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Interferon-Gamma Receptor Signaling Plays an Important Role in Restraining Murine Ovarian Tumor Progression

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ABSTRACT

Immune cell-derived cytotoxic pathways have been implicated in antitumor immune responses. The goal of this study is to characterize how these cytotoxic pathways influence ovarian cancer development. We have utilized the TgMISIIR-TAg transgenic mouse model which expresses the transforming SV40 TAg in the ovary, leading to spontaneous development of ovarian tumors that closely mimic human epithelial ovarian cancer. To test how perforin (Prf1), granzyme B (GzmB) and interferon-gamma (IFNg) impact tumor occurrence and progression, we bred the TgMISIIR-TAg transgene into *Prf1*$^{-/-}$, *GzmB*$^{-/-}$, and *IFNgR1*$^{-/-}$ mice. The transgenic females developed peritoneal tumors at 9-15 weeks and succumbed at 184 ± 37 days of age with 100% penetrance (n=41). Knockout of these cytotoxic genes does not affect tumor occurrence. However, loss of function in the IFNg signaling pathway significantly expedited tumor progression with all of the *IFNg R1*$^{-/-}$ TgMISIIR-TAg females succumbing to tumor outgrowth at 167 ± 27 days of age (p=0.0074, n=24). In contrast, loss of function of Prf1 or GzmB did not significantly impact tumor progression and host survival. Since tumor cells in the *IFNg R1*$^{-/-}$ TgMISIIR-TAg mice are *IFNg R1* deficient, we used the implantable MOSEC (mouse ovarian surface epithelial cell) tumor line to validate that *IFNg R* signaling in host immune cells but not in tumor cells impacts tumor progression. Indeed, when the IFNg -responsive MOSEC cells were inoculated, *IFNg R1*$^{-/-}$ mice exhibited significantly higher tumor burden compared to WT mice. Furthermore, a MOSEC-splenocyte co-culture system confirmed that *IFNg R1*$^{-/-}$ immune cells were less effective than WT immune cells in controlling MOSEC tumor growth *in vitro*. Together, these results indicate that the *IFNg R* signaling pathway plays an important role in restraining murine ovarian tumor progression.

Keywords: Cytotoxic pathways, Interferon-gamma (IFNg), Perforin (Prf1), Granzyme B (GzmB), Tumor immunity, Ovarian cancer.

INTRODUCTION

The intricacy of immune activation versus immune suppression in the tumor environment affects clinical outcomes. Several major immune cell-derived cytotoxic pathways have been shown to contribute to antitumor immune responses [1-3]. For example, the perforin/granzyme pathway was believed to be critical for immune surveillance against several types of blood cancers and epithelial malignancies [4-7]. Perforin and granzymes were shown to be key effector molecules for cytotoxic T cells and natural killer (NK) cells to eliminate transformed tumor cells [8-11]. Interferon-gamma (IFNg) has also been shown to be broadly involved antitumor immune response mediated by both T cells and innate immune cells [1,12]. Interestingly, these same cytotoxic pathways have recently been implicated in suppressing antitumor immune responses [13]. Several studies have revealed that perforin and granzyme B can be used by regulatory T cells to suppress immune responses in mechanisms involving damaging antigen presenting cells or effector lymphocytes [14-17].

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IFNg has also been implicated in a complex network of immune suppression that involves myeloid derived suppressor cells, regulatory T cells and indoleamine 2,3-dioxygenase, [18-20] although IFNg has the ability to directly inhibit tumor-induced regulatory T cell proliferation [21,22].

The opposite impacts of suppressive immune cells versus antitumor immune cells have been documented in both ovarian cancer patients and mouse models. While intratumoral CD8+ T cell infiltration and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer, accumulation of myeloid-derived suppressor cells in the tumor environment leads to weakened tumor immunity [23,24]. However, the cytotoxic molecular pathways described above have not been carefully examined in ovarian cancer models. Therefore, we have studied the roles of these cytotoxic pathways in mouse models of ovarian cancer. We hypothesized that the perforin/granzyme and IFNg pathways contribute to tumor immunity during ovarian tumor progression. We have utilized a transgenic ovarian cancer model developed by Dr. Denise Connolly [25]. The TgMISIIR-TAg transgenic mice specifically express the transforming SV40 TAg in the ovary and epithelium of the female reproductive tract, leading to spontaneous development of ovarian tumors that closely mimic human epithelial ovarian cancer [25,26]. To test whether perforin (Prf1), granzyme B (GzmB) and interferon-gamma (IFNg) are involved in tumor development in this model, we have bred the TgMISIIR-TAg transgene into Prf1$^{-/-}$, GzmB$^{-/-}$, and IFNg R1$^{-/-}$ mice and monitored tumor occurrence, progression and mouse survival. Results from this transgenic model indicate that loss of function in the IFNg signaling pathway significantly expedited tumor progression while loss of function of Prf1 or GzmB did not significantly impact tumor progression. In addition, we have used the IFNg -responsive MOSEC ovarian tumor cell line to demonstrate that IFN signaling pathway in the host immune cells plays an important role in restraining ovarian tumor progression.

MATERIALS AND METHODS

Mouse colonies and tumor cell line

The TgMISIIR-TAg transgenic mice in the C57BL/6J strain were obtained from Dr. Denise Connolly at Fox Chase Cancer Center [25]. *Prf1*$^{-/-}$ and *GzmB*$^{-/-}$ mice in the C57BL/6J strain have been generated and maintained as previously described [9,15,27]. Wild-type (WT) and *IFNg R1*$^{-/-}$ mice in the C57BL/6J strain were purchased from the Jackson laboratory. MOSEC tumor cell line was obtained from Dr. Kunle Odunsi at Roswell Park Cancer Institute. MOSEC cells were transduced with a retroviral vector to express luciferase and used for bioluminescence imaging to measure tumor burden as previously described [15,21,27,28]. All mice were maintained in SPF housing, and all experiments were conducted in accordance with the animal care guidelines at Roswell Park Cancer Institute, using protocols approved by animal studies committee.

Transgene breeding, genotyping and monitoring tumor development

The TgMISIIR-TAg transgenic mice were crossed with the *Prf1*$^{-/-}$, *GzmB*$^{-/-}$, and *IFNg R1*$^{-/-}$ mice respectively to breed the TgMISIIR-TAg transgene into the *Prf1*$^{-/-}$, *GzmB*$^{-/-}$, and *IFNG R1*$^{-/-}$ colonies. Presence of the transgene was confirmed by PCR amplification of a 773-bp fragment of the large *TAg* using the TAg F4 forward primer (5′-TGCATGGTGTACAACATTCC) and the TAg R1 reverse primer (5′-TTGGGACTGTGAATCAATGCC) as previously described [25]. Prf1 locus was genotyped by PCR with a forward primer (5′-TGGTCTGGTGGACTACAGCCTGGA) and a reverse primer (5′-CCTGAACTCCTGGCCACCAAAGA), which produces a 300bp fragment for WT allele and a 1500bp fragment for the knockout allele. GzmB locus was genotyped by PCR with a forward primer (5′-ACACAAGTACTCAGAAGACGTCA) and reverse primer (5′-TGAACACTGGGGAACCACT), which produces a 690 bp fragment for WT allele and a 2.2 kb fragment for the knockout allele. *IFNG R1* locus was genotyped by separate PCR with a common forward primer (5′-TTG TTT GAT CCA TTC TTT AAA TTG) paired with a reverse primer (5′-GCT TCT TTG AAG GGC TGG A) that produces a 310bp fragment for WT allele, and paired with another reverse primer (5′-AAT GGA GGG AGC ACA GTT TG) that produces and a 450bp fragment for the knockout allele. The resultant four genotypes of TgMISIIR-TAg, *Prf1*$^{-/-}$ TgMISIIR-TAg, *GzmB*$^{-/-}$ TgMISIIR-TAg, and *IFNg R1*$^{-/-}$ TgMISIIR-TAg female mice were evaluated weekly for tumor development. After 12 weeks of age, all transgenic females were monitored daily for intraperitoneal tumor formation and accumulation of ascites fluid (detected by abdominal swelling). Time of death was recorded when tumor-bearing mice that became moribund were sacrificed.

Bioluminescence imaging *in vivo* and *in vitro*

WT and *IFNg R1*$^{-/-}$ mice were injected intraperitoneally with 2.5 x 10^6 luciferase-expressing MOSEC tumor cells. Bioluminescence imaging was performed to monitor tumor burden *in vivo* as previously described [15,21,27,28]. To measure MOSEC tumor growth *in vitro*, various doses of MOSEC cells were cultured in 48-well plates with a total volume of 1ml media for 92 hours, and then 20 µl D-Luciferin (15mg/ml) was added into each well, and bioluminescence imaging was performed to measure tumor

burden in each well. Tumor burden was expressed as photon flux (photons/sec).

MOSEC tumor and splenocyte co-culture

2 x 10^6 spleen cells isolated from WT and *IFNg R1*$^{-/-}$ mice were mixed with various doses (4000, 2000, and 1000) of luciferase-expressing MOSEC cells respectively, and co-cultured in 48-well plates with a total volume of 1ml media in each well. 92 hours later, 20 µl D-Luciferin (15mg/ml) was added into each well, and bioluminescence imaging was performed to measure tumor burden in each well as described above.

RESULTS

Global deficiency of IFNg R1, but not Prf1 or GzmB, significantly expedites TgMISIIR-TAg-driven tumor progression.

The TgMISIIR-TAg transgenic mice specifically express the transforming SV40 TAg in the ovary and epithelium of the female reproductive tract, leading to spontaneous development of ovarian tumors [25,26]. To test whether perforin (Prf1), granzyme B (GzmB) and interferon-gamma (IFNg) are involved in controlling tumor development driven by the TgMISIIR-TAg transgene, we have bred the TgMISIIR-TAg transgene into *Prf1*$^{-/-}$, *GzmB*$^{-/-}$, and *IFN R1*$^{-/-}$ mice and monitored tumor development and mouse survival. The transgenic females developed peritoneal tumors at 9-15 weeks and succumbed at 184 ± 37 days of age with 100% penetrance (n=41). Loss of function in the IFN signaling pathway significantly expedited tumor progression with all of th *IFNg R1*$^{-/-}$ transgenic females succumbing to tumor outgrowth at 167 ± 27 days of age (p=0.0074, n=24) (**Figure 1A**). In contrast, loss of function of Prf1 or GzmB did not significantly impact tumor progression as the *Prf1*$^{-/-}$ transgenic females succumbed to tumor outgrowth at 187±27 days of age (p=0.2505, n=23) and the *GzmB*$^{-/-}$ transgenic females succumbed at 191 ± 29 days (p=0.4954, n=38) (**Figure 1B-1C**). It is clear that global loss of function of these cytotoxic pathways does not affect tumor occurrence driven by the TgMISIIR-TAg transgene, as 100% of the mice expressing the transgene developed ovarian tumor in all four genotypes of mice. However, these results highlight the importance of the IFNg R signaling pathway for limiting tumor progression and prolonging survival of the tumor-bearing mice.

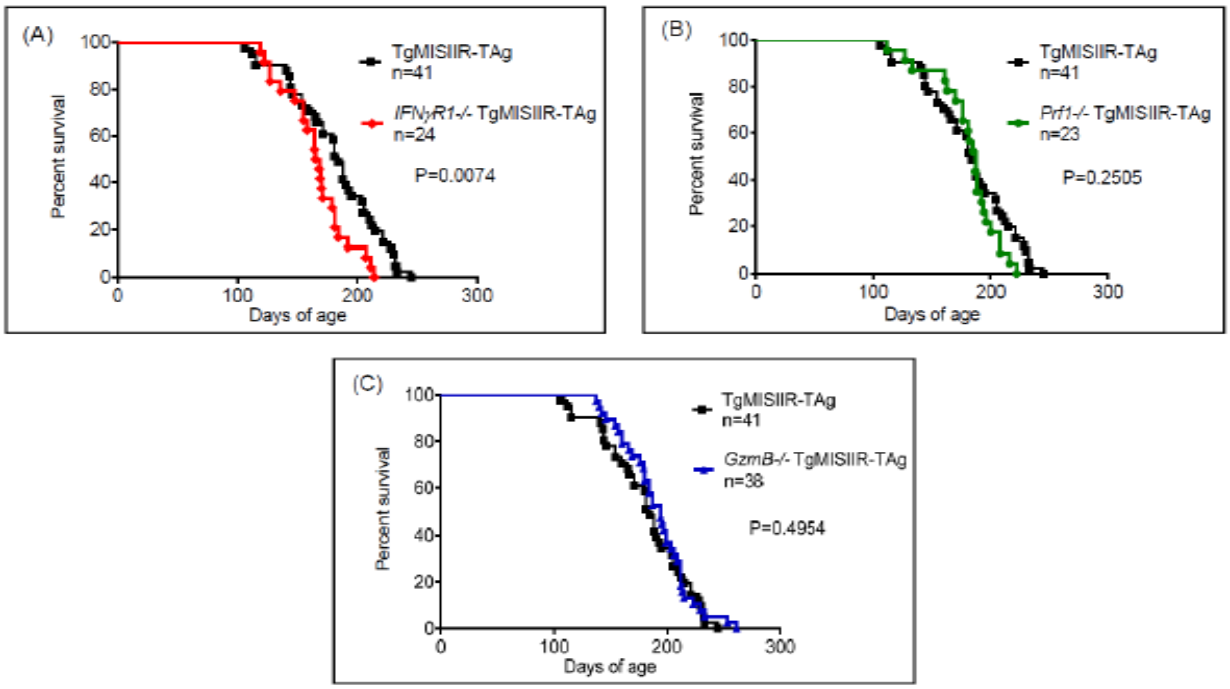

Figure 1. Global loss of function of IFNg R1, but not Prf1 and GzmB, accelerates ovarian tumor progression driven by the TgMISIIR-TAg transgene. TgMISIIR-TAg transgene was bred into strain-matched *IFNg R1*$^{-/-}$, Prf1$^{-/-}$, GzmB$^{-/-}$ mice, and tumor development and mouse survival were monitored as described in Materials and Methods. Summarized data are shown as Kaplan–Meier survival curves of *IFNg R1*$^{-/-}$ TgMISIIR- TAg mice (A), *Prf1*$^{-/-}$ TgMISIIR-TAg mice (B), and *GzmB*$^{-/-}$ TgMISIIR-TAg mice. (C) bearing ovarian tumors analyzed against TgMISIIR-TAg mice. Mantel-Cox test was performed to determine statistical significance.

IFNg R1$^{-/-}$ mice control MOSEC tumor growth less efficiently than WT mice

It is important to note that in the *IFNg R1*$^{-/-}$ TgMISIIR-TAg mice described above, both the immune cells and the SV40 TAg transformed tumor cells are *IFNg R1*$^{-/-}$. Since *IFNg R1*$^{-/-}$ mice can still produce IFNg , it is therefore likely that loss of response to IFN by the *IFNg R1*$^{-/-}$ ovarian tumor cells or by the *IFNg R1*$^{-/-}$ immune cells accounts for the accelerated tumor progression. To test a hypothesis that loss of function of the IFNg R signaling pathway in immune cells may dampen antitumor response against the ovarian tumor cells, we employed the established MOSEC ovarian tumor cell line, which was transformed through repeated passages of mouse ovarian surface epithelium cells *in vitro* [29]. The transformed MOSEC tumor cells were able to develop into peritoneal ovarian tumors after inoculation into syngeneic C57BL/6 mice [29]. We first confirmed that the MOSEC tumor cells were able to directly respond to IFNg. Various doses of recombinant IFN protein added to cell culture media were able to suppress MOSEC cell proliferation consistently (**Figure 2A**), indicating that MOSEC cells have a functional IFNg R signaling pathway. We also tried to measure potential tumor cell killing by IFN treatment. However, no significant cell death was observed by the methods of trypan blue, annexin V and 7AAD staining (data not shown). Even with IFN treatment, tumor cells were still proliferating, but at slower rates compared the non-treated control culture (**Figure 2A**). These results indicate that IFNg treatment inhibits MOSEC tumor cell growth without causing substantial cell death.

Next, to investigate the effect of IFNg R signaling pathway in the host mice on ovarian tumor progression, we inoculated *IFNg R1*$^{-/-}$ and WT C57BL/6 mice with luciferase-expressing MOSEC tumor cells. Using bioluminescence imaging to serially monitor tumor burden *in vivo*, we have observed significantly increased tumor growth in *IFNg R1*$^{-/-}$ mice compared to that in WT mice (**Figure 2B**). These results with the MOSEC tumor model are consistent with the finding with the TgMISIIR-TAg transgenic model. Furthermore, these data indicate that IFNg R signaling in the host, presumably in the host immune system, plays an important role in limiting ovarian tumor growth.

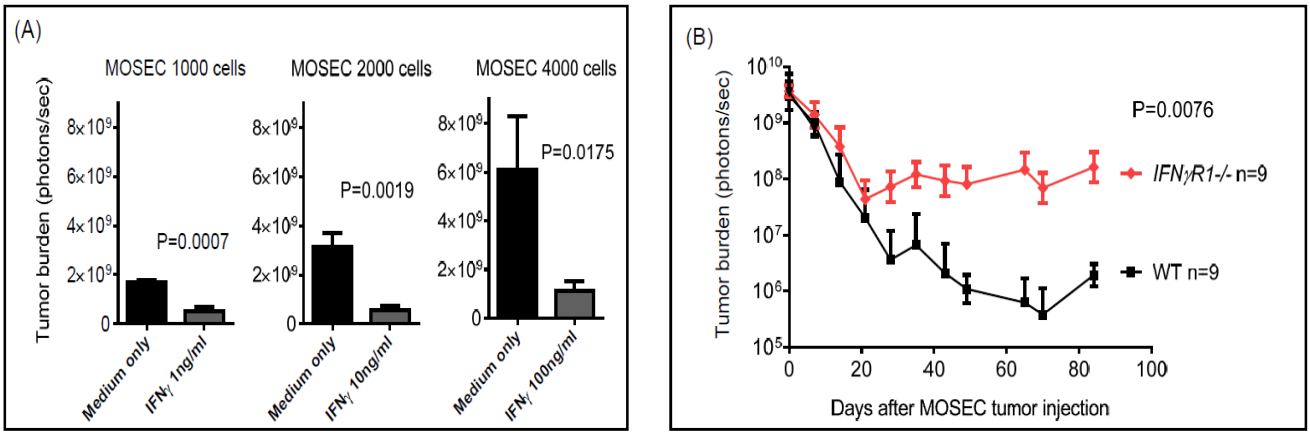

Figure 2. IFNg R1$^{-/-}$ mice control MOSEC tumor growth less efficiently than WT mice. (A) MOSEC tumor cells are responsive to *IFN* . Different doses (1000, 2000, and 4000) of luciferase-expressing MOSEC tumor cells were cultured with various doses (1ng, 10ng, and 100ng) of recombinant IFNg in 48-well plates with a total volume of 1ml media in each well for 92 hours, and then 20 µl D-Luciferin (15mg/ml) was added into each well, and bioluminescence imaging was performed to measure tumor burden. Two-tailed t-tests were performed to determine statistical significance. (B) WT and *IFNg R1*$^{-/-}$ mice were injected intraperitoneally with 2.5 x 10^6 luciferase-expressing MOSEC tumor cells, and tumor burden was measured by bioluminescence imaging. Summary data are shown as mean ± SD, with 9 mice in each group. Two-way ANOVA was performed to determine statistical significance.

IFNg R1$^{-/-}$ spleen cells have diminished ability to control MOSEC tumor growth *in vitro* compared to WT spleen cells

To further define whether IFNg R signaling in the host immune system is responsible for controlling ovarian tumor growth, we isolated spleen cells from WT and *IFNg R1*$^{-/-}$ mice and tested their ability to control tumor growth *in vitro*. As expected, spleen cells added to the MOSEC cell culture were able to inhibit tumor cell growth (**Figure 3**). Furthermore, *IFNg R1*$^{-/-}$ spleen cells showed reduced ability to control MOSEC tumor growth compared to WT spleen cells. This *in vitro* co-culture system confirms that the IFNg R signaling pathway in the host immune cells is important

for an effective antitumor response against ovarian tumor cells.

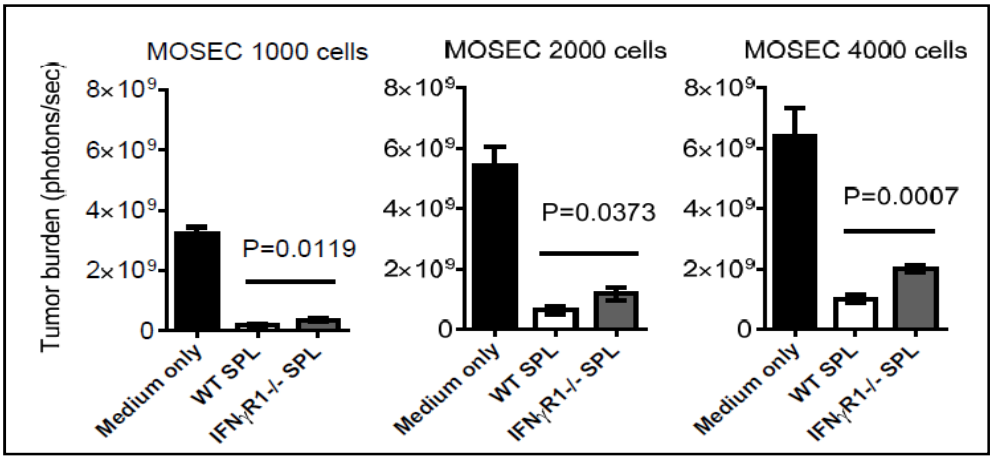

Figure 3. *IFNg R1*$^{-/-}$ spleen cells have diminished ability to control MOSEC tumor growth *in vitro* compared to WT spleen cells 2 x 10^6 spleen cells isolated from WT and *IFNg R1*$^{-/-}$ mice were mixed with different doses (1000, 2000, and 4000) of luciferase-expressing MOSEC tumor cells, and co-cultured in 48-well plates with a total volume of 1ml media in each well. Tumor cells were also cultured with media only without spleen cells as a negative control. 92 hours later, 20 µl D-Luciferin (15mg/ml) was added into each well, and bioluminescence imaging was performed to measure tumor burden. Two-tailed t-tests were performed to determine statistical significance.

DISCUSSION

It remains challenging to diagnose ovarian cancer at early stages of disease because of lack of symptoms and an effective screening system. Due to the often late diagnosis at advanced stages of disease and limited efficacy of surgical and chemotherapeutic strategies, ovarian cancer remains the most frequent cause of death from gynecologic malignancy [29]. As promising new treatment modalities, cancer immunotherapy strategies based on cancer vaccines and adoptive cellular transfer are being actively investigated. This study aims to provide a better understanding of how major cytotoxic molecular pathways in the immune system impact ovarian cancer development and progression, which will be useful for developing effective immunotherapy. We have provided solid evidence to show that while global deficiency of *GzmB, Prf1, or IFNg R* does not affect ovarian tumor occurrence driven by the aggressively transforming SV40 TAg, global loss of function of the IFNg R signaling pathway, but not *Prf1 or GzmB,* significantly accelerates tumor progression.

However, we believe that this information has to be considered with caution because it has recently been revealed that these cytotoxic pathways can be employed not only by effector lymphocytes engaged in antitumor immune responses but also by immune suppressor cells involved in dampening antitumor responses. For example, global deficiency of *GzmB* or *Prf1* could disable their function in not only effector lymphocytes but also regulatory T cells [14-17], resulting in opposite effects that could neutralize the overall impact on tumor development. Likewise, global deficiency in the IFNg R signaling pathway could have even broader effects because almost all innate and adaptive immune cell types express functional IFNg receptor [18-22]. Although global deficiency of the IFNg R signaling pathway could impact the functions of effector immune cells as well as suppressor immune cells, this work with ovarian cancer models shows that the oval impact of the IFNg R signaling pathway has a positive effect on antitumor immune responses. In line with our work, a previous study of ovarian cancer patients shows that loss of IFNg R in ovarian tumor cells independently predicts poor prognosis [30], which may help explain our observation since the tumor cells in our SV40-driven spontaneous model are also deficient for IFNg R. However, our study with the IFNg -responsive MOSEC model presents a similar phenotype, suggesting that IFN R deficiency in host immune cells is at least partially responsible for accelerated tumor progression. Our previous study reveals that the numbers of mature $CD4^+$, $CD8^+$ T cells and $CD4^+Foxp3^+$ Treg cells are not altered in naive IFN $R^{-/-}$ versus WT mice. However, tumor inoculation induces a higher expansion of Treg cells in IFNg $R^{-/-}$ versus WT mice due to IFNg -mediated inhibition of Treg cell expansion [21], which may partially contribute to the increased tumor burden in the IFNg $R^{-/-}$ mice. In contrast to these studies that describe a positive impact of IFNg on tumor immunity, a recent study with murine melanoma model shows that IFNg actually inhibits peptide vaccine-induced tumor immunity by inducing expression of high levels of noncognate MHC-I

and PD-L1 molecules on tumor cells [31]. While the discrepancy may result from many variables including the different tumor types and distinct immunogenicity of the different tumor models, we must acknowledge that IFNg signaling may play more complex roles than simply promoting antitumor immune responses.

In summary, due to the complex functions of these cytotoxic pathways in multiple immune cell compartments that may have differential and even directly opposite impacts on tumor immunity, it will be necessary to develop compartment-specific deficiency of these cytotoxic pathways. Further investigation with such improved models will provide deeper mechanistic understanding of how these molecular pathways mediate interactions between individual immune cell types and cancer cells, which may reveal novel targets that can be manipulated to improve the safety and efficacy of immune-based cancer therapy.

DISCLOSURE OF CONFLICT OF INTEREST

The authors have no potential conflict of interest to disclose.

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