



Review

Main NK cell receptors and their ligands: regulation by microRNAs

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Abstract: The NK cells functions are finely tuned by several kinds of inhibitory and activating receptors, whose pattern of expression characterizes different NK subpopulations and varies with the cell activation status. MicroRNAs have an important role in tightly regulating the expression of NK receptors and, analogously, the expression of their ligands in target cells. The relevance of the microRNA-mediated control is highlighted by the dysregulation of these pathways observed in cancer and virus-infected cells. Here we review our current knowledge of the microRNAs involved in the regulation of NK receptors, as well as that of the corresponding cellular ligands.

Keywords: NK cells; microRNAs; NK receptors; NK receptor ligands; TGF- β 1

1. Introduction

Natural Killer (NK) cells are innate lymphoid cells endowed with several effector and regulatory functions, including cytotoxic activity against virus infected and tumor-transformed cells. Although there is a growing knowledge of the phenotypic heterogeneity of the NK cell population [1], two major subsets of circulating NK cells have been described, CD56^{bright} CD16^{low/neg} and CD56^{dim} CD16^{pos} cells, which have different function and tissue distribution. CD56^{bright} NK cells, which release large amount of soluble factors in response to pro-inflammatory cytokines, are poorly represented in peripheral blood, while largely populating secondary lymphoid organs. Conversely, CD56^{dim} cells, which are highly cytotoxic effectors, represent the largest percentage of circulating

NK cells.

The NK cell functions are finely regulated by a variety of germline-encoded inhibitory and activating receptors and by different cytokines, produced in the early or in the late phase of immune responses. All these cytokines other than potentiate NK cell responsiveness towards transformed cellular targets, stimulate the production of chemokines and cytokines (IFN- γ , TNF- α) crucial for shaping both innate and adaptive immunity [1,2].

The main inhibitory NK receptors include: Killer-cell immunoglobulin-like receptors (KIRs), CD94/NKG2A and LILRB1, MHC class I-specific receptors that physiologically maintain self-tolerance; PD-1, TIM-3 and LAG-3 that belong to the immune checkpoint family, inhibitory pathways that are induced/up-regulated during immune responses and limit the duration and amplitude of the immune responses, thus minimizing tissue damage. Activating signals are mediated by a plethora of activating receptors and co-receptor including NKp46, NKp30 and NKp44 (collectively termed Natural Cytotoxicity Receptors, NCR), NKG2D and DNAM-1. The activating receptors recognize non-MHC ligands up-regulated or *de novo* expressed by cells upon cellular stress, tumor transformation or viral infection. The modality of NK cell activation, well depicted by the rheostat model [3,4], depends on the cytokine milieu as well as on the presence/absence and surface densities of the different ligands on neighboring healthy or altered cells.

Different mechanisms regulate the expression of receptors, on effector NK cells, or ligands on potential targets. Among these, microRNA-mediated mechanisms that regulate gene expression at the post-transcriptional level. MicroRNA (miRNAs) are small RNAs, processed from stem-loop regions of longer RNA transcripts, which can interact with the 3'-Untranslated Region (3'-UTR) of target mRNAs via sequence complementarity, thus down-regulating gene expression through translation repression and/or mRNA degradation [5]. The importance of this kind of regulation is underlined by the fact that tumors and viruses evolved mechanisms to negatively affect NK activity acting through these pathways.

This review synthetically describes the current knowledge of miRNAs targeting molecules fundamental for the NK cell effector functions and trafficking.

2. NK cell receptors and miRNAs

Several miRNAs have been described that target different NK receptor pathways. These include activating receptors (NKG2D), the DAP12 signaling molecule, inhibitory receptors (KLRG1), immune checkpoint receptors (TIM-3, PD-1 and CTLA-4), chemokine and cytokine receptors (CX₃CR1, IL-2R γ). Interestingly, TGF- β 1, an immune-modulatory cytokine highly produced and activated in the tumor microenvironment [6], has been shown to hamper NK cells activity down-regulating different receptors via distinct miRNA pathways (Figure 1).

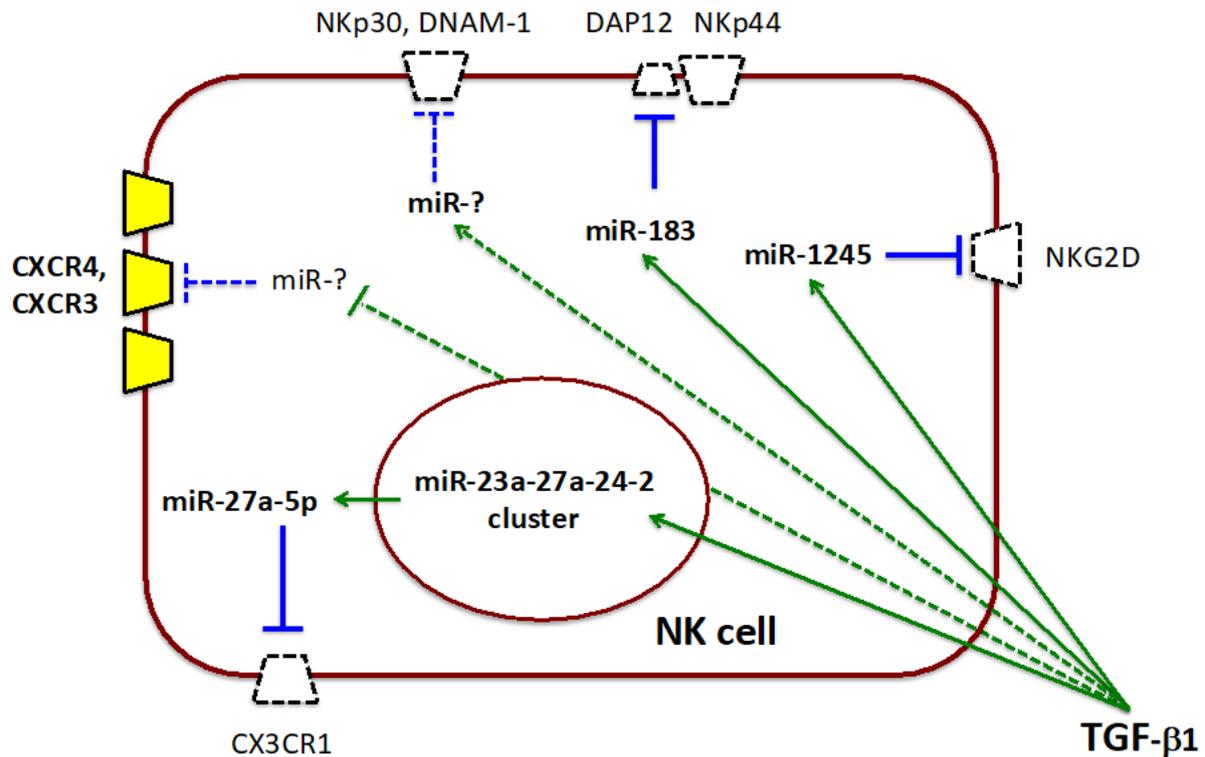


Figure 1. TGF- β 1 modifies the receptors expression in human NK cells. The TGF- β 1 contained in the tumor microenvironment reduces the expression of NKG2D, DAP12/NKp44 and CX₃CR1 in NK cells via different miRNA-mediated pathways. The involvement of miRNAs in the TGF- β 1-mediated up-regulation of CXCR4 and CXCR3 [9] or down-regulation of NKp30 [8] and DNAM-1 [91] remains to be determined.

2.1. NKG2D

NKG2D (CD314) is a C-type lectin-like receptor expressed on NK and CD8⁺ T cells. It is an activating receptor able to detect and promote the elimination of damaged, transformed, and pathogen-infected cells by recognizing stress-inducible ligands belonging to the MIC (MICA and MICB) and ULBP (ULBP1–6) families in humans [7]. *In vitro* studies showed that the surface expression of NKp30 and NKG2D is significantly down-regulated by conditioning NK cells with TGF- β 1 or TGF- β 2 [8,9]. Moreover, subsequent studies highlighted the presence *in vivo* of NKG2D^{low} NK cells in cancer patients, ascribable to TGF- β activity. One mechanism responsible for the TGF- β -mediated NKG2D down-regulation has been associated to the impairment of transcriptional and translational level of DAP10, the signaling subunit associated with human NKG2D [10]. Another mechanism has been showed by Espinoza et al. [11] who reported that TGF- β 1 causes an increase of miR-1245 in NK cells. Overexpression of miR-1245 induced a down-regulation of NKG2D at the cell surface level, but had no effect on other activating receptors such as NKp30, NKp44 and NKp46. Moreover, NK cells overexpressing miR-1245 had lower cytotoxicity against target cells expressing NKG2D ligands. MiR-1245 was shown to directly target and down-regulate the NKG2D mRNA.

2.2. DAP12

DAP12 (DNAX activating protein of 12 kDa) (also termed killer cell activating receptor-associated protein, KARAP) is a transmembrane protein containing an immune tyrosine-based activation motif (ITAM) in its cytoplasmic domain [12]. DAP12 is a fundamental signaling molecule that mediates a broad array of biological functions by associating with different activating receptors expressed in cells of lymphoid and myeloid lineage [12]. These receptors include the activating isoform of KIRs, CD94/NKG2C, and NKp44, the latter being expressed by NK cells upon activation. Donatelli et al. [13] reported that TGF- β 1 down-regulates the DAP12 level in NK cells. MiR-183, which was up-regulated by TGF- β 1, was shown to directly target DAP12 mRNA. NK cells overexpressing miR-183 had reduced DAP12 (both mRNA and protein) as well as decreased NKp44 surface levels. NK92 cells transduced with miR-183 were less efficient in killing Raji cells (which do not express NKG2D ligands) with respect to control lentiviral transduced cells. Interestingly, NK cells infiltrating lung cancers were shown to have a diminished DAP12 expression.

2.3. KLRG1

KLRG1 is a C-type lectin-like inhibitory receptor, containing an immune tyrosine-based inhibitory motif (ITIM), which binds to members of the cadherin family leading to inhibition of NK and T cell function. It is predominantly expressed by NK cells with a mature phenotype [14] and it is strongly induced by viral and other infections [15]. Cipolla et al. [16] reported that miR-584-5p targets KLRG1 and that overexpression of miR-584-5p in peripheral blood mononuclear cells (PBMC) causes a decrease of KLRG1 mRNA expression. Interestingly, a 3'-UTR KLRG1 polymorphism, rs1805672, associated to the autoimmune disease Pemphigus foliaceus, abolishes the binding of the miRNA, thus causing the loss of the miRNA-mediated control of KLRG1 expression. Authors suggested a link between the loss of the miRNA-target interaction and predisposition to the autoimmune disease.

2.4. TIM-3

TIM-3 belongs to the T cell immunoglobulin and mucin domain (TIM) family. It is expressed on the surface of several immune cells, including NK cells. Upon activation by the C-type lectin galectin-9 ligand, TIM-3 functions as an inhibitory receptor on NK cells by reducing their cytotoxicity and cytokine production [17]. Cheng et al. [18] reported that TIM-3 and T-bet expression in NK cells from chronically HCV-infected patients is up-regulated compared to NK from healthy controls, while miR-155 is down-regulated. The opposite regulation of TIM-3/T-bet and miR-155 was recapitulated *in vitro* by incubating primary NK cells or the NK92 cell line with Huh-7 hepatocytes expressing HCV. MiR-155 transfected in NK92 cells reduced TIM-3 and T-bet and enhanced IFN- γ expression. Authors suggest that TIM-3 expression is indirectly regulated by miR-155.

2.5. PD-1

PD-1 is an immune checkpoint receptor evolutionary related to the CD28 family. It recognizes

two cellular ligands, PD-L1 and PD-L2, and can be expressed on several cell types including T, B, NK, NKT and myeloid cells. Regarding NK cells, its expression seems to be confined to terminally differentiated NKG2A^{neg} KIR^{pos} CD57^{pos} NK cells, whose antitumor activity can be partially restored *in vitro* by antibodies disrupting the PD-1/PD-Ls interactions [19]. Timing in the expression of PD-1 is in line with the PD-1-dependent regulatory activity, which occurs in the later phases of the immune response [20]. To date, no miRNAs modulating PD-1 have been described in NK cells. Nevertheless, miR-4717, regulating only PD-1 variants containing a specific polymorphism in the 3'-UTR, has been described in total lymphocytes [21]. Moreover, miR-138 has been reported to modulate CTLA-4 and PD-1 expression in mouse and human CD4⁺ T cells [22], and miR-28 has been described to regulate PD-1 in mouse CD4⁺ T cells [23].

2.6. CTLA-4

CTLA-4 (CD152) is an immune checkpoint receptor binding the CD80 and CD86 ligands with higher affinity than CD28. It is expressed in Treg and activated T cells, while its expression in NK cells has been detected in mouse, but not in human [24]. CTLA-4 is targeted by miR-155, and in stimulated naive T_H cells transfection with the miR-155 precursor down-regulates CTLA-4 expression [25]. Moreover, as for PD-1, CTLA-4 is regulated by miR-138 in mouse and human CD4⁺ T cells [22].

2.7. CX₃CR1

CX₃CR1 is a chemokine receptor expressed by different immune cell types, including monocytes, dendritic cells (DCs), T, and NK lymphocytes, which binds CX₃CL1 (also known as fractalkine). CX₃CR1, with other chemokine receptors, drives, at steady state, NK cell localization in peripheral tissues, and promotes their migration under inflammatory conditions [26]. Moreover, it has been shown that CX₃CR1, CXCR4 and S1P5 regulate NK cells homing and migration from the bone marrow (BM) [27,28]. It has been demonstrated that the surface expression of CX₃CR1 was down-regulated in NK cells co-cultured with SH-SY5Y neuroblastoma (NB) cells under trans-well conditions. Notably, CX₃CR1 expression was reduced in NK cells from NB patients compared with healthy controls [9]. TGF-β1 released by NB cells has been shown to be responsible for the observed CX₃CR1 down-regulation, as also confirmed *in vitro* using recombinant TGF-β1. Looking for the mechanisms involved, TGF-β1 was found to determine a significant increase of miR-27a-5p, which targeted the CX₃CR1 mRNA in NK cells [29]. Up-regulation of miR-27a-5p was due to the TGF-β1-dependent up-regulation of miR-23a-27a-24-2 cluster, which encodes, among others, miR-27a-5p. As expected, inhibition of miR-27a-5p expression in NK cells caused an up-regulation of the CX₃CR1 mRNA. Conversely, a reduction of CX₃CR1 surface expression was observed in CX₃CR1-expressing HEK293T cells following their treatment with miR-27a-5p mimic. In agreement with the original findings [9], NK cells cultured in the presence of SH-SY5Y NB cells exhibited a significant up-regulation of miR-27a-5p and down-regulation of CX₃CR1 mRNA [29].

2.8. IL-2R_γ

IL-2R_γ is a transmembrane protein belonging to the cytokine receptor gene superfamily. It is the

γ component of multiple cytokine receptors, including those for IL-2, -4, -7, -9, -15, and -21, which are expressed on the surface of lymphocytes and other hematopoietic cells. Lack of IL2R γ due to mutations of the encoding gene is responsible for X-linked severe combined immunodeficiency (X-SCID), a cellular and humoral immunodeficiency with a near-complete absence of NK and T cells and nonfunctional B cells [30]. Yun et al. [31] reported that miR-583 targets IL-2R γ . Differentiating NK cells transfected with miR-583 mimics showed a decreased surface expression of IL-2R γ and defective differentiation. In particular, the miR-583 overexpressing NK cells showed decreased NKG2D, NKp30 and NKp46 expression and reduced capacity to kill target cells and to produce IFN- γ [31].

3. NK receptor ligands and miRNAs

A number of miRNAs have been identified as regulators of ligands of NK cell receptors. Targeted molecules include ligands for MHC class I-specific (HLA-G, HLA-C, HLA-E) and non-specific (MICA, MICB, ULBPs) receptors, as well as the immune checkpoint ligands PD-L1, B7-H3, CD80 and CD86. In cancer cells modification of miRNA expression may represent a common mechanism of escape from the NK-mediated immune surveillance (Figure 2).

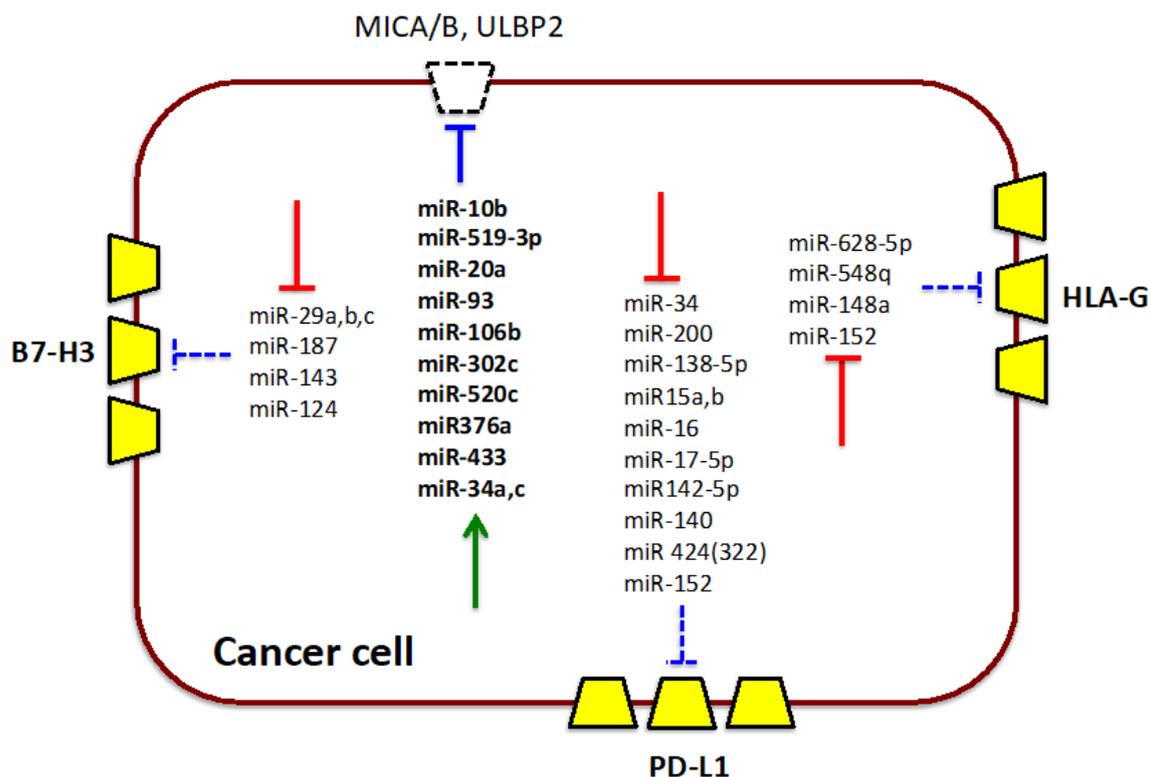


Figure 2. Altered ligands expression in tumor cells. Cancer cells can hamper NK cytotoxicity by up- or down-regulating a number of miRNAs, which reduce the expression of ligands of activating receptors or increase that of inhibitory ones, respectively. The latter includes the immune checkpoint ligands PD-L1 and B7-H3.

3.1. NKG2D ligands

NKG2D ligands MICA, MICB, and ULBPs can be regulated by several miRNAs, of cellular or viral origin [32] that are potentially able to protect cancer cells and virus infected cells from NK cell cytotoxicity [7,32,33]. Stern-Ginossar et al. [34] reported 7 miRNAs regulating MICA and MICB expression (miRNAs miR-17-5p, miR-20a-5p, miR-93-5p, miR106b-5p, miR-372, miR373-3p, miR-520d-3p). Overexpression of these miRNAs *in vitro* and *in vivo* resulted in down-regulation of MICA and MICB expression and in lower susceptibility to NKG2D-dependent killing by human NK cells. On the other hand, three different viral miRNAs (miR-UL112, miR-BART2.5p and miR-K12-7), all derived from herpesviridae, targeted MICB, while a miRNA from polyoma viruses JCV and BKV targeted ULBP3 [32,33]. Two miRNAs (miR-376a and miR-433) were shown to regulate MICB and to decrease NK-mediated cytotoxicity against colon carcinoma cells [35]. MiR-34a and miR-34c were reported to target ULBP2 and, when overexpressed in a melanoma cell line, down-regulated its expression at the protein level. Moreover, miR-34c transfected melanoma cells displayed reduced susceptibility to NK cells cytotoxicity [36]. Other miRNAs (miR-10b, miR-519-3p, miR-20a, miR-93, miR-106b, miR302c, miR-520c), which were found overexpressed in various tumors, have been shown to down-regulate MICA, MICB and ULBP2, and, consequently, to reduce the NK cell-mediated killing of target cells [37–42].

3.2. HLA-G

HLA-G is a non-classical HLA class Ib molecule, expressed mainly in the placental trophoblasts, where is thought to provide immune protection to the embryo from the decidual NK cells, the major lymphocyte population at the fetal-maternal interface. HLA-G is also expressed by tumors and virus-infected cells [43]. HLA-G is recognized by LILRB1 and KIR2DL4 inhibitory receptors on human NK cells [44] and its soluble form has been described to regulate the chemokine receptor repertoire [45]. Several miRNAs down-regulate HLA-G expression: miR-148a, miR-152 (belonging to the same miRNA family), miR-133a, miR-548q, miR-628-5p and miR-365 [44,46–48]. An inverse correlation was reported between miR-628-5p and HLA-G in primary renal cell carcinoma tumors and cell lines [49]. In the case of miR-148a, miR-152 and miR-548q, it has been experimentally shown that they induce in target cells HLA-G down-regulation and increased susceptibility to the cytolytic activity of LILRB1⁺ NK cells [44,47,49]. The long noncoding RNA HOTAIR has been shown to bind miR-148a in cervical cancer [50] and miR-152 in gastric cancer cells [51], thus up-regulating HLA-G. TGF- β 1 has been reported to cause a down-regulation of miR-152 inducing an up-regulation of HLA-G in gastric cancer cell lines [52].

3.3. HLA-C

HLA-C are classical HLA class I molecules recognized by inhibitory KIRs. In particular, KIR2DL1 binds C2 allotypes characterized by a lysine in position 80, whereas KIR2DL2 and KIR2DL3 recognize C1 allotypes sharing an asparagine in position 80 [53]. Interestingly, miR-148a is able to bind not only HLA-G, but also HLA-C. Kulkarni et al. [54] reported that a polymorphism in the HLA-C 3'-UTR regulates binding capability of miR-148a to its target site, resulting in relatively low HLA-C surface expression of alleles that bind this miRNA and high expression of HLA-C

alleles that escape post-transcriptional regulation.

3.4. *HLA-E*

The non-classical HLA-E binds a restricted subset of peptides encoded by the leader sequence of other class I molecules. It is recognized by the inhibitory CD94/NKG2A and activating CD94/NKG2C heterodimeric receptors, both expressed by T cells and NK cells [55]. The human cytomegalovirus [HCMV] encodes UL40, a peptide stabilizing HLA-E and driving the proliferation of CD94/NKG2C⁺ NK cells [56]. Interestingly, Nachmani et al. [57] showed that the short form of the RNA-editing enzyme ADAR1 induced editing of miR-376a in HCMV⁺ cells. Edited miR-376a down-regulated HLA-E expression rendering HCMV infected cells susceptible to elimination by NK CD94/NKG2A⁺ NK cells.

3.5. *PD-L1*

PD-L1 and PD-L2 are the two ligands of the PD-1 inhibitory receptor, which is expressed by activated NK and T cells [58,59]. Under physiological conditions, the PD-1/PD-Ls interactions control the duration and amplitude of immune responses, preventing autoimmune reactions. In cancer cells the expression of PD-Ls plays a crucial role in immune evasion. Several miRNAs regulating PD-L1 have been described, suggesting a tight control of its expression. In cancer cells PD-L1 expression is accompanied by miRNAs down-regulation [60–62]. MiR-513 has been the first miRNA reported to regulate PD-L1 and its inhibition in cholangiocytes induced PD-L1 protein expression [63]. In acute myeloid leukemia samples an inverse correlation was reported between miR-34 and PD-L1 expression and miR-34 was found to regulate PD-L1 expression in leukemia cell lines [64]. An inverse correlation was also reported between miR-200 and PD-L1 expression in lung adenocarcinoma samples. MiR-200 suppressed PD-L1 expression in murine and human mesenchymal lung cancer cell lines, while its down-regulation increased PD-L1 expression causing CD8⁺ T cells immune suppression [65]. MiR-138-5p was also found to target PD-L1. An inverse correlation was reported between miR-138-5p and PD-L1 expression in human colorectal cancer (CRC) samples and miR-138-5p overexpression suppressed CRC cell tumorigenicity [66]. Other miRNAs have been described as regulators of PD-L1 in various cancer cells, miR-15a, miR-15b and miR-16 in malignant pleural mesothelioma cell lines [67], miR-17-5p in metastatic melanoma [68], miR-142-5p in a pancreatic cancer cell line [69], miR-140 in non-small cell lung cancer (NSCLC) cell lines [70]. MiR-424(322) regulated PD-L1 and CD80 in ovarian cancer and high levels of this miRNA correlated with increased progression-free survival of patients [71]. Expression of miR-152 was low in gastric carcinoma compared to controls, and miR-152 mimics inhibited PD-L1 expression in gastric cancer cell lines with increased T cell proliferation and function [72]. MiR-324-5p and miR-338-5p targeted PD-L1 and dendritic cells expressing these miRNAs displayed reduced surface expression of PD-L1 [73].

3.6. *B7-H3*

B7-H3, a member of the B7 superfamily [74,75], is the orphan ligand of an inhibitory receptor expressed by human NK and CD8⁺ T cells [74,76]. B7-H3 is considered as a new member of the

immune checkpoint family [77]. Indeed, it has been shown to inhibit cytotoxicity and protect neuroblastoma (NB) (and other tumor cells) from NK cell-mediated lysis [74,76]. B7-H3 also promotes tumor invasiveness [78]. In line with these pro-tumoral properties, high B7-H3 expression correlates with poor prognosis in several tumor histotypes [78,79]. B7-H3 expression is regulated by several miRNAs. Xu et al. [80] reported that miR-29a, b, c are down-regulated in several B7-H3 expressing tumor tissues compared to normal tissues. MiR-29a targets B7-H3 in neuroblastoma cell lines [80] and down-regulation of miR-29a in central nervous system NB metastasis is associated with up-regulation of different molecules, including B7-H3 [81]. MiR-29c expression was inversely correlated to B7-H3 expression in melanoma [82] and was reported to target B7-H3 in breast cancer, where miR-29c expression correlated with increased survival [83]. Another miRNA targeting B7-H3 is represented by miR-187. MiR-187 was down-regulated in clear cell renal cell carcinoma [ccRCC] compared to normal tissues. Overexpression of miR-187 (as well as silencing of B7-H3) decreased cell migration in ccRCC cell lines [84]. Interestingly, in CRC cell lines, miR-143 was down-regulated by TGF- β 1, thus causing the up-regulation of both its targets B7-H3 and B7-H4 and leading to tumor immune evasion [85]. MiR-124 targeted B7-H3 in osteosarcoma [OS] cells and inhibited their growth and invasive ability. Moreover, miR-124 was down-regulated in OS clinical specimens, with an inverse correlation between miR-124 and B7-H3 protein expression [86].

3.7. CD80 and CD86

The expression of CD80 and CD86, CTLA-4 ligands, can be modulated by miRNAs. Besides miR-424(322) that regulates both PD-L1 and CD80 (see above) [71], miR-134 has been shown to bind CD86 mRNA suppressing its expression. Transfection of miR-134 in a melanoma cell line caused reduction of CD86 at the mRNA and protein level [87].

4. Conclusion

What clearly emerges from the recent literature on the regulation of NK receptors and ligands expression is a central role of miRNAs in the maintenance of a delicate equilibrium that can be heavily perturbed by tumor transformation or viral infection.

To escape NK cell immunosurveillance, tumor and virus infected cells evolved mechanisms to deregulate the expression of genes coding for molecules important for effector/target interactions [88]. These mechanisms often use the miRNA machinery. Pathological cells can affect NK-mediated recognition by up- or down-regulating miRNA targeting activating or inhibitory receptor ligands, respectively. Tumor cells can also act on NK cells by producing immunomodulatory factors, such as TGF- β 1 that, influencing the expression of different receptors via distinct miRNA-pathways, realizes a combined inhibitory action. Other mechanisms can add levels of complexity to the action of the miRNA machinery, as the long noncoding RNAs sequestering miRNAs or the polymorphisms in the 3'-UTR of miRNA target genes.

The link between miRNA derangement and unpaired NK immunosurveillance makes miRNAs potential therapeutic targets [89]. MiRNA mimics or anti-miRs can be used to increase or decrease the activity of miRNAs with tumor suppressive or oncogenic function. However, susceptibility to degradation in biological fluids and poor delivery to target sites might severely hamper the use of miRNAs in therapy. Chemical modifications of the miRNA backbone and the use of specific vehicles,

as nanoparticles or minicells, have been developed to confer stability to the miRNAs. Moreover, specific molecules, such as antibodies, can be conjugated to the miRNAs or to the vehicles to ensure an efficient and specific delivery. Avoiding toxicities and off-target effects are additional challenges for a miRNA-based therapeutic approach.

In spite of these difficulties, two different phase I clinical trials treating cancer patients have been performed using miRNAs as therapeutic agents. The former study (NCT01829971) used miR-34 mimics encapsulated in lipid nanoparticles, without the association with specific targeting molecules, to treat patients affected by several solid tumors and hematological malignancies. Unfortunately, it was terminated due to immune related serious adverse events. In the latter study (NCT02369198), miR-16 mimics encapsulated in minicells targeted to EGFR-positive cancer cells by an anti-EGFR antibody have been used to treat patients with malignant pleural mesothelioma and non-small cell lung cancer. The study was regularly completed, establishing a dose of miRNA mimic which was well tolerated and accompanied by early signs of antitumor activity [90].

Therefore, combination of targeted miRNA-based therapies with other therapeutic interventions represents a possible strategy to reduce tumor aggressiveness and to potentiate NK cell-mediated responses against cancer.

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Conflict of interest

All authors declare that they have no conflict of interest in this paper.

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