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FOCUSSED RESEARCH REVIEW

Natural killer cells control metastasis via structural editing of primary tumors in mice

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Abstract

Natural killer (NK) cells are innate immune lymphocytes which express an array of activating and inhibitory receptors. These receptors bind a large spectrum of ligands, which are expressed on stressed, malignantly transformed or virally infected cells, as well as on bacterial, fungal, and parasitic pathogens. The decision on whether or not to kill the target is based on the integration of activating and inhibitory signals sent downstream from NK cell receptors. One of the most prominent NK cell activating receptor families is the family of natural cytotoxicity receptors (NCRs) which includes NKp30, NKp44, and NKp46. NKp46 is the only NCR to have a fully functional mouse orthologue denoted Ncr1. Despite a large body of evidence highlighting its importance in the clearance of both solid and liquid tumors, the membrane-bound tumor ligand for NKp46 and its mouse orthologue Ncr1 is still unknown. Here we review the discovery of a novel role for NKp46/Ncr1, not only in tumor clearance but also in prevention of metastasis by structural editing of primary tumors.

Keywords NK cells · NKp46 · Ncr1 · IFNγ · FN1 · TIMO2018

Abbreviations

FN1	Fibronectin1
IFNγ	Interferon gamma
KO	Knock out
MHC	Major histocompatibility complex
NCR	Natural cytotoxicity receptor
NK	Natural killer
RCM	Reflectance confocal microscopy
TNFα	Tumor necrosis factor alpha

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Introduction

Natural Killer (NK) cells were first discovered in the late 1960s and were named for their ability to kill target cells without prior stimulation [1]. NK cells express an array of non-rearrangeable, germline-encoded activating and inhibitory receptors. For every target, the sum between the inhibitory and activating signals transmitted by these receptors will determine whether the target will be spared or eliminated [2]. For example, somatic cells can express low levels of activating ligands, but, since major histocompatibility complex (MHC) class I molecules are expressed in higher levels, the overall signal will be inhibitory and NK cells will not eliminate somatic cells [2]. Upon various stresses such as heat-shock [3], malignant transformation [4, 5] or viral infection [6, 7], the expression of activating ligands on the stressed cell will increase and the overall activating signal will overcome the inhibitory signal, thus ensuring that aberrant cells are eliminated by NK cells. NK cells will form an immunological synapse with the target cell marked for clearance, then release cytotoxic granules. These cytotoxic granules contain granzyme and perforin. Perforin mediates granzyme entry to the target cell which in turn induces cell death [8].

One of the most important and vastly studied groups of NK activating receptors is the Natural Cytotoxicity Receptor

(NCR) family, which includes, in humans, NKp30, NKp44 and NKp46 [2]. NKp46 is the only NCR member to have a fully functional mouse orthologue called Ncr1 [2]. Members of the NCR family have a very wide range of cellular and pathogen-derived ligands. For example, NKp46/Ncr1 can bind the Hemagglutinin protein of influenza virus [9], the Sigma1 protein of reovirus [10], the EPA 1,6 and 7 proteins of *Candida glabrata* [11] but also have an unknown membrane-bound tumor ligand which allows recognition and elimination of tumor cells [12, 13].

Interestingly, NKp46/Ncr1 engagement by target cells does not always result in perforin or granzyme-mediated direct killing. It can also lead to the secretion of the inflammatory cytokines Tumor Necrosis Factor alpha (TNFα) and Interferon gamma (IFNγ) [14]. The importance of NKp46/Ncr1 in the immune response against cancer was first demonstrated in vivo when Ncr1 knock out (KO) mice were generated [15]. Ncr1 KO mice were significantly impaired in their ability to clear lymphomas [16]. NKp46/Ncr1 was identified as a crucial player in the recognition and reactivity of NK cells against solid tumors as well. Engagement of NKp46/Ncr1 by the unknown ligand on tumor cells lead to degranulation towards and lysis of the target in different tumor mouse models [12, 16, 17].

Ncr1 KO mice suffer from higher metastatic rates

When challenging Ncr1 heterozygous (het, which still have one functioning allele of Ncr1 and are therefore fully immune competent) or KO mice with B16 melanoma subcutaneously, *Glasner* et al. noticed that there was no significant difference in tumor volume between the groups. But, when looking at the rate of metastasis, there was a significant difference between the groups: Ncr1 KO mice had significantly higher metastasis incidence than Ncr1 het mice [17, 18]. This was puzzling, how can the absence of a receptor affect the metastasis rate so dramatically, yet have no apparent effect on the primary tumor volume? *Glasner* et al. excised the tumors from the mice and checked how well NK cells were activated in their presence. Although Ncr1 KO NK cells were less activated than Ncr1 het NK cells in the presence of B16 tumors excised from mice, there was no significant difference in NK cell cytotoxicity elicited by tumors originating from Ncr1 het or KO mice.

To further study the phenomenon of increased metastasis in the presence of same volume primary tumors, *Glasner* et al. checked to see whether there was a difference in ligand expression between tumors residing in Ncr1 het and KO mice. Expression levels of the unknown tumor ligand of Ncr1 were similar on tumors excised from Ncr1 het or KO mice. Additionally, the biochemical properties of the ligand were the same between tumors originating from Ncr1 het and KO mice. The unknown ligand was sensitive to trypsin and proteinase K treatment but resistant to treatment with neuraminidase.

NK cell-derived IFNγ as a key player in B16 metastasis formation

Glasner et al. subsequently checked whether primary tumors had different immune cell infiltration between Ncr1 het and KO mice. There was no difference in the levels of infiltrating T cells, B cells, NK cells, neutrophils and macrophages in the primary tumors of both Ncr1 het and KO mice.

Since NK cells specialize in the secretion of the inflammatory cytokines TNFα and IFNγ, *Glasner* et al. hypothesized that the difference in metastasis incidence could be due to differential secretion of these cytokines. To test this hypothesis, Ncr1 het and KO NK cells were co-incubated with B16 tumor cells and TNFα or IFNγ secretion was quantified. Ncr1-sufficient NK cells secreted significantly higher levels of both TNFα and IFNγ, as compared to Ncr1-deficient NK cells.

The fact that B16 cells only express the IFNγ receptor and not the TNFα receptor, suggested that the effect seen in vivo might be mediated by IFNγ secreted by NK cells. To investigate this hypothesis, *Glasner* et al. inoculated IFNγ and TNFα KO mice with B16 and monitored tumor progression and metastasis formation. Astonishingly, IFNγ KO mice had significantly higher metastasis incidence, comparable only to the Ncr1 KO mice, while TNFα KO mice had lower rate of metastasis, similar to that of Ncr1 het mice. Since T cells can also be the source of IFNγ, anti-CD3 (to deplete T cells) and anti-NK1.1 (to deplete NK cells) antibodies were administered to mice prior to tumor inoculation. Astonishingly, higher metastasis was only observed in the NK cell- but not in the T cell-depleted mice. This indicated that indeed NK cell-derived IFNγ mediates the observed effect and plays a major role in metastasis formation in B16 melanoma.

Primary tumor architecture changes upon exposure to IFNγ

If it is not the size or of the primary tumor which determines the metastatic outcome, what is it that accounts for the significant differences seen between mice expressing or lacking Ncr1?

Reflectance confocal microscopy (RCM) is a novel method used by dermatologists to asses and determine the malignancy of skin lesions suspected to be melanoma. This method employs imaging of the internal structure of the lesion. The system uses several different structural criteria

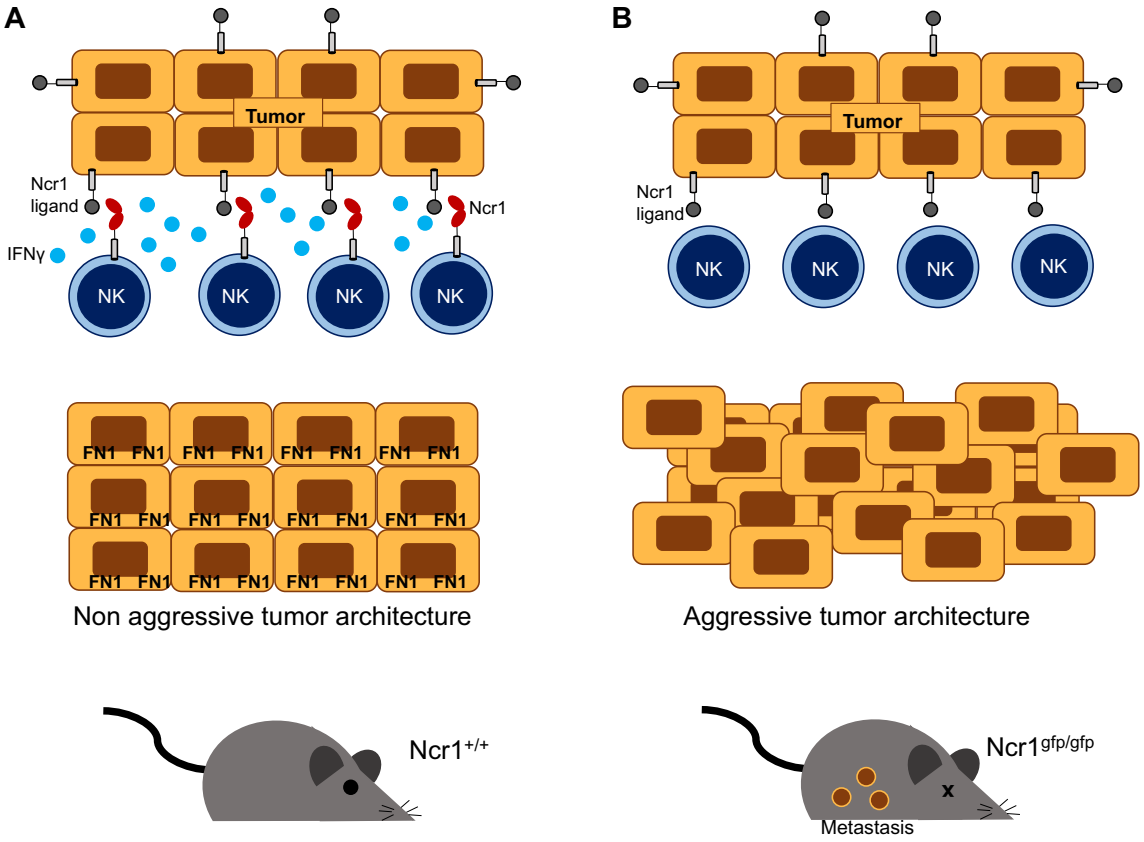

Fig. 1 Ncr1 engagement leads to IFNγ secretion and architectural editing of primary tumors. **a** In the presence of Ncr1, primary tumor membrane-bound ligand triggers IFNγ secretion from NK cells which leads to upregulation of FN1 in tumor cells. This results in structural editing of primary tumor leading to a less aggressive architecture, which results in less metastasis. **b** In the absence of Ncr1, IFNγ is not secreted by NK cells, leading to tumors with more aggressive architecture which results in metastasis

to describe the lesion's architecture. Malignant lesions will score higher on all criteria than benign lesions. This indicates internal disorder and abnormal architecture in malignant lesions, compared to benign lesions which present with a more organized architecture. RCM-mediated diagnosis of skin lesions was found to be significantly more accurate than conventional diagnosis [19]. Importantly, B16 melanoma lesions imaged with RCM look very similar to human melanoma lesions and can be scored using the same set of criteria. To better understand the differences between the primary tumors of Ncr1 het and KO mice, *Glasner* et al. employed RCM imaging. Tumors in Ncr1 KO and IFNγ KO mice scored significantly higher in 4 RCM criteria, indicating higher level of architectural disorder, compared to Ncr1 het mice.

To investigate the mechanism behind these differences, *Glasner* et al. treated B16 melanoma cells with recombinant IFNγ and performed bulk RNA sequencing. A 24-fold increase was detected in the expression of *Fibronectin1* (FN1) gene, in B16 cells treated with IFNγ. Fibronectin1 is a key participant in important cellular processes such as adhesion, actin cytoskeleton and integrin-linked kinase signaling pathways [20]. Inoculation of FN1 knock down (KD) B16 cells into Ncr1 sufficient mice abrogated the protective effect of Ncr1, and these mice had the same metastasis incident as Ncr1 KO and IFNγ KO mice, although primary tumors were of the same volume. RCM assessment of same size primary tumors showed that the KD of FN1 was sufficient to damage the internal structure arrangement which resulted in higher RCM scoring, even in Ncr1 sufficient mice (Fig. 1).

Finally, recombinant IFNγ treatment of Ncr1 KO or IFNγ KO mice significantly improved RCM scoring of primary tumors, resulting in overall more organized tumor architecture. The same phenomenon was observed when B16 melanoma was inoculated into mice overexpressing Ncr1.

Discussion

Taken together, these results suggest a novel mechanism of NK cell-mediated structural editing of primary tumors. In this model, B16 tumors engage Ncr1 on NK cells via an

unknown membrane-bound ligand. This results in NK cell secretion of IFNγ which increases tumor levels of FN1. The higher expression of FN1 in the primary tumor results in architectural changes and a more organized structure, which is characteristic of less aggressive tumors and ultimately results in significantly less metastasis. This effect is completely abolished in the absence of Ncr1 or IFNγ.

Since the time they were discovered, almost 50 years ago, NK cells were famous for their ability to kill tumor cells without prior stimulation, thus participating in the first line of defense against malignant transformation. The above results bring to light a new immunological role for NK cells which are not mediated by direct killing, but by cytokine secretion which results in structural editing of primary tumors. By increasing the expression levels of FN1 through IFNγ secretion, and without affecting primary tumor size, NK cells shape the tumor to become less aggressive and to form less metastasis.

Author contributions Batya Isaacson wrote the first draft and Ofer Mandelboim edited the manuscript. Both authors revised the manuscript and approved the final version of this paper.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interests.

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