on MDSCs were increased in the early stage after cryo-thermal therapy, NKG2D-NKG2DL (RAE1) might be associated with NK-cell-MDSC contact. Moreover, we identified that NKG2D was mostly expressed on NK cells, and RAE1 was mostly expressed on MDSCs (figure 4E), confirming that the NKG2D-NKG2DL axis mediated the interaction between NK cells and MDSCs. To verify whether the activation of NK cells after cryo-thermal therapy was dependent on NKG2D, NKG2D was blocked by i.p. injection of anti-NKG2D monoclonal antibodies in vivo before and after therapy, as shown in figure 1C. We found that most of the mice (75%) with blockade of NKG2D relapsed within 14 days after cryo-thermal therapy, while only one mouse (25%) in the isotype group recurred (figure 4F), suggesting that blocking NKG2D severely attenuated antitumor immunity induced. Moreover, cryo-thermal therapy induced necrosis in tumor tissue, which was abrogated by blocking NKG2D (figure 4G). These results revealed that NKG2D mediated antitumor immunity induced by cryo-thermal therapy. As shown in figure 4H1, although the percentage of NK cells in spleen in the anti-NKG2D group was not significantly changed, the proportion of mature NK-cell subset ($CD11b^{+}CD27^{+}$) and the absolute number of NK cells in blood was decreased. In addition, the expression of IFN-γ, perforin, granzyme B, and NKG2D were significantly decreased in the anti-NKG2D group (figure 4J). These data demostrated that NKG2D mediated the activation of NK cells with high expression of IFN-γ, perforin, granzyme B, and NKG2D after cryo-thermal therapy.

Nk cell-mediated regulation of MDSCs and modulation of the T-cell response depends on NKG2D

Because the NKG2D-NKG2DL axis is critical for the crosstalk between NK cells and MDSCs and NKG2D blockade impaired NK cells, we further studied the changes in MDSCs after NKG2D blockade. As described previously, MDSCs in the isotype group and anti-NKG2D group were isolated by MACS, and 3′ RNA sequencing was performed. As shown in figure 5A–B, MDSCs in anti-NKG2D group underwent robust transcriptional changes compared with those in the isotype group. GO term analysis revealed

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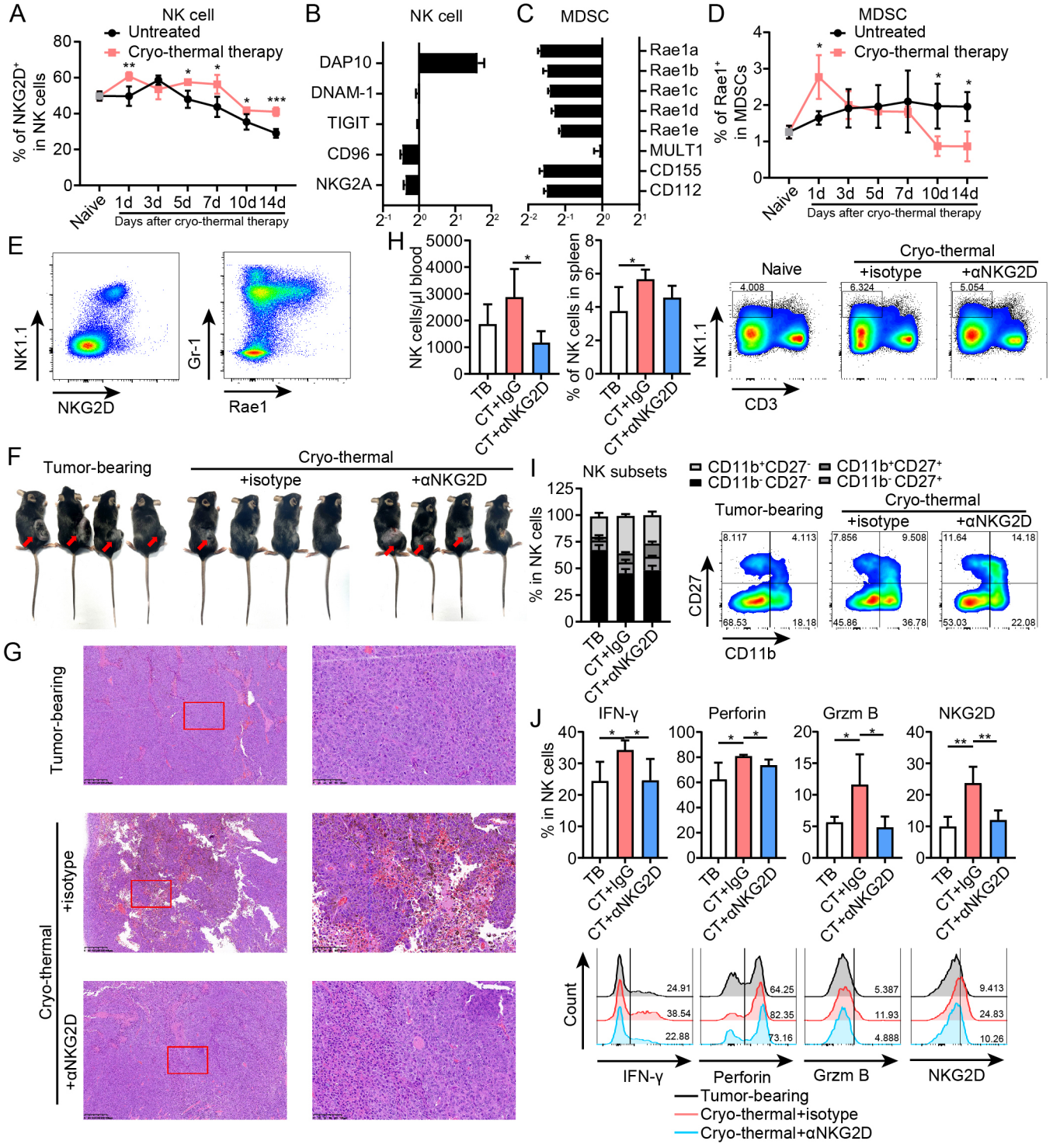

Figure 4 Change and role of NKG2D-NKG2DL. (A) Expression of NKG2D in NK cells. (B) Activation and inhibitory receptor and related molecule expression of NK cells. (C) Activation and inhibitory ligand expression of MDSCs. (D) Expression of NKG2D ligand (RAE1) in MDSCs. (E) Coexpression of NK1.1 with NKG2D and Gr-1 with RAE1 in immune cells. (F) Tumor regression and relapse after NKG2D blockade. (G) H&E staining of tumors. (H) NK cells proportion in spleen and absolute number in blood. (I) Activation and maturation of NK cells. (J) Cytokine and receptor expression in NK cells. All data are shown as the mean ± SD n=4 for each group. *p<0.05, **p<0.01, ***p<0.001. MDSC, myeloid-derived suppressor cell; NK, natural killer.

that MDSCs in the anti-NKG2D group showed significant downregulation of the immune response to cytokines (figure 5C). Hallmark GSEA of MDSCs in the anti-NKG2D group revealed that the inflammatory response and response to IFN-γ were significantly downregulated in MDSCs (figure 5D,E). Interestingly, the MHC I pathway in MDSCs in the anti-NKG2D group was not significantly changed, but the MHC II pathway was markedly suppressed (online supplemental figure S6). These results

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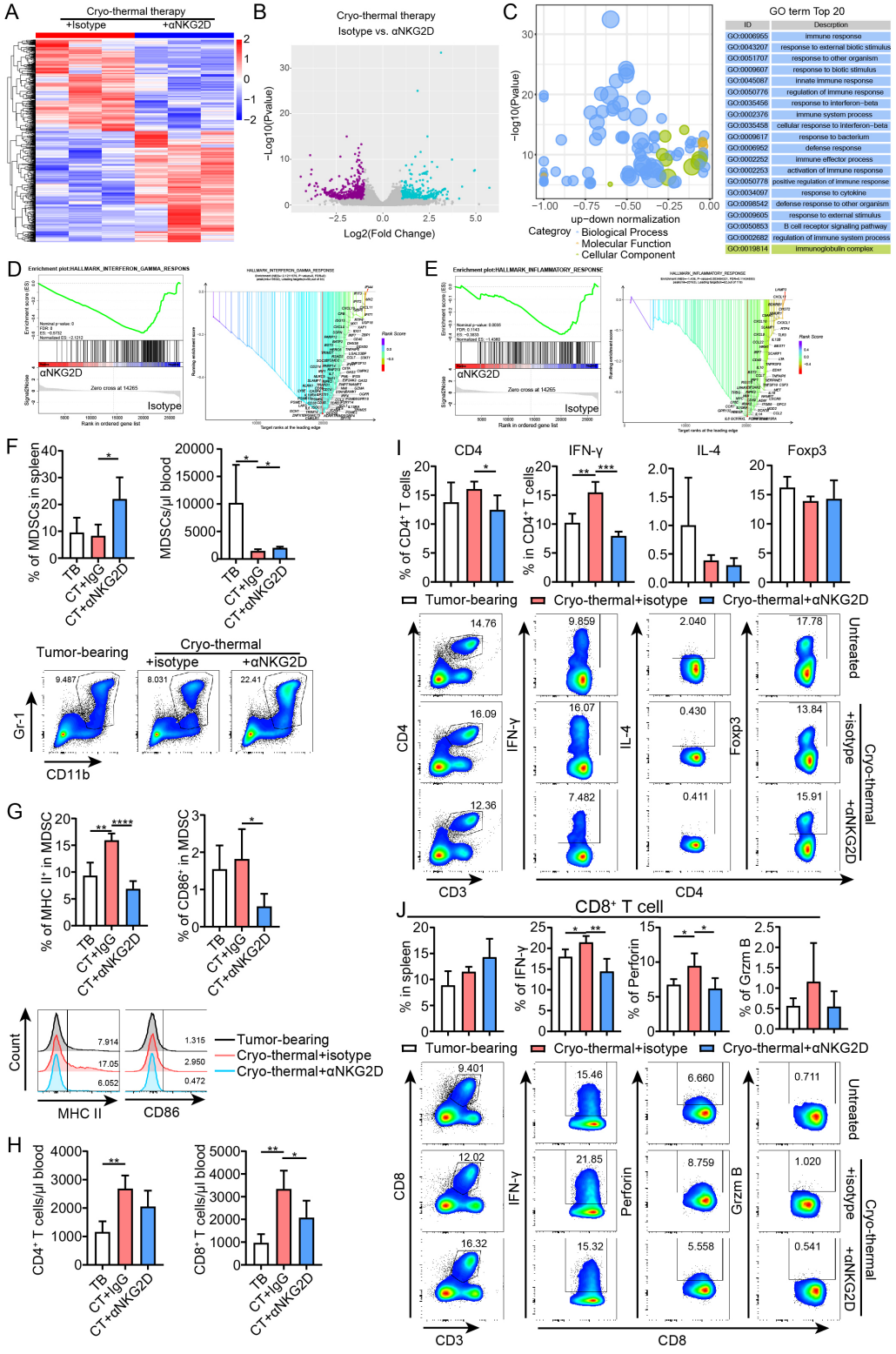

Figure 5 NKG2D-NKG2DL engagement is involved in MDSC regulation and the T-cell immune response. MDSCs were isolated by MACS and analyzed by mRNA-seq. (A) Heatmap of differentially expressed genes, encompassing sample and gene clustering. (B) Volcano plot of gene expression change of MDSCs from cryo-thermal treated mice over untreated mice, encompassing p value and fold change. (C) Z score bubble map of GO term top 20 of MDSCs. (D) Individual GSEA enrichment plot for the hallmark IFN-γ response gene set (left), GSEA dot plot (right). (E) Individual GSEA enrichment plot for the hallmark inflammatory response gene set (left), GSEA dot plot (right). (F) MDSCs proportion in spleen and absolute number in blood. (G) Maturation of MDSCs. (H) Absolute number of $CD4^+$ and $CD8^+$ T cells in blood. (I) Frequency and subsets of $CD4^+$ th cells. (J) Frequency and cytokines of $CD8^+$ CTL cells. All data are shown as the mean±SD n=4 for each group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. CTL, cytotoxic T lymphocyte; GSEA, gene set enrichment analysis; MASC, magnetic-activated cell sorting; MDSC, myeloid-derived suppressor cell.

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indicated that the NKG2D-NKG2DL axis promoted the differentiation of inflammatory MDSCs and converted MDSCs into potent APCs. The percentage of MDSCs in spleen and absolute number in blood in the anti-NKG2D group was significantly increased while the expression of MHC II and CD86 were decreased (figure 5F,G). These data further confirmed that the decreased levels and maturation of MDSCs were mediated by NKG2D-NKG2DL. Then, an in vitro study was performed to determine the role of the NKG2D-NKG2DL axis in the direct regulation of MDSCs by NK cells. NK cells and MDSCs were cocultured in the presence of anti-NKG2D antibodies or isotype as described above. As shown in online supplemental figure S7A, NK-cell-mediated killing of MDSCs was significantly abolished by NKG2D blockade. Moreover, the level of CD86 on MDSCs induced by activated NK cells was decreased by NKG2D blockade (online supplemental figure S7B). Taken together, these results revealed that NKG2D-NKG2DL mediated the reprogramming of MDSCs by NK cells; specifically, activated NK cells with high level of NKG2D directly inhibited the accumulation of MDSCs via the NKG2D-NKG2DL axis and promoted the maturation of MDSCs via IFN-γ secreted by NK cells in response to NKG2D.

Because the NKG2D-NKG2DL axis mediates NK-cell activation and reprogramming of MDSCs, changes in T cells were further analyzed on NKG2D blockade. The absolute number of $CD8^+$ T cells in blood in the anti-NKG2D group was significantly lower (figure 5H). Meanwhile, the percentage of $CD4^+$ T cells and the level of Th1 cells were significantly decreased by NKG2D blockade (figure 5I). In addition, the proportions of Th2 and Tregs were not obviously changed after blockade of NKG2D (figure 5I). Moreover, the levels of IFN-γ and perforin in $CD8^+$ T cells were significantly decreased in the anti-NKG2D group (figure 5J). Collectively, these results suggested that MDSCs regulated by activated NK cells via NKG2D-NKG2DL triggered Th1-dominant $CD4^+$ T-cell immunity to inhibit tumor recurrence and metastasis.

NKG2D is correlated with T-cell infiltration and clinical outcomes

To further identify whether NKG2D-NKG2DL correlated with the clinical immune landscape and outcomes, we used TIMER to comprehensively examine the immunological, clinical, and genomic features of tumors.[26] The infiltration of resting NK cells and MDSCs was positively correlated with high risk (figure 6A). Moreover, a higher level of NKG2DL, which represents chronic NKG2D tolerance of NK-cell immunity, was positively correlated with the infiltration of resting NK cells and MDSCs and high risk (figure 6B and online supplemental figure S8E, F, I). Similarly, our data showed the expression of NKG2DL (RAE1) in untreated mice continuously increased with time (figure 4D). The levels of IFN-γ and NKG2D (*KLRK1*) had positive correlations with low risk in most cancer types, including BRCA and melanoma (figure 6B, online supplemental figure S8A–D). The level of IFN-γ and NKG2D was highly positively correlated, indicating that NK cells were activated (online supplemental figure S8H). Moreover, the levels of IFN-γ and NKG2D were highly positively correlated with the infiltration of NK cells but negatively correlated with the infiltration of resting NK cells and MDSCs (online supplemental figure S8G). Further analysis showed that patients with synchronous high levels of NK cells and IFN-γ or NKG2D expression had the best clinical outcomes (figure 6C,D). In addition, IFN-γ and NKG2D were positively correlated with $CD4^+$ and $CD8^+$ T-cell infiltration, confirming our findings in the animal model (figure 6E,F). These results indicated that NKG2D-NKG2DL mediated the interaction between NK cells and MDSCs, which was related to the T-cell immune response and clinical outcomes, suggesting that NKG2D could be a prognostic biomarker for cancer therapy.

DISCUSSION

In previous study, $CD4^+$ Th1 cells were shown to mediate long-term antitumor immune memory after cryo-thermal therapy,[19] but the mechanism by which cryo-thermal therapy induces a $CD4^+$ Th1-dominant immune response is still unknown. In this study, we revealed that activated NK cells with high expression of NKG2D in the early stage reduced *MDSC* expansion via the NKG2D-NKG2DL axis and reprogrammed MDSCs toward a mature phenotype via IFN-γ in response to NKG2D, leading to enhanced Th1-dominant immunity to inhibit tumor recurrence and metastasis. Clinical database analysis of multiple cancers also showed that the level of NKG2D, IFN-γ and NK-cell infiltration and in tumor tissues was positively associated with T-cell infiltration and better clinical outcomes. Our present study not only demonstrated that NK cells activated by cryo-thermal therapy reprogramed MDSCs toward a mature phenotype, triggering Th1-dominant immunity, but also suggested that NKG2D could be a prognostic biomarker for tumor therapy and that NKG2D-NKG2DL in NK-cell-MDSCs offered new opportunities in cancer treatment. Current study was restricted in the specific melanoma tumor model, additional studies about other cancer types would be performed in near future.

Although MDSCs mainly resulted in tumor immunosuppression, our previous studies revealed that cryo-thermal therapy could reset immunosuppression toward antitumor immunity.[17 27] MDSCs can be further differentiated into mature neutrophils, macrophages and DCs, which promote T cell immunity through antigen presentation and cytokine production.[5] However, the complex nature of MDSCs presents a substantial challenge for therapeutic targeting. Currently, different therapeutic strategies aim to eliminate MDSCs or abrogate their protumor activities, such as depleting MDSCs, inhibiting MDSC recruitment and suppressive activity, and promoting MDSC differentiation.[28] Several studies have shown that MDSCs can acquire a mature phenotype and differentiate into mature myeloid cells, charactering by MHC-II, CD80

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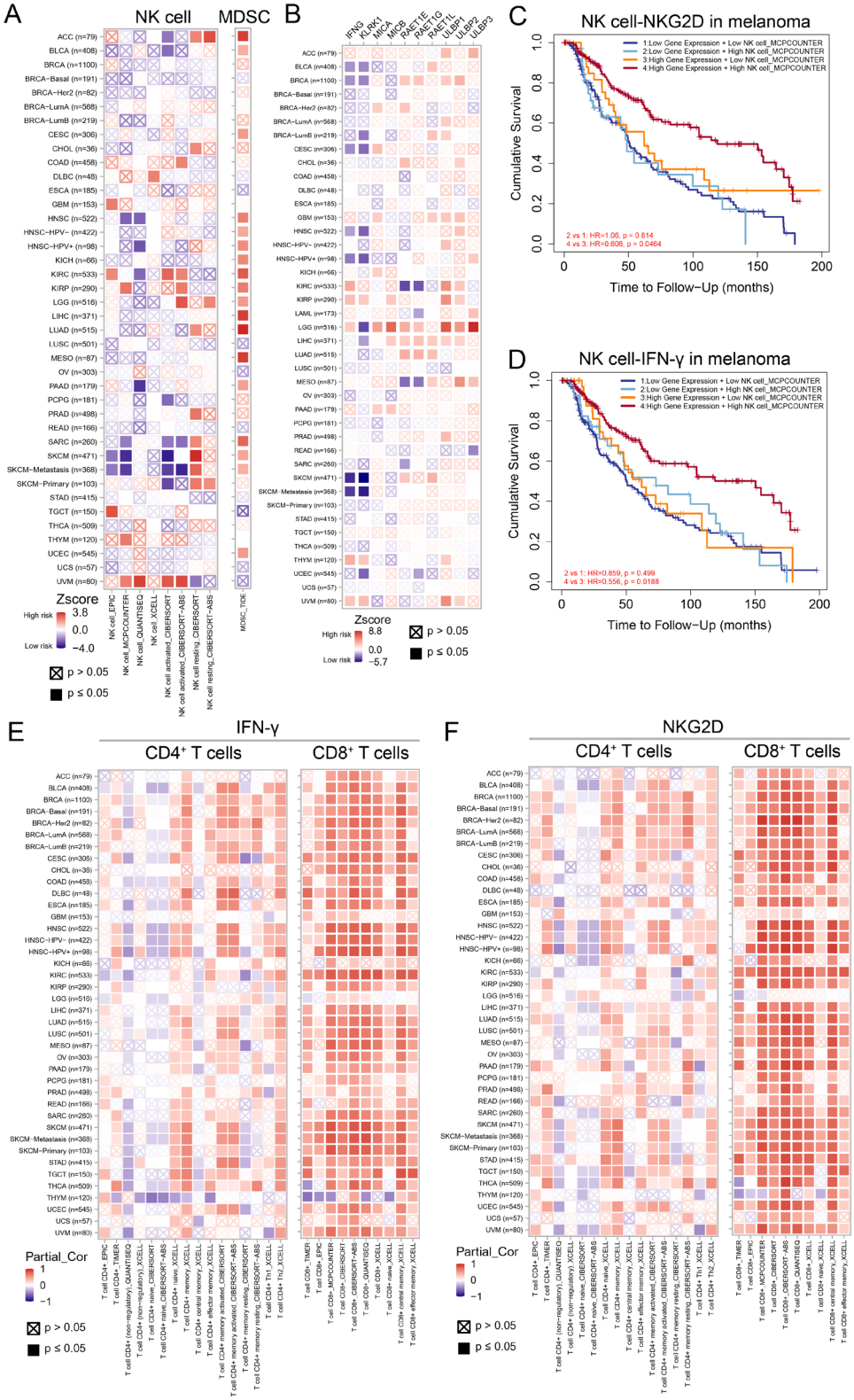

Figure 6 Clinical outcomes associated with MDSC and NK-cell infiltration and T-cell infiltration associated with IFN-γ and NKG2D expression in tumor tissue. (A) Heatmap showing the associations between clinical outcomes and the infiltration of NK cells and MDSCs across multiple cancers from the TIMER2.0 database. (B) Heatmap showing the associations between clinical outcomes and the expression of *IFNG* (IFN-γ), *KLRK1* (NKG2D), and NKG2DLs across multiple cancers from the TIMER2.0 database. (C, D) *IFNG*, *KLRK1,* and NK-cell infiltration exerted a synergistic effect in predicting melanoma survival from the TIMER2.0 database. The Kaplan-Meier survival curve shows that the combination of *IFNG* (C) or *KLRK1* (D) and NK-cell infiltration better predicts patient survival. (E, F) Heatmap showing the associations between T-cell infiltration and the expression of *IFNG* (E) and *KLRK1* (F) across multiple cancers from the TIMER2.0 database. The figures were created using the TIMER2.0 database (http://timer.cistrome.org/). MDSC, myeloid-derived suppressor cell; NK, natural killer.

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and CD86 and overexpression of CD11b.[29 30] The mature CD11b$^+$ MHC II$^+$ cells are able to induce allogeneic antitumor T-cell stimulation and infiltration.[31] Our results showed that activated NK cells could promote the maturation of MDSCs with MHC II and CD86 increase via high expression of IFN-γ, which triggered a Th1-dominant antitumor immune response. Interestingly, we found that NKG2D-NKG2DL-mediated NK-cell-MDSC contact could also increase the expression of CD86 on MDSCs, but the detailed mechanism requires further study.

The crosstalk between NK cells and MDSCs has received attention in recent years, but most studies focused on the immunosuppression of MDSCs on NK cells.[32 33] Studies on crosstalk between NK cells and MDSCs inducing antitumor immunity are rather rare. NK cells can be activated by M-MDSCs and conversely eliminate MDSCs through perforin.[34] Tumor-associated neutrophils could suppress tumor growth efficiently in the presence of normal NK cells.[35] NK cells expressing NKG2D were introduced into chimeric activating receptors and could eliminate MDSCs and rescue impaired chimeric antigen receptor (CAR) T cell activity against solid tumors.[36] Our results revealed that activated NK cells could eliminate MDSCs via NKG2D-NKG2DL and reprogram MDSCs toward a mature phenotype via IFN-γ. However, whether or how MDSCs affect NK cells after cryo-thermal therapy should be further examined. Interestingly, our study showed that NK-cell-mediated killing of MDSCs was inhibited by IFN-γ (online supplemental figure S3), suggesting that in addition to promoting MDSC maturation, IFN-γ could protect mature MDSCs from being killed by NK cells, which would explain why the viability of MDSCs was higher after being cocultured with NK cells expressing higher level of IFN-γ after cryo-thermal therapy (online supplemental figure S2D). Thus, our results revealed a universal role of NK cells in regulating host antitumor immune response and controlling tumor progression by regulating MDSCs.

Strategies targeting the NKG2D-NKG2DL axis for cancer immunotherapy have been developed in recent years.[25 37 38] NKG2DL is usually expressed in response to cellular stress and in transformed and virus-infected cells, acting as an alarm or indicator of inflammation.[39] Because NKG2DL^{high} tumor cells can be effectively killed by NK cells, strategies that focus on enhancing NKG2D expression in immune cells, increasing the expression of NKG2DL in tumor cells, and eliminating soluble NKG2DL could effectively activate the antitumor immune response.[25 38] In addition, the NKG2D-NKG2DL axis is widespread in the interaction of lymphoid and myeloid cells in healthy and disease conditions, indicating its role in immune regulation.[40] RAE-1-expressing myeloid cells can be efficiently killed by activated NK cells.[41–43] Tumor-infiltrating macrophages would upregulate the NKG2D ligand RAE-1δ by tumor-derived CSF-1 and interacted with NK cells to augment NK-cell functional responses.[44] Thus, the NKG2D-mediated myeloid-NK-cell interaction would have dual effects on the antitumor response. Cryo-thermal therapy activated NK cells with increased expression of IFN-γ, perforin and granzyme B via NKG2D, further reprogramming MDSCs to a mature phenotype via the NKG2D-NKG2DL axis, suggesting that cryo-thermal therapy is a novel strategy to target the NKG2D-NKG2DL axis in NK cells and MDSCs to induce potent antitumor immunity. The mechanism that cryo-thermal therapy induces NKG2D and NKG2DL upregulation will be furtherly studied.

With the development of adoptive T-cell therapy, the importance of CD4$^+$ T cells has been recognized due to their powerful immunostimulatory properties in augmenting the endogenous immune response, while CD8$^+$ T cells only have direct cytotoxicity against tumor cells.[45–47] Recently, a study on CAR-T-cell therapy showed that decade-long leukemia remission was mediated by the predominance and persistence of CD4$^+$ T cells.[48] Thus, activating CD4$^+$ T cells to achieve a long-term antitumor immune memory is crucial in cancer immunotherapy.[49] Our previous study showed that cryo-thermal therapy activated a Th1-dominant antitumor immune response, mediating strong systemic and long-term antitumor immunity and skewing other immune cells toward an antitumor immune phenotype.[19] In this study, we showed that mature MDSCs triggered a CD4$^+$ Th1-dominant antitumor immune response but limited the CD8$^+$ T-cell response. Moreover, MDSCs reprogrammed by activated NK cells could further promote the Th1 response while inhibiting the induction of Th2 and Treg subsets. Thus, targeting NK-cell activation or MDSC maturation to modulate T-cell immunity in cancer immunotherapy could be a potential strategy in clinical tumor therapy. However, many studies have shown that NK cells can also directly regulate the T-cell response,[50] and whether it happens after cryo-thermal therapy should be further studied.

Chronic NKG2D engagement by NKG2DL impairs the function of NK cells, leading to the silencing of NKG2D-mediated responses.[51] In this study, RAE1 was briefly upregulated after cryo-thermal therapy in the early stage and decreased slightly in the late stage, while it was continuously increased in the untreated group and significantly higher than that in the cryo-thermal group in the late stage (figure 4D). However, the expression of NKG2D on NK cells remained the same from untreated mice, suggesting that although the expression of RAE1 on MDSCs was significantly increased, it did not activate NK cells in untreated mice and even induced immune tolerance of NK cells.

CONCLUSION

In summary, we revealed that NK cells were activated by cryo-thermal therapy and that activated NK cells not only eliminated MDSC accumulation through NKG2D engagement but also promoted the maturation of MDSCs through IFN-γ in response to NKG2D, resulting in the induction of CD4$^+$ Th1-dominant antitumor immunity. Our present study showed that cryo-thermal-activated NK cells could reprogram the tumor-immune environment to foster CD4$^+$ Th1-dominant durable antitumor immunity, which could be used to develop a novel tumor immunotherapeutic strategy.

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Contributors PP, YL, SW, JW, ZZ, PD and JZ performed the experiments. PL coordinated the project. PL, and PP designed the experiments. The manuscript was written by PP and revised by PL, and LXX. LXX is responsible for the overall content as guarantor. The guarantor accepts full responsibility for the finished work and/or the conduct of the study, had access to the data, and controlled the decision to publish. All authors reviewed the manuscript.

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Competing interests None declared.

Patient consent for publication Not applicable.

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Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information.

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