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REGULATION OF NK CELL ACTIVATION BY STIMULATORY AND INHIBITORY RECEPTORS IN TUMOR ESCAPE FROM INNATE IMMUNITY

Peng Wu [1], Haiming Wei [1], Cai Zhang [3], Jian Zhang [2], and Zhigang Tian [1, 2, 3]

[1] Institute of Immunology, School of Life Sciences, University of Science and Technology of China, Hefei, Anhui Province 230027, China; [2] Institute of Immunopharmacology and Immunotherapy, School of pharmaceutical Sciences, Shandong University, Jinan, Shandong 250021, China, [3] Institute of Basic Sciences, Shandong Academy of Medical Sciences, Jinan, Shandong 250062, China

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1. ABSTRACT

Recent years have witnessed our major progresses in understanding the membrane-bound receptors on NK cells. Although functional studies of ligands for these NK cell receptors provide good opportunities to study roles of NK cells in anti-tumor and anti-virus immunosurveillance, little was known about how these ligands expression were modulated under physiological and pathological conditions. Several recognition models have been proposed to explain such an issue, which include "missing-self", "induced-self" and "modified-self" recognition model. Here stimulatory immunoreceptor - mediated NK cell activation will be reviewed, and possible recognition mechanism by which NK cells operate during interaction with target cells will be discussed in details. Tumor escape from NK cell-mediated immunosurveillance will also be further addressed.

2. INTRODUCTION

NK cells constitute an important component of the innate immune system, providing surveillance against certain viruses, intracellular bacteria, and transformed cells (1-3). They regulate both innate and acquired immune responses by exerting cell-mediated cytotoxicity and by release of various cytokines (such as IFN-gamma, GM-CSF and TNF-beta) and chemokines (e.g., MIP-1 family and RANTES) (4, 5). In spite of their functional relevance in host defenses, NK cells remained mysterious for many years. However, this situation has now dramatically changed by the major progresses in understanding the membrane-bound receptors on NK cells (as demonstrated in table 1). As the "missing-self" recognition model guided, several classes of NK inhibitory receptors have been identified, which include the Ly49 receptors (6), the killer immunoglobulin-like receptors (KIRs) (7, 8), the leukocyte

Table 1. Functions and ligands of human NK cell receptors

Receptor	ligand(s)	Expression	Function
Natural cytotocixity receptors			
NKp46	Unknown (oligosaccharide moieties of glycoproteins and glycolipids?)	Resting and activated NK cells	Stimulatory
NKp 30	Unknown (oligosaccharide moieties of glycoproteins and glycolipids?)	Resting and activated NK cells	Stimulatory
NKp44	Unknown	Activated NK cells	Stimulatory
NKp 80	Unknown	Resting and activated NK cells/$CD3^+CD56^+$ T cells	Co-stimulatory
Killer immunoglobulin-like receptors (KIR)			
KIR2DL1 (p58.1)	HLA-Cw2, 4, 5, 6	NK, T cells	Inhibitroy
KIR2DL2 (p58.2)	HLA-Cw1, 3, 7, 8	NK, T cells	Inhibitroy
KIR3DL1 (p70)	HLA-Bw4	NK, T cells	Inhibitroy
KIR3DL3 (p140)	HLA-A3, -A11	NK, T cells	Inhibitroy
KIR2DS1 (p50.1)	HLA-Cw2,4,5,6	NK, T cells	Stimulatory
KIR2DS2 (p50.2)	HLA-Cw1,3,7,8	NK, T cells	Stimulatory
KIR2DS4 (p50.3)	Unknown	NK, T cells	Stimulatory
Immunoreceptors of NKG2 family			
CD94/NKG2A	HLA-E	NK, CTL	Inhibitroy
CD94/NKG2C	HLA-E	NK, CTL	Stimulatory
CD94/NKG2E	HLA-E	NK, CTL	Stimulatory
NKG2D	MICA, MICB, ULBPs	NK, gamma delta T, $CD8^+$ T cells	Stimulatory
CD2 receptor family			
2B4 (CD244)	CD48	All NK cells ,a $CD8^+$ T cell subset and monocytes	Co-stimulatory
NTB-A	Homophilic	All NK cells ,T cells and B cells	Co-stimulatory
DNAM-1 (CD226)	PVR (CD155), Nectin-2 (CD112)	All NK cells, T cells and monocytes	Co-stimulatory
CS1 (CRACC)	Homophilic	CTL, activated B cells, NK cells and mature DCs	Co-stimulatory

immunoglobulin-like receptors (LIRs) (9, 10) and the CD94/NKG2 receptors (11-13). These receptors recognize allelic determinants that are shared by groups of HLA-class I alleles or sense the overall expression of MHC class I molecules, respectively (14, 15). On the other hand, as K. Kärre indicated, stimulatory receptors were not only possible but also actually required within the framework of missing-self recognition (16). The search of such receptors led to the identification and molecular characterization of NCRs, which include NKp30, NKp44 and NKp46, and NKG2D. Meanwhile several co-stimulating receptors such as 2B4, NTB-A and NKp80 were also identified (15, 17).

Now, there is a common view that NK cell effector functions are regulated by integrated signals across the array of stimulatory and inhibitory receptors engaged upon interaction with target cell surface ligands (18). Although functional studies of ligands for some of these NK cell receptors provide good opportunities to study how NK cells discriminate between normal cells and cells in danger, little was known about how their ligands expression were modulated under physiological and pathological conditions. On one hand, down regulation of ligands for inhibitory NK receptors leads to decline of inhibitory effect on NK cells. As "missing-self" recognition model predicted (16), tumor cells losing or altering MHC class I antigen expression obviously escape detection by cytotoxic $CD8^+$ T cells, but they are at a greater risk of elimination by NK cells (19). Even nontransformed cells that lack MHC class I molecules as a result of gene targeting events are sensitive to destruction by NK cells (20). Actually, on the other hand, the optimal activation of NK cells requires the "on" signal that is mediated through stimulatory receptors, which in turn demands the expression of the ligands for NK stimulatory receptors on target cells. It will be of great interest to investigate the expression pattern of ligands for stimulatory receptor of NK cells and its possible application in medical setting. Disappointedly, unidentified NCR ligands still made modulation of NK cells cytotoxicity function a mystery and until now, most of our knowledge about NK stimulatory receptor came from researches concentrated on one of them – NKG2D.

Here, possible recognition mechanisms by which NK cell operated during recognition of its targets *via* NKG2D and potential NCRs-mediated stimulating signals will be reviewed. Additionally, we will discuss the possible underlying mechanisms of tumors escaping from NK stimulatory receptor-mediated natural cytotoxicity.

3. STIMULATORY IMMUNORECEPTORS-MEDIATED NK CELL ACTIVATION

3.1. "Stress inducible" NKG2D ligands-mediated NK cell activation

NKG2D is a stimulatory receptor, which belongs to NKG2 family, expressed as disulphide-linked homodimer on most NK cells, $CD8^+$ alpha beta T cells and gamma delta T cells. Several ligands for NKG2D have been identified including the MHC class I like molecules MICA and its close relative MICB, ULBPs and the recently

described RAET1 (also termed ULBP4) in human, Rae1alpha-Rae1epsilon, minor histocompatibilty antigen H60 and Mouse UL16-binding-protein-like transcript 1 (Mult1) in mouse (21). All of them contain the alpha1 and alpha2 MHC class I domains called minimum class I folding unit, which consist of a beta sheet platform surrounded by a pair of alpha helices (22). However, none associate with beta2-microglobulin and, with the exception of human MICA and MICB, most of the corresponding genes are in a gene cluster separate from the MHC (23). Each ligand is likely distinct in terms of the tissue expression pattern, level of expression, and thermodynamics of receptor binding, which may ultimately govern the effort response (24).

Upon interaction with ligands on tumor cells, NKG2D-induced signals markedly enhances the sensitivity of tumor cells to NK cells *in vitro* and immune destruction *in vivo*, which can be inhibited by blocking with an anti-NKG2D antibody or antibodies directed to its ligands (25-28). Depending on the levels of NKG2D ligands expressed on target cell surface, killing activity induced by NKG2D pathway can overwhelm the inhibitory signals delivered by the inhibitory receptors with its MHC class I ligands *in vitro* (25, 29, 30). Target cells (with normal MHC class I expression) that are resistant to NK cell attack can become sensitive by transferring NKG2D ligands (25, 31). Cross-linking of NKG2D receptor on NK cells leads to mobilization of Ca^{2+} (32), production of cytokines such as IFN-gamma (29, 30, 32-34), GM-CSF (31, 34), TNF-alpha (33), TNF-beta (31) as well as chemokines like macrophage inflammatory protein-1 (MIP-1) (33,34). All these experimental data indicated that NKG2D could serve as an important immunoreceptor in the recognition of target cell by NK cells.

Investigation reveals that expression modulation of these various NKG2D ligands are different from each other although all ligands expression are absent or present at low levels in normal cells and can be up-regulated in pathological conditions (27, 35, 36). Significantly, the heat shock elements identified in *MICA* promoter (37) shed lights on modulation of ligands expression for NK cell activating receptor. MICA protein was considered as stress-inducible as reports showing that its expression could be up-regulated after being treated with heat shock and other cellular stress such as virus and bacterial infection with Herpes virus, M*ycobacterium* and E*scherichia coil* (28, 38, 39). In addition, up-regulated MICA expression in various tumor cells (38,40) indicated that MICA can function as signals of cellular stress and trigger a range of immune effector mechanisms, or in other words, NK cell can sense and kill cells that suffered from cellular stress *via* NKG2D-mediated activating signals.

According to all the data above, a novel recognition model mediated through NKG2D called 'induced-self' model has been proposed and could be viewed as a significant complement to the 'missing-self' hypothesis (23, 41). Disappointedly, other ligands for NKG2D such as ULBP1, 2, and 3 in human, and Rae1 and H60 in mouse do not have heat shock element in their promoters and their expression could not be up-regulated after heat shock treatment (21). Therefore, the signaling events that are responsible for the up-regulation of these ligands expression by tumour cells are not known. Although MIC molecules have limited expression pattern by epithelial and vascular endothelial cells, the constitutive expression pattern of ULBP molecules by a broader array of tissues at the mRNA level made such a recognition model more confusing (31). It has been observed that levels of mRNA expression for certain NKG2D-ligands often do not correlate with the amounts of protein expressed on the cell surface demonstrating that NKG2D-ligands expression is not only regulated transcriptionally, but also post-transcriptionally at cell surface (42, 43). Meanwhile, limited information is available about how MIC expression is regulated, particularly in specific tumors. It seems possible that in tumors it is the transformation process itself that induces molecules such as the NKG2D ligands so that the genomic upheaval of tumorigenesis is directly translated into enhanced immune recognition (44). A universal question also comes when so many NKG2D ligands have been identified: are these ligands redundant in NKG2D-mediated stimulating signals or they play different roles in different tissues or under different conditions? Cerwenka A. *et al.* proposed that it is possible that nature has created NKG2D-ligands diversity and polymorphisms not in redundancy, but rather as a strategy to foil viral diseases (22). On the contrary, Michael Gleimer proposed that the cellular immune system could potentially benefit from this diversity by being able to differentiate between the types and degrees of stress that a cell is experiencing (24). However, when we mentioned NKG2D ligands up-regulation, we speak of stress-inducible with little ideas about how it exactly happened.

3.2. Oligosaccharide moieties of glycoproteins and glycolipids as possible ligands for NCRs

The natural cytotoxicity receptors (NCRs), including NKp46, NKp30 and NKp44, are also important stimulatory receptors. NCRs play crucial roles in the NK-mediated recognition and killing of most target cells (17, 45, 46). The lysis of the majority of tumour cells by NK cells is mediated through the NCRs and the surface density of NCRs that is expressed by NK cells of a given individual strictly correlates with their cytotoxicity activity (47). However, how NCRs modulate NK cell cytotoxicity function is still unknown because ligands for NCRs have not been identified. Recently, putative cellular ligands for NCRs have been discovered showing that NKp30 and NKp46 proteins recognize target cell surface heparan sulfate proteoglycans (HSPGs) as cellular ligands and that 6-O-sulfation and N-acetylation state of the glucose building unit affect this recognition and lysis by NK cells. Whether the ligands are particular HSPGs, unusual heparan sulfate epitopes, or a complex of HSPGs and either other protein or lipid moieties remains to be further explored (48).

How are structures like carbohydrates involved in recognition of target cells by NK cells? Similar examples illustrating involvement of oligosaccharide structure in modulation of natural killer cytotoxicity were also

identified. For example, a diversity of high-affinity oligosaccharide ligands was identified for NKR-P1, a membrane protein on NK cells (49). In mice it was found that soluble sulfated mono- and polysaccharides interfere with the interaction of Ly-49 molecules, a family of regulatory receptors expressed on murine NK cells, to murine class I ligands (50). It is possible that the oligosaccharides on class I molecules are sulfated and participate in Ly-49A binding. Chikara Ohyama *et al*. showed that the CD94 receptor was solely responsible for the recognition of sialyl Lewis x over-expressed on tumor cells (51). The author also showed that moderate amounts of sialyl Lewis x lead to tumor metastasis, whereas expression of high levels of sialyl Lewis x leads to an NK cell attack on tumor cells, demonstrating that expression of different amounts of sialyl Lewis x results in entirely different biological consequences. Therefore, it seems feasible to believe that oligosaccharides could serve as ligands for NK cell receptors. In fact, in mammalian cells, a large number of secretory proteins, whether membrane bound or soluble, are asparagine (N)-glycosylated and aberrations in cell surface carbohydrates are often associated with malignant transformation and other pathological conditions (52-54). The carbohydrate epitopes, resulting from either incomplete synthesis or neosynthesis, accumulate in high density, possibly in a novel conformation, at the tumor cell surface (55). Based on these observations, carbohydrate vaccines are being developed for the active immunotherapy of cancer. Potential targets include members of the ABH Lewis blood group family, members of the mucin core family (e.g., Tn, sialyl Tn and T-F) and gangliosides (e.g., GM2) (56). Meanwhile, the oligosaccharide structures on target cell may also influence the binding of NK cell to its target, thus in turn influence sensitivity to NK lytic activity (57-59). Study performed by Patricia B. Ahrens showed that the presence of high mannose-type glycans on K-562 cells correlates with increased binding of effectors and a greater susceptibility to lysis and further conformed the theory hypothesized that NK cells discriminate targets on the basis of the glycoproteins present on target cell surface (60).

However, the contribution of carbohydrates as ligands for NCRs in recognition of target cells by NK cells cannot be fully assessed until more information is available concerning the cellular ligands for NCRs to be identified and pathways that signal specific oligosaccharide structure changes in cancer cells. Moretta *et al*. discussed previously that the cellular ligands for NCRs appear to be expressed on both normal and tumor cells whose expression could be modified by cellular stress, cell activation or tumour transformation (45). Aberrant oligosaccharide moieties of glycoproteins and glycolipids may satisfy such a requirement as ligands for NK receptors. It is feasible to believe that aberrant glycosylation may serve as hallmarks of cancer cells that could be used by NK cells to distinguish cancer cells from the healthy ones. Evidences showed that expression of aberrant oligosaccharide moieties of glycoproteins and glycolipids is a typical characteristic of essentially all animal and human tumors, irrespective of the carcinogenic mechanism. It has been observed long before that NK cells discriminate between targets on the basis of the glycoproteins present on the target cell surface (57-59). Yet, the exact identity of these target cell recognition sites remains obscure.

3.3. Heat shock protein recognized as “modified-self” by NK cells

Another recognition model of NK cell that proposed by Eric O. Long: the “modified-self” model was exemplified by CD94/NKG2 receptors (61). Human HLA-E and its murine functional counterpart Qa-1 bind to nonameric peptides derived from the signal sequence of other class ？molecules and was expressed as membrane-bound ligands for both CD94/NKG2A and CD94/NKG2C, which in turn inhibit or activate NK cells respectively (62). However, the amino acid sequence of the peptide in HLA-E and Qa-1 is known to affect binding by CD94/NKG2A (63, 64). Michaëlsson *et al*. showed that HLA-E loaded with a peptide from the signal sequence of stress protein HSP60 does not bind to CD94/NKG2A or CD94/NKG2C thus prevents recognition by inhibitory CD94/NKG2A (65). Enhanced surface expression of HLA-E was also observed in stressed cells and did not result in increased protection from NK cells. In mice, in the absence of MHC class I leader peptides, Qa-1 can be stabilized on the cell surface by heat shock treatment (66). Therefore, it seems that stress-induced modification of HLA-E would activate NK cells due to loss of inhibition.

In agreement with such a discovery, observations in animal tumor models have demonstrated the importance of molecule chaperones like HSPs in tumor recognition and subsequent tumor regression (67). Besides the ability to carry tumor antigens and present to professional APCs to generate protective immunity against a series of tumor challenge (68), it was discovered that HSPs were unusually expressed on plasma membrane of tumor cells (69-71) that could be recognized by NK cells as a tumor-selective recognition structure (72). Interaction of HSP70 itself with receptor on NK cells results in activation of cytolytic and proliferative function. It is intriguing that the receptor binds to membrane-bound HSP70 is neither CD91, which has been described as a common receptor for HSP90s and HSP70s on antigen-presenting cells, nor lipopolysaccharide receptor CD14 who acts as a coreceptor for HSP70-mediated signaling in human monocytes, since both of them are not expressed on NK cells (72).

3.4. “self” or “non-self”: how to be recognized by NK cells?

Unlike T cells, NK cells killing of virus-infected or malignant transformed cells do not need pre-sensitization and are independent of a MHC restricted manner. However, quiescent, circulating NK cells do not kill autologous cells under normal conditions and do not lyse all tumor targets with equal efficacy. Disappointingly, the mechanisms that regulate NK cell function as a first line of defense against infection and transformation have exceeded our expectations in terms of their sophistication and complexity (61). Now, there is a common view that activation of NK cells results from the integration of multiple positive and negative signals mediated through the array of stimulatory and inhibitory receptors engaged upon

interaction with target cell surface ligands (18). Although recent identification and molecule characterization of surface receptors of NK cells shed new light on how NK cell functions, there are still many unresolved questions about the expression modulation of ligands for NK cell receptors. Several recognition models have been proposed to explain such an issue, which include "missing-self" recognition model, "induced-self" recognition model and "modified-self" model. These recognition models addressed are surely helpful, but still made us confused as one might possibly speculate that the recognition strategies used by NK cells are diverse (23). However, the fact that NK cells, under certain circumstances, destroy normal autologous cells suggests that target ligands for triggering receptors are not necessary anything foreign and they could be expressed or redistributed after normal cells infected by viruses or undergoing transformation (73). It means that a universal recognition mechanism must be existed to regulate natural killing activity. Such a recognition mechanism will have no more help to illustrate exactly how target cells are recognized and killed, but will possible clue us to find and define the "headstream" of molecule changes cell makes after transitions from a normal cell to a danger one.

From unicellular prokaryotes to multicellular eukaryotes, cells experience relentless bombardment from environmental stress stimuli of all flavors-physical, chemical, and biological (24) which could be named as cellular stress. Cellular stress here may include extremes of temperature, high levels of radiation, toxic conditions that lead to the accumulation of non-native proteins and denaturation of essential cellular proteins, exposure to heavy metals, transformation, and virus infection. It has been observed that cells that suffered from cellular stress could make some kinds of responses to infectious pathogens or environmental assaults that lead to changes in gene expression pattern, cell metabolism, organization of cytoskeleton and redistribution of membrane-bound ligands. One of them is the pathway that leads to a rapid synthesis of a suit of proteins referred to as "stress proteins" or "heat shock proteins" which could be viewed as a major indicator of cellular stress. As addressed before, HSPs could play vital roles in tumor recognition and might be recognized by NK cells directly or as a complex with signal peptides binding to HLA-E. In addition, MICA protein could co-express with HSPs in cells suffered from cellular stress as the promoter of *MICA* has heat shock element in it (37). It is also possible that cellular stress also influence cell metabolism that may alter oligosaccharide moieties of glycoproteins and glycolipids, which in turn may serve as ligands for NCRs. Moreover, the repertoire of peptides presented by classical MHC class I molecules could be changed in cells suffering cellular stress (24). Although not being clearly defined, different MHC class I-peptide complex may bind the same NK receptor with different affinity thus in turn alter the balance between activating signals in NK cells after engagement of stimulatory receptors and the inhibitory signals induced by engagement of inhibitory receptors for MHC class I molecules. In conclusions, although ligands for different NK receptors have different molecule nature, expression of all these ligands may possible be modulated by cellular stress.

In such a notion, changes that sensed by NK cells as danger signals occurred as positive reaction made by cells that suffered from cellular stress in contrast to the viewpoint that changes detected by NK cells can be viewed as consequences of viruse infection and cellular transformation. The acquirement of such ligands on cells in danger can be a sign of cell response to infectious pathogens and transformation that alarm the danger bells to immunity and lead to the clearance of them from normal body. In the past, it was argued that cellular transformation did not provide sufficient proinflammatory or danger signal to alert the immune system to the presence of a developing tumor. Recently, Gavin P. Dunn *et al*. concluded that danger signals, such as uric acid, may arise from the inherent biology of the tumor itself which will verify the idea that cells in danger could response positively to cellular stress (44). Therefore, in such a notion, roles NK cell plays in innate immunity may not be restricted in anti-tumor and anti-virus immunity, because any cell that suffered from cellular stress and could not be repaired would be recognized and cleared by NK cells. This notion reinforced the commonly accepted idea that NK cells constitute an important component of the innate immune system.

3.5. Questions need to be illustrated

Absolutely, until now, not a single hypothesis could explain how NK cell functions in immunosurveillance. Several questions still need to be illustrated here in detail.

First, is the diversity of NKG2D ligands. Several possible explanations have been proposed as discussed before (22, 24). It is possible that different signal pathways modulate diverse NKG2D-ligands expression when cells suffer from cellular stress, which would occur in different tissues or under different circumstances. However, the possibility that NKG2D-ligands diversity occurring as strategies to counteract virus infection also could not be excluded. Another possibility also exists that different soluble NKG2D ligands have different roles in modulating cell surface NKG2D expression. It has been observed that tumor shedding of MICA systemically impairs the responses of $CD8^+$ alpha beta T cells and presumably NK cells (detailed discussed below). In addition, one human ULBP family member was shown to be secreted from transferred CHO cells, implying that tumor cells might secret additional soluble NKG2D-ligands (22, 74). It have been clearly defined that the endocytosed growth factor receptor tyrosine kinases (RTKs), such as EGFR/ErbB1, undergo a sorting process, which determines receptor fate and signal intensity (75, 76). The receptors can be targeted to the lysosome for degradation, which terminates receptor signals. Alternatively, the internalized receptors can be recycled back to the cell surface for continued ligand binding and signaling (76–80). The relative efficiency of lysosomal sorting versus recycling is a key determinant of the signaling potency of RTKs (81). In contrast, the clear process of soluble NKG2D-ligands-induced NKG2D

internalization was unknown. We supposed that different soluble NKG2D-ligands function distinctively in such process, which will further determine the fate of NKG2D-mediated stimulatory signal upon natural killing cytotoxicity.

Another question also comes when pathogen genome encoded molecules were identified as stimulatory NK receptor ligands. Ly49H, a member of the Ly49 family, is a stimulatory receptor expressed by approximately half of NK cells in certain mouse strains. It binds to a product of m157 of mouse cytomegalovirus (MCMV) blockade of which prevents early control of MCMV infections (23, 82-84). In human, the haemagglutinin of influenza virus and the haemagglutinin neuraminidase of parainfluenza virus were identified as putative ligands for NKp44 and NKp46 receptors (85). According to these data, one might speculate that NK cells may directly recognize pathogen genome encoded molecules through membrane-bound receptors. Although we can not exclude such a possibility, it is also possible that such ligands expressed could be a strategy virus used to fool the immunity despite results showing that Ly49H functions as a pathogen-specific receptor that enables NK cells to limit early-stage MCMV infections and to undergo considerable proliferation (23). The roles of pathogen genome encoded molecules play in the process of modulation of natural killing activity must be further researched and re-valuated in details.

4. ESCAPE OF NK CELL-MEDIATED IMMUNOSURVEILLANCE

The expression of ligands for stimulatory receptors may stand for an effective barrier to formation and growth of tumor cells *in vivo.* However, tumors have developed different strategies to escape.

The presence of MIC on many progressive tumors (including breast, lung, gastric, renal, colon, ovarian, prostate carcinomas, and melanomas) suggested that MIC or NKG2D might be functionally impaired, thereby promoting immune evasion. Elegant studies accomplished by Veronika Groh *et al.* recently demonstrated that NKG2D expression was down-regulated on T cells and NK cells in some tumor patients, as compared to tumor-free individuals (86). Certain tumor cells released a soluble form of MICA by proteolytic cleavage with metalloproteases, which in turn impaired cell surface expression of functional NKG2D receptors (22, 87, 88). Therefore, soluble MIC molecules released by tumor cells could be taken as a novel tumor-evasion strategy. Recently, we also found that recombinant soluble MICA can down-regulate the expression of NKG2D on NK cell line (NK92) and freshly isolated NK cells and therefore decreased the cytotoxicity of NK cells to $MICA^+$ tumors (our unpublished data). Jennifer D. Wu demonstrated recently that a significant correlation of MIC shedding and deficiency in NK cell function with the grade of disease in prostate cancer (89). They found that, although MIC expression was prevalent in prostate carcinoma, membrane-bound MIC was predominant only in low-grade cancers and significant serum levels of soluble MIC were detected in higher-grade cancers, indicating that prostate tumors counteract MIC-stimulated, NKG2D-mediated immunity *via* MIC shedding. Importantly, serum levels of soluble MIC and a loss of NKG2D-mediated NK cell function significantly correlated with the degree of disease in prostate cancer patients. They summarized that two possible negative effects may result from MIC shedding. One effect was the loss of predominant surface localization of MIC in high-grade prostate carcinomas. The second was soluble MIC-induced impairment of NK cell surface NKG2D expression (89).

In mice, some experiments also revealed that interaction of RAE-1 and NKG2D on NK cells induced dysfunction of activated NK cells (90). NKG2D on NK cells was modulated by the interaction with RAE-1 *in vivo* in the local microenvironment. Initial interactions between NK cells and NKG2D ligand-bearing cells may trigger killing and cytokine production. However, prolonged exposure to ligands may desensitize NK cells and render them functionally anergic (90). This has implications in the context of tumor development because secretion of soluble NKG2D ligands by tumors or prolonged exposure of NK cells to ligand-bearing tumors may render them dysfunctional. This may contribute to tumor escape from NK cell and $CD8^+$ T cell-mediated immune surveillance.

Notably, Ekaterina S. Doubrovina reported that internalization of receptor *in vivo* and i*n vitro* is not limited to NKG2D but was also observed for the chemokine receptors CXCR1 and CCR7 (91). NK cells from colorectal cancer patients with elevated serum levels of soluble MIC are lack expression of activating NKG2D and chemokine CXCR1 receptors, both of which are internalized. Furthermore, natural cytotoxicity receptor NKp44 and chemokine receptor CCR7 are also down modulated in IL-2-activated NK cells co-cultured in MIC-containing serum - an effect secondary to the down-modulation of NKG2D and not directly caused by physical association with soluble MIC. These results suggested that soluble MIC-mediated down modulation of the triggering receptor NKG2D is rapid and precedes down-modulation of chemokine receptors. The authors proposed that the causal relationship between soluble ligand-mediated down-modulation of NKG2D and other receptors may reflect NKG2D-mediated general inactivation of the NK cells which was confirmed by the similar findings of activating receptor down modulation in NK cells from patients with acute leukemia (91). Recently, we also found that after incubation NK92 or freshly isolated NK cells with recombinant soluble MICA for 24hs, down-regulated expression of NKG2D, NKG2A/B and KIR2DL1 and decrease of the cytotoxicity of NK cells to $MICA^+$ tumors were observed (Our unpublished data). All the data suggested that multiple and complex mechanisms were involved in soluble MICA induced NK receptor internalization, which remain to be explored. Further investigation and clear definition of mechanisms involved may provide new methods to restore MIC-NKG2D–mediated immunity and have clinical significance in cell-based adaptive immunotherapy for cancer treatment.

Although some researches have indicated that cytokines such as IL-2, IL-15, IFN-alpha could up-regulate

the expression of NKG2D (29, 32, 34, 92), mechanisms that control the expression of NKG2D receptor were still illusive and the possible regulatory factors needed to be clearly defined in details. In contrast, some tumors secreted immunosuppressive cytokines, such as TGF-beta or IL-10, which could impair the expression of NK cell receptors and down-regulate NK cell or T cell effector function (93). TGF-beta and IL-10 can enhance the expression of CD94/NKG2A on T cells and NK cells. Recently, Castriconi R *et al*. found that TGF-beta1 could down-regulate the expression of NKG2D and NKp30 on freshly isolated NK cells from peripheral blood and cultured NK clones (94). Therefore, tumor itself would provide signals required for inhibitory receptor expression while inhibit the expression of stimulatory receptors by tumor-specific CTL and NK cells, thus result in imbalance of NK cell receptors, suggesting the existence of another mechanism of tumor escape from NK and CTL-mediated control. Recently, we found that in addition to the up-regulating effect of classical and non-classical MHC molecules on tumor cells, IFN-gamma could down-regulate the expression of stimulatory receptor NKG2D and up-regulate the expression of inhibitory receptors NKG2A/B and KIR2DL1 on NK cells and then down-regulate NK lysis (95). Our results are supported by Malmberg *et al*. who also found that IFN−gamma could protect short-term ovaian carcinoma cell lines from CTL lysis through a CD94/NKG2A-dependent manner (96). The negative effect of IFN-gamma on NK cells may reflect the self-regulatory effect of NK cells, which prevents NK cells from over-activation. Ross ME and his colleagues found that after co-stimulation of human NK cells with IL-2 and IL-12, IL-12 induces human NK cell IFN-gamma production, followed by NK cell apoptosis and a decline in IFN-gamma production (97). Therefore, cytokines that activate innate immune response may also serve to limit it *via* apoptosis. Based on the results above-mentioned, we postulate that IFN-alpha and IFN-gamma exert opposing effect on modulation of NK cell function, especially on NKG2 expression. The finding that IFN-gamma down-regulates the function of NK cells might partly explain why the use of IFN-gamma has not been more successful than IFN-alpha/beta in the clinical setting and therefore may have important biological implications (98-101).

5. CONCLUSIONS AND PERSPECTIVES

Recent years have witnessed our major progress in understanding NK cell receptors and ligands they recognize. However, the mechanisms that regulate NK cell function as a first line of defense against infection and transformation remains to be determined. Regulation of NK cell lysis is still illusive, especially about expression modulation of ligands for stimulatory NK receptors. More and more results suggested that cellular stress induced structure changes, which include gene expression alteration, modification and re-distribution of membrane-bound proteins, could serve as ligands for NK stimulatory receptors. The acquirement of such ligands on cells in danger could be a sign of cell responses to infectious pathogens and transformation that alarm the danger bells to immunity and lead to the clearance of them from normal body. In such a notion, changes that sensed by NK cells as danger signals occurred as positive reaction made by cells that suffered from cellular stress in contrast to the viewpoint that changes detected by NK cells can be viewed as consequences of viruses infection and cellular transformation to escape the immunosurveillance. Although such an idea is still questionable and the signal pathways that involved are still illusive and need to be illustrated, it would help to understand and characterize the molecule events that define a “non-self” cell.

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Abbreviations: NK cell: natural killer cell, KIRs: killer immunoglobulin-like receptors, NCRs: natural cytotocixity receptors, HSPs: heat shock proteins, MCMV: mouse cytomegalovirus, HSPGs: surface heparan sulfate proteoglycans, MICA: MHC class I chain-related protein A, APCs: antigen presentation cells, ULBPs: UL16-binding proteins, Mult1: Mouse UL16-binding-protein-like transcript 1

Key Words: NK cells, stimulatory immunoreceptors, cellular stress, tumor, NKG2D, NCRs, heat shock protein, immunosurveillance, review

Send correspondence to: Dr. Zhigang Tian, Institute of Immunology, School of Life Sciences, University of Science and Technology of China, Hefei 230027, Anhui Province, PR China, Tel: (086)-551-360-7379, Fax: (086)-551-360-6783, E-mail: tzg@ustc.edu.cn

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