Targeting cytokine signaling checkpoint CIS activates NK cells to protect from tumor initiation and metastasis

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ABSTRACT

The cytokine-induced SH2-containing protein CIS belongs to the suppressor of cytokine signaling (SOCS) protein family. Here, we show the critical role of CIS in suppressing natural killer (NK) cell control of tumor initiation and metastasis. Cish-deficient mice were highly resistant to methylcholanthrene-induced sarcoma formation and protected from lung metastasis of B16F10 melanoma and RM-1 prostate carcinoma cells. In contrast, the growth of primary subcutaneous tumors, including those expressing the foreign antigen OVA, was unchanged in Cish-deficient mice. The combination of Cish deficiency and relevant targeted and immuno-therapies such as combined BRAF and MEK inhibitors, immune checkpoint blockade antibodies, IL-2 and type I interferon revealed further improved control of metastasis. The data clearly indicate that targeting CIS promotes NK cell antitumor functions and CIS holds great promise as a novel target in NK cell immunotherapy.

ABSTRACT

The cytokine-induced SH2-containing protein CIS belongs to the suppressor of cytokine signaling (SOCS) protein family. Here, we show the critical role of CIS in suppressing natural killer (NK) cell control of tumor initiation and metastasis. Cish-deficient mice were highly resistant to methylcholanthrene-induced sarcoma formation and protected from lung metastasis of B16F10 melanoma and RM-1 prostate carcinoma cells. In contrast, the growth of primary subcutaneous tumors, including those expressing the foreign antigen OVA, was unchanged in Cish-deficient mice. The combination of Cish deficiency and relevant targeted and immuno-therapies such as combined BRAF and MEK inhibitors, immune checkpoint blockade antibodies, IL-2 and type I interferon revealed further improved control of metastasis. The data clearly indicate that targeting CIS promotes NK cell antitumor functions and CIS holds great promise as a novel target in NK cell immunotherapy.

Introduction

A new fourth pillar of cancer treatment, immunotherapy, particularly targeting immune checkpoint inhibitors, is now complementing surgery, radiotherapy and chemotherapy. Many forms of immune suppression in the local tumor microenvironment (TME) prevent what would otherwise be an effective innate and adaptive immune response to cancer. Recently, novel combination therapeutic approaches aimed at re-activating tumor-specific T cells demonstrated a significant portion of late-stage melanoma patients undergoing complete remission. Indeed, the majority of these clinically effective immune checkpoint-specific therapies (e.g., ipilimumab, nivolumab, pembrolizumab, and so on) are monoclonal antibodies directed at inhibitory receptors (e.g., CTLA-4 and PD-1) on tumor-infiltrating T cells. However, a significant proportion of cancer patients remains non-responsive or relapses after these contemporary treatments. We contend that other immune cell types such as natural killer (NK) cells must also be targeted to fully realize the potential of immunotherapy. NK cells express an array of germ-line encoded activating and inhibitory receptors that scan for altered protein expression patterns and are extremely efficient producers of effector molecules including cytotoxic granules and cytokines (IFN-γ). Currently, major efforts are being undertaken to target receptors on NK cells, but until now the immune checkpoints have been largely restricted to surface inhibitory receptors such as inhibitory killer immunoglobulin-like receptors, NKG2A, and CD96.

The cytokine-induced SH2-containing protein (Cish, encoding CIS) belongs to the suppressor of cytokine signaling (SOCS) protein family and functions as a classical negative feedback loop to inhibit cytokines such as IL-2, IL-3, and erythropoietin. SOCS genes are induced following cytokine receptor engagement and activation of the JAK/STAT signaling cascade. They primarily function as adaptors for an E3 ubiquitin ligase complex and inhibit cytokine signaling by binding to the receptor complex and/or the associated JAK protein tyrosine kinases, targeting them for proteasomal degradation. In addition, SOCS1 and SOCS3 can directly inhibit JAK enzymatic activity.

NK cell development, proliferation, survival, and activation are dependent on the cytokine IL-15. We recently demonstrated that IL-15 promotes NK cell survival directly by inducing STAT5 phosphorylation and binding to the 3'UTR of the anti-apoptotic gene Mcl1. In this study, we also noted that...
IL-15 stimulation of NK cells resulted in STAT5 binding to Cish. We have recently shown that Cish deletion in mouse NK cells renders them hyperresponsive to IL-15 as revealed by enhanced JAK1/STAT5 activity, proliferation, survival, IFN-γ production, and cytotoxicity. Furthermore, we identified JAK1 as a novel target for CISH-mediated kinase inhibition and E3 ligase activity.\(^1\) Consequently, we demonstrated a critical role for CISH in limiting NK cell control of experimental lung metastasis.\(^1\) Cish deficiency is also associated with enhanced CD4\(^{+}\) T cell proliferation and greater susceptibility to T-cell-mediated allergic asthma.\(^12\) Cish-deficient CD8\(^{+}\) T cells were reported to be hyperproliferative with enhanced activity against B16 melanoma.\(^13\) However, the importance of CISH in immune responses to solid tumors and hematological cancers is still unknown.

Here, we now define the important role of CISH in suppressing NK cell control of tumors, in some settings, but not others. In parallel, T cell-mediated control of primary experimental tumors was generally not altered in Cish-deficient mice. Combining Cish deficiency with contemporary targeted and immunotherapies, which promoted NK cell function, further improved control of metastasis. Our data strongly suggest that CISH is an attractive target for NK cell-based immunotherapy.

**Results**

**Loss of Cish protects against experimental metastasis and carcinogen-induced tumors**

IL-15-mediated activation of JAK1/3 and STAT5 is critical to NK cell survival in vivo, and Cish is a STAT5 target gene in NK cells. We noted that the loss of CISH significantly enhanced NK cell survival in vivo and in vitro where IL-15 concentrations were limiting (Fig. S1A and B). To investigate the role of CISH in NK cell responses against metastasis, C57BL/6 Cish\(^{-/-}\) and control wild-type (WT) mice were challenged intravenously with B16F10 melanoma or RM-1 prostate carcinoma cells. Cish\(^{-/-}\) mice exhibited significantly lower numbers of lung metastasis (Fig. 1A) and survived twice as long as WT mice (Fig. 1B). Enhanced killing of B16F10 by Cish\(^{-/-}\) NK cells was also observed in vitro (Fig. S1C). Interestingly, depletion of CD8\(^{+}\) T cells did not impact on the number of RM-1 lung metastases, whereas the protection observed in Cish\(^{-/-}\) mice was lost upon NK cell depletion or neutralization of IFN-γ (Fig. 1C). These results confirm the role of CISH in downregulating NK cell responses and indicate that the genetic ablation of Cish protects against metastasis formation in an NK cell- and IFN-γ-dependent manner. Importantly, the protection from metastasis formation translated into significantly prolonged survival of Cish\(^{-/-}\) mice. In concert, Cish\(^{-/-}\) mice were highly resistant to methylcholanthrene (MCA)-induced fibrosarcoma formation (Fig. 2A and S2). Depletion of NK cells or neutralization of IFN-γ again significantly reduced the survival of WT and Cish\(^{-/-}\) mice and completely abolished the protective effect of Cish deficiency (Fig. 2B). The extent of protection from experimental metastasis and de novo carcinogenesis observed in Cish\(^{-/-}\) mice is impressive and clearly indicated that targeting CISH holds promise as a novel target for NK-cell-based immunotherapy. These results are consistent with the IL-15 induction of CISH expression in NK cells and the observation that the loss of Cish renders NK cells hypersensitive to IL-15.\(^1\)

![Figure 1](image-url)
Cish deficiency does not have a major impact on the progression of hematopoietic malignancies

Besides limiting metastasis, NK cells are known to suppress some hematopoietic malignancies. In particular, NK cells control the clearance of MHC class I-deficient cells, such as the RMA-S cell line. To our surprise, Cish−/− and WT mice appeared equally susceptible to the intraperitoneal (i.p.) injection and growth of luciferase+ RMA-S lymphoma cells (Fig. 3A and S3A). When injected with lower numbers of the parental RMA-S lymphoma cell line, Cish−/− and WT mice showed superimposable disease latencies (Fig. 3B). In line, the growth of primary RMA-S tumors after subcutaneous injection was similar in Cish−/− and WT mice (Fig. S3B). In order to increase the immunogenicity of the cell line further, we used a NKG2D ligand-expressing RMA-S variant. When injected subcutaneously, RMA-S-Rae1β cells gave rise to a solid tumor, which was rejected in 70.0% of WT mice (14/20). Interestingly, a higher proportion of Cish−/− mice (89.5%, 17/19) successfully rejected the tumor (Fig. 3C and D). The protective effect of Cish deficiency in this model could not be explained by differential infiltration of NK cells into the tumor (Fig. S3C–F).

Next, we challenged WT and Cish−/− mice with the Vk/C3 MYC multiple myeloma cell line Vk12653. In this model, the myeloma growth can be followed by measuring the serum g-globulin levels. Three and five weeks after the transplant, WT and Cish−/− mice

Figure 2. Cish-deficient mice are protected from MCA-induced tumor development. (A) Groups of 15 B6.WT (Cish+/+) and 18 B6.Cish−/− male mice were inoculated s.c. in the hind flank with 300 μg of MCA in 0.1 mL of corn oil. Mice were then monitored for fibrosarcoma development over 250 d, and data were recorded as the percentage tumor free mice (tumors > 3 mm in diameter were recorded as positive). (B) Groups of 16–18 B6.WT (Cish+/+) and 14 B6.Cish−/− male mice were inoculated s.c. in the hind flank with 300 μg of MCA in 0.1 mL of corn oil. Mice were treated with either 250 μg hamster cIg, 50 μg anti-asialoGM1 (anti-asGM1; NK cell depletion) or 250 μg anti-IFN-γ antibodies injected i.p. on days −1, 0, 7, 12, 24, 28, 35, and 42. Mice were monitored for fibrosarcoma development over 200 d. Tumors were measured every week with a caliper as the product of two perpendicular diameters (mm²). Mice were euthanized when the tumor reached > 150 mm² in diameter. Statistically significant survival differences between the groups were determined by the Log-rank Mantel–Cox test (A) followed by the Bonferroni correction for multiple testing (B) (‘p < 0.05; ‘‘p < 0.001; ‘‘‘p < 0.0001).

Figure 3. Lack of Cish does not have a major impact on the surveillance of lymphoma. (A) Groups of 10 B6.WT (Cish−/−) and B6.Cish−/− mice were injected i.p. with 5 × 10⁴ luciferase+ RMA-S cells. Mice were monitored for tumor development by in vivo imaging (see Fig. S3). The Kaplan–Meier plot summarizes two independent experiments (p = 0.22; Log-rank Mantel–Cox test). (B) Groups of 10 B6.WT (Cish−/−) and B6.Cish−/− mice were injected i.p. with 1 × 10⁶ parental RMA-S cells. Mice were monitored for tumor development and were euthanized at the point of abdominal swelling and discomfort. The Kaplan–Meier plot shows survival curves of WT and Cish−/− mice (p = 0.96; Log-rank Mantel–Cox test) (C, D) Groups of 9–10 B6.WT (Cish−/−) and B6.Cish−/− mice were injected s.c. with 1 × 10⁶ RMA-S-Rae1β cells. Tumors were measured every 2–3 d with a caliper as the product of two perpendicular diameters (mm²). Shown are (C) the tumor growth curves of individual mice and (D) the rejection rate summarized from two independent experiments.
displayed similar serum \( \gamma \)-globulin levels (Fig. 4A), and consistent with the reported role of NK cells in this model, NK cell-depletion raised serum \( \gamma \)-globulin levels (Fig. S4A). In concert, similar numbers of malignant CD155\(^+\) plasma cells were observed in the spleen and bone marrow of WT and Cish\(^{-/-}\) mice (Fig. 4C and D). An in depth analysis of NK, CD4\(^+\), and CD8\(^+\) T cell numbers and activation did not reveal any obvious differences between WT and Cish\(^{-/-}\) mice at 5 weeks post-myeloma challenge (Fig. S4B–E). Accordingly, in a survival experiment, WT and Cish\(^{-/-}\) mice succumbed to multiple myeloma with comparable disease latency. NK cell depletion significantly reduced the survival of multiple myeloma-bearing WT and Cish\(^{-/-}\) mice (Fig. 4E). Thus, despite the notion that NK cells control many hematopoietic malignancies, we observed a variable degree of CIS-dependent protection depending on the tumor model. Whereas Cish deficiency did not seem to have a major impact on the surveillance of RMA-S and Vk12653 multiple myeloma, the rejection of RMA-S-Rae1\(\beta\) tumors was more efficient in Cish\(^{-/-}\) mice compared to WT mice.

**T-cell-mediated control of tumors does not appear CIS dependent**

Although NK cells are known to be important in controlling tumor metastases and hematopoietic malignancies, NK cell infiltration of, and their effectiveness against, transplanted mouse subcutaneous or established tumors is limited. Such tumors are often controlled at some level by effector and regulatory T lymphocytes. Different groups have shown the importance of CIS in the function of CD4\(^+\) and CD8\(^+\) T cells. In line with these studies, we observed enhanced proliferation of anti-CD3/anti-CD28-stimulated Cish\(^{-/-}\) CD4\(^+\) and CD8\(^+\) T cells compared to WT controls (data not shown). However, Cish deficiency did not alter the growth kinetics of B16F10, B16-OVA, or MC38-OVA tumors compared to WT mice (Fig. 5A–C). The expression of strong foreign antigens such as ovalbumin is known to increase the immunogenicity of tumor cells and engage T cell responses. The clearance of these tumor cells is reportedly controlled by the OVA-specific CD8\(^+\) T cells. In contrast, when injected subcutaneously with the immunogenic MCA-induced fibrosarcoma, MCA1956, Cish\(^{-/-}\) mice showed better tumor control than WT mice (Fig. 5D). Whereas only 1/10 WT mice spontaneously rejected the tumor, 5/10 Cish\(^{-/-}\) mice successfully cleared the primary tumor by day 30 after transplant (Fig. 5E). Depletion of NK cells or CD8\(^+\) T cells accelerated tumor growth and abrogated the differences seen in WT and Cish-deficient mice (Fig. 5E). Whether NK cells control the growth of MCA1956 tumors directly or whether they play an indirect role by modulating T cell activity remains to be elucidated. Taken together, our data suggest Cish deficiency primarily alters the natural growth of subcutaneous tumors where NK cells are critical.

**Cish-deficient NK cells do not have heightened response to TLR agonists**

Cish-deficient NK cells are hypersensitive to IL-15 resulting in enhanced NK cell cytotoxicity and IFN-\(\gamma\) release that can
This raises the issue of safety of CIS blockade and whether CIS might generally regulate NK cell response to inflammatory cytokines, in particular those regulated by JAK1. In the context of bacterial and viral infection, pattern recognition pathways including Toll-like receptors (TLR) are activated and induce myeloid cells to produce vast amounts of pro-inflammatory cytokines, such as IL-12, IL-18, and type I IFNs that can activate NK cells. The TLR agonists lipopolysaccharide (LPS) and CpG oligonucleotides signal via TLR-4 and TLR-9, respectively, and when administered in high doses induce a lethal endotoxicosis. WT and Cish−/− mice were challenged with increasing doses of LPS and showed equal severity of endotoxicosis by means of body weight loss (Fig. 6A) and survival (Fig. 6B). Six hours after the i.p. administration of a low dose (0.2 mg/30 g mouse) of LPS, NK cell proportions and absolute numbers were significantly reduced in spleens of Cish−/− and WT mice alike. Interestingly, whereas WT NK cells showed reduced expression of the IL-2Rβ chain (CD122) in response to LPS, Cish−/− NK cells retained normal CD122 levels. Irrespective, a similar fraction of WT and Cish−/− NK cells expressed the activation marker CD69 and produced IFN-γ (Fig. 6C). In addition, the cytokine release induced in the first 6 h after LPS challenge was similar in WT and Cish−/− mice (Fig. 6D).

Intraperitoneal injection of 0.1 mg/30 g mouse CpG DNA induced a similar reduction of NK cells in spleens of WT and Cish−/− mice as analyzed after 6 h. In line with the results from the LPS challenge, Cish−/− NK cells retained high expression of CD122, whereas WT NK cells showed a significant fall in CD122 expression levels. Interestingly, CpG challenge induced fewer Cish−/− NK cells to produce IFN-γ (Fig. 7A). Although the CpG-induced cytokine release was similar in both WT and Cish−/− mice, a reduced level of IFN-γ at 6 h and a slightly reduced level of KC (IL-8) were observed in Cish−/− mice at 3 h (Fig. 7B).

Collectively, our data suggest that inhibiting CIS may be a safe way to enhance NK-cell-mediated tumor surveillance without provoking exaggerated NK cell responses and cytokine induction.

**NK cell-dependent anti-metastatic therapies are more effective with Cish deficiency**

We next sought to compare Cish deficiency with contemporary immunotherapies, including immune checkpoint blockade (anti-PD-1, anti-CTLA-4, and anti-CD96) and cytokines (IFN-αβ and IL-2) promoting NK cell function, in the B16F10 experimental metastasis model. Notably, the Cish-deficient mice were more resistant to B16F10 lung metastases than the WT mice treated with a regimen of anti-PD-1, type I IFN (IFN-αβ) or IL-2 (Fig. 8A). All of these immunotherapies have been used with some degree of success in the treatment of advanced human melanoma. Although the level of B16F10 metastasis
was low in cIg-treated Cish−/− mice, both type I IFN and IL-2 treatment appeared to further reduce metastasis (Fig. 8A). A further experiment assessed a higher dose challenge with B16F10, and here it became apparent that both the anti-PD-1/anti-CTLA-4 combination and IL-2 were more effective than cIg in the Cish−/− mice (Fig. 8B). In particular, low dose IL-2 was ineffective in WT mice, but effective in Cish−/− mice. This improved effect of IL-2 in Cish−/− mice compared with WT mice was also observed in the RMA-S i.p. lymphoma model (Fig. 8C). This is of interest, since NK cell-mediated control in WT and Cish−/− mice was equivalent in untreated (Fig. 3A and B) or control treated mice (Fig. 8C). An additional experiment performed in a second experimental metastasis model, RM-1, indicated the superior anti-metastatic activity of Cish deficiency combined with anti-PD-1/anti-CTLA-4 or anti-CD96 treatment (Fig. 8D). The superior activity of anti-CD96 in Cish−/− mice was also observed in the B16F10 experimental metastasis model (Fig. 8E). Collectively, these experiments indicated that the CIS regulation of NK cell anti-metastatic activity was independent of the anti-metastatic activities of immune checkpoint antibodies. Interestingly, although treatment with anti-PD-1/anti-CTLA-4 also significantly inhibited the growth of subcutaneous B16F10 tumors, there was no significant difference in treatment outcome between WT and Cish−/− groups (Fig. S6). In this model, CD8+ T cells are critical for anti-PD-1/anti-CTLA-4 efficacy.

Ultimately, we challenged the mice with the BRAFV600E-mutant metastatic melanoma cell line LWT1 and again observed significantly less lung metastasis in Cish−/− mice. This metastasis was further reduced by treating with the BRAF-inhibitor PLX4720 (Fig. 8F), as reported previously in WT mice.21 As shown in Fig. S7A, the combined effect of Cish deficiency and BRAF inhibition is mediated by NK cells, as the depletion of NK cells completely abolished the protective effect of the treatment. The more clinically relevant combination of BRAF and MEK inhibition was extremely effective in Cish−/− mice (Fig. S7B). In summary, these data indicate that targeting CIS combines well with other immunotherapies, and CIS holds great promise as a novel target in NK cell immunotherapy.

Discussion

Our previous study showed that Cish deletion in mouse NK cells rendered them hyperresponsive to IL-15 as revealed by enhanced JAK1/STAT5 activity, proliferation, survival, IFN-γ production, and cytotoxicity ex vivo.11 Loss of Cish also protected mice from experimental lung metastasis of several tumor types.11 In an effort to better understand the role of CIS in tumor control, we have now significantly extended the analysis of Cish−/− mice to include challenge with solid and hematopoietic tumors of various origins and immunogenicities in various
locations (including lung, subcutaneous, bone marrow, spleen, and peritoneum). These experiments suggest that not all tumors are significantly controlled by Cish depletion, and as a general rule, the impact of Cish deletion appears restricted to a subset of those tumors where NK cells are critical. We have very limited evidence to support the CIS control of T cell-mediated antitumor immunity, but the complexities of regulatory and effector T cell populations may complicate the picture, and we cannot yet rule out a role for CIS deficiency in regulating T cell antitumor activity.

Given the importance of CIS for IL-15 signaling and the lack of impact of Cish deletion on NK cell response to two major TLR agonists, NK cells critically regulated by IL-12, IL-18, and type I IFN are less likely to be influenced by Cish deletion. This may well apply to the TME where NK cells may engage directly with tumor cells or in crosstalk with antigen-presenting cells, and IL-12, IL-18, and type I IFN are influential. Perhaps in scenarios where NK cell survival maintained by IL-15 is critical, CIS will be more important. Tumor models that were unaffected by Cish deficiency (such as RMA-S in the peritoneum and growing subcutaneously or multiple myeloma in the bone marrow) might be characterized by low levels of IL-15 in the microenvironment, or factors such as IL-12, IL-18, type I IFN might predominate in regulating NK cell behavior. Certainly IL-2 was more effective in Cish<−/−> mice than WT mice when challenged with RMA-S i.p., despite the lack of importance of CIS in natural NK cell-mediated control of RMA-S in the peritoneum. It is notable that tumor loss of IL-15 correlates with a higher risk of tumor recurrence and reduced patient survival. It now remains to better understand the relationship and relative importance of IL-15 and CIS in regulating immune cell control of different tumors. Answering these questions will require new tools, such as CIS and IL-15 reporter mice, to determine whether CIS is only critical when IL-15 signaling is induced. Our data so far suggest that in NK cells CIS is specifically important for IL-15 signaling, whereas it appears to be dispensable for TLR signaling where IL-12 and IL-18, rather than IL-15, are critical for NK cell activation. However, we have only assessed a few mouse hematopoietic tumors in our study, so we cannot yet conclude that IL-15 and CIS are not relevant in these settings. The relative importance of these molecules will undoubtedly be influenced by the tumor site and microenvironment (including antigen presenting cell composition). It was of great interest to us that fibrosarcoma growth initiated by the carcinogen MCA, was so strongly inhibited altogether, delayed, or slowed in Cish<−/−> mice. This effect was clearly NK cell and IFN-γ-dependent and the findings are very important given that, until this time, the impact of Cish deletion on the process of de novo tumor formation had not been

Figure 7. Cish<−/−> mice respond similarly to WT mice to CpG challenge. Groups of 5 B6.WT (Cish<+/+>) and B6.Cish<−/−> mice were injected i.p. with 0.1 mg CpG/30 g mouse. (A) Six hours after the injection of CpG, spleens were analyzed for the presence (fraction and absolute numbers) and activation (expression of CD122, CD69, and IFN-γ) of NK cells by flow cytometry (gated on live CD45<+>TCRβ<+>NK1.1<+>DX5<+>NKp46<+>). Three naive B6.WT (Cish<−/−>) and B6.Cish<−/−> mice were used as untreated control. Shown is the summary of two independent experiments represented by the median ± interquartile range of 10 mice per group. Statistically significant differences between WT and Cish<−/−> groups as indicated were determined by one-way ANOVA with the Tukey post-test (\(p < 0.05\); \(\ast p < 0.01\); \(\ast\ast p < 0.001\)). (B) Serum cytokine levels were determined 10 d before (time point 0), and 1, 3, and 6 h after the i.p. injection of CpG. Shown is the summary of two independent experiments represented by the mean ± SEM of 10 mice per group. Statistically significant differences between WT and Cish<−/−> groups as indicated were determined by the Mann–Whitney U test (\(p < 0.05\)).
evaluated. This determination should now encourage a much deeper assessment of IL-15 and CIS regulation in situations wherein inflammation may contribute to tumorigenesis.

The additional important findings in this paper concern the enhanced anti-metastatic activities of both those tested targeted therapies and immunotherapies (immune checkpoint antibodies and cytokines) in Cish−/− mice. Anti-CTLA-4 and/or anti-PD1 and anti-CD96 approaches were still effective in Cish−/− mice, indicating the independence of CIS and immune checkpoint regulation of NK cell anti-metastatic activity. Similarly, IFN-αβ and IL-2 were also effective independently of CIS and indeed low dose IL-2, which was completely ineffective in WT mice, was substantially effective in Cish−/− mice. Importantly, targeted therapies such as the BRAF inhibitor, PLX4720, have been shown to mediate their anti-metastatic effect in mice via NK cells.23 Here, we have demonstrated that this activity on NK cells is independent of the function of CIS and indeed exceptional tumor metastatic control can be realized by inhibiting BRAF, MEK, and CIS in combination. These data provide further impetus to better understand the role of IL-15 and CIS in TMEs and to generate specific inhibitors for CIS to complement an armamentarium of NK cell-based cancer therapies.24

**Figure 8.** Combining Cish deficiency and checkpoint inhibitors or cytokine stimulation shows an improved anti-metastatic effect. (A) Groups of 5–6 B6.WT (Cish+/+) or B6. Cish−/− mice were injected i.v. with 2 × 10⁸ B16F10 melanoma cells and treated with either control lg (cIg) (250 μg i.p. on days 0, 3, and 6), anti-PD-1 (250 μg i.p. on days 0, 3, and 6), mouse IFN-γ (25 μg i.p. on days 0, 1, 2, and 3) or recombinant IL-2 (10,000 IU i.p. on days 0, 1, 2, and 3). (B) Groups of 5–11 B6.WT (Cish+/+) or B6.Cish−/− mice were injected i.v. with 7.5 × 10⁸ B16F10 melanoma cells and treated with either cIg (250 μg i.p. on days 0, 3, and 6), anti-PD1/anti-CTLA-4 combination (250 μg i.p. each on days 0, 3, and 6), or recombinant IL-2 (10,000 IU i.p. on days 0, 1, 2, 3, and 4). Individual mice are shown by each symbol and the results are plotted as mean ± SEM. Statistically significant differences as indicated were determined by one-way ANOVA with the Tukey post-test (for multiple comparisons) (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001).

**Material and methods**

**Mice**

C57BL/6J WT mice were purchased from the Walter and Eliza Hall Institute for Medical Research and/or bred in-house at the QIMR Berghofer Medical Research Institute. C57BL/6Mcl1CreNcr1iCre10 and C57BL/6 Cish-deficient mice (Cish−/−; generously provided by James Ihle, St. Jude Children’s Research Hospital, Memphis, USA) were bred at the QIMR Berghofer Medical Research Institute and used between 6 and 16 weeks. All mice were sex- and age-matched and groups of 5–11 mice were used per experimental assay. All experiments were approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee.

**Experimental tumor models**

B16F10, B16-OVA, RM-1, and MC38-OVA cell lines were maintained in cDMEM media containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamax. The luciferase+ RMA-S, parental RMA-S and RMA-S-Rae1β lymphoma, MCA1956 fibrosarcoma (derived from a B6.WT female mouse, kind gift of Robert Schreiber), and LWT1 melanoma cell lines were cultured in cRPMI media containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM...
glutamax, HEPES, and sodium pyruvate. The in vivo growth of luciferase+ RMA-S cells was monitored as previously described. Subcutaneous and intravenous injections were performed in a volume of 100 μL or 200 μL plain media, respectively. Primary transplant and experimental metastases models and the MCA-induced fibrosarcoma model were performed as previously described. The presence of monoclonal immunoglobulin in the blood serum was determined by serum protein electrophoresis (HYDRASYS, Sebia Hydragel), and the percentage of the γ-globulin fraction was quantified using the Phoresis (8.6.1) software.

In vivo treatments

Some groups of mice were treated with cIg (2A3 or 1–1 or polyclonal rabbit), anti-CD96 (3.3, rat IgG1), anti-PD-1 (CD279) (RMP1-14, rat IgG2a), anti-CTLA-4 (CD152) (UC10-4F10, hamster IgG, kindly provided by Jeffrey Bluestone), recombinant human IL-2, mouse IFN-γ, hamster IgG, kindly provided by Gideon Bollag, Plexxikon Inc.), or MEK inhibitor (MEKi, trametinib provided by the Royal Brisbane and Women's Hospital Oncology Pharmacy) as indicated. Some mice additionally received either: anti-CD8 (53.5.8) to deplete CD8+ T cells; anti-asialoGM1 to deplete NK cells; or anti-IFN-γ (H22) as previously described. To induce endotoxicosis, groups of five female mice were injected intraperitoneally with varying doses of LPS (0.2, 0.5, or 1 mg/30 g body weight, obtained from Sigma-Aldrich) or 0.1 mg CpG DNA/injection, spleens were harvested and erythrocyte-depleted single-cell suspensions were immediately analyzed via flow cytometry for the presence and activation (IFN-γ production, CD69, and CD122 expression) of NK cells. In order to pool two independent experiments, the mean fluorescent intensities of CD122 were normalized to the expression on naive B6.WT NK cells in the respective experiment.

Flow cytometry

Single-cell suspensions of spleens or bone marrow were depleted from erythrocytes and stained with monoclonal antibodies in phosphate buffered saline (PBS) containing 1% (v/v) FBS and 2.5 mM EDTA. Dead cells stained by Zombie Yellow or 7-AAD (BioLegend) were excluded from analysis. Absolute cell numbers were determined using the BD liquid counting beads (BD Biosciences). Antibodies specific for B220 (RA3-6B2), CD4 (RM4-5), CD8a (53–67), CD45.2 (104–2, Miltenyi), CD49b (DX5, ebioscience), CD69 (H1L2F3), CD122 (TM-β1, BD), CD138 (281–2), CD155 (TX56), IFN-γ (XMG1.2), NK1.1 (PK136, BD or BioLegend), NKp46 (29A1.4), and TCRβ (H57-597) were purchased from BioLegend unless stated otherwise.

Intracellular IFN-γ staining was performed by using the BD Cytofix/CytoPerm kit. The data were acquired on a LSR Fortessa 4 laser (BD Biosciences, San Jose, CA) and analyzed with FlowJo Version 10.0.8.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software V7.01. Statistical tests used were the Mann–Whitney U test, one-way ANOVA with the Tukey post-test (for multiple comparisons) and Log-rank Mantel–Cox test (for comparison of 2 survival curves, followed by Bonferroni correction for comparison of > 2 survival curves). Data were considered to be statistically significant if the p value was equal to or less than 0.05.

Disclosure and potential conflict of interest

MJS has research agreements with Medimmune and Bristol Myers Squibb. Other authors declare that they have no conflict of interest.

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