NKG2D and its ligands: active factors in the outcome of solid organ transplantation?

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The role of natural killer (NK) cells in solid organ transplantation is not well established, although several recent reports highlight the importance of the activating receptor NKG2D and its ligands in the development of rejection during transplantation. The human NKG2D ligands (MICA and MICB) are induced in allografts during acute and chronic rejection, and the presence of anti-MICA antibodies is correlated with a higher incidence of rejection. The binding of these ligands to its receptor NKG2D activates NK cells, enhances the functions of effectors, and allows NK cells to function as a bridge between innate and adaptive immunity associated with the transplantation. In fact, blockage of NKG2D with the anti-NKG2D monoclonal antibodies prolongs graft survival and prevents CD28-independent rejection in heart and skin allograft mouse models. Furthermore, the current immunosuppressive therapies can modulate the expression of NK cell receptors and consequently the effector functions of NK cells. That is particularly important during the first few months after transplantation, when the susceptibility to opportunistic viral infections is higher and NKG2D has an essential role. In this review, we analyze in detail the potential role of the NKG2D-activating receptor and its ligands in the immune responses during the outcome of solid organ transplantation. These findings open a new pathway for therapeutic intervention that can contribute to tolerance in solid organ transplantation.

Kidney International Supplements (2011) **1,** 52–57; doi:10.1038/kisup.2011.13

KEYWORDS: MICA; NKG2D; natural killer cells; rejection; solid organ transplantation

TO CITE THIS ARTICLE:
Suárez-Álvarez B, Fernández-Sánchez A, López-Vázquez A *et al*. NKG2D and its ligands: active factors in the outcome of solid organ transplantation? *Kidney Int Sup* 2011; **1**: 52–57.

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Natural killer (NK) cells, described by several groups in the early 1970s, are important components of the innate immune system and can kill target cells without a requirement for a prior exposure to these targets, in contrast to cytolytic T cells.[1,2] These cells mediate their effector functions through a complex integration of inhibitory and activating receptors that interact with different ligands expressed on host cells. Activated NK cells produce cytolytic molecules (perforin and granzymes) that can directly kill allogeneic cells and proinflammatory cytokines (tumour necrosis factor-α/β, interferon (IFN)-γ) that activate the adaptive immune responses involved in the rejection.

The main inhibitory receptors, such as the killer Ig-like receptors, leukocyte Ig-like inhibitory receptors, and the C-type lectin receptor superfamily (CD94/NKG2A), recognize self-MHC class I molecules that are expressed on healthy cells and protect them from NK-induced lysis. In contrast, the activating receptors bind mainly to stress-induced molecules, or molecules found in pathological situations, and lead to killing of target cells. The major activating NK receptors in humans include the NKG2D, CD16, and the natural cytotoxic receptors (NKp46, NKp44, and NKp30). A great number of excellent reviews had been published recently that describe the structure and function of NK-related receptors.[3]

The ability of NK cells to recognize and kill allogeneic cells without prior sensitization is explained by the concept of 'missing self'.[4] Inhibitory receptors expressed on NK cells interact with self-MHC class I molecules, deliver an inhibitory signal, and lead to inhibition of NK activity. Thus, target cells that lost major histocompatibility complex (MHC) class I expression by virus infection or malignant cell transformation become sensitive to NK cell destruction. However, it is now known that the activation of NK cells requires more than the absence of inhibitory signals, and target cells have to express specific ligands for activating receptors. The NKG2D-activating receptor recognizes several ligands that are induced in response to cellular damage, activate NK cell functions, and lead to the lysis of target cells. In comparison with the missing self-hypothesis, this process was called 'induced or stressed' self-hypothesis.

During development, NK cells experience a process of functional maturation referred to as 'NK-cell education' that involves the interaction with self-MHC class I molecules via inhibitory receptors. Two models have been proposed to explain this process. The 'licensing' hypothesis postulates that the engagement of MHC class I to inhibitory receptors on NK cells during development allows these cells to become competent effector cells.[5] Although the 'disarming' model suggests that the absence of MHC class I molecules on normal cells would chronically stimulate NK cells and render them anergic, recent studies demonstrated that the educational process of NK cells is regulated by the number and the affinity of each inhibitory receptor for self-MHC class I molecules.

During bone marrow transplantation, NK cells have a major role in the rejection of donor bone marrow cells, but the impact of donor and recipient NK cells in solid organ allografts is a controversial point even today. First, studies showed that heart transplants in $Rag^{-/-}$ mice, which are deficient in T and B cells, but maintain an intact NK cell population, are accepted indefinitely and survive throughout the length of the experiment, suggesting that NK cells are not sufficient to reject a heart allograft. However, recent studies highlight the ability of NK cells to promote graft injury or rejection.[6–9] The cellular microenvironment and the interactions with other cells (dendritic cells (DCs), T and B cells) may modulate the immune responses developed against the donor and may contribute to these ambiguous effects of NK cells. Furthermore, the functions of NK cells are determined ultimately by integrated signals obtained from both inhibitory and activating receptors, which are expressed in a stochastic pattern. The inhibitory and activating receptor profiles on NK cells aid in identifying the many subsets of NK cells with different functional properties. Taken together, NK cells may have some functional plasticity during transplantation, so that they can contribute to promoting rejection or inducing tolerance on the basis of the surrounding milieu.

As we have previously reported, NKG2D is one of the most potent activating receptors expressed on NK cells, and induces a potent cytotoxic response in humans through its association with the adaptor molecule DAP10 (ref. 10). The interaction of NKG2D with its ligands has been involved in multiple processes and has an important role in infections, tumors, and autoimmune diseases. Recently, several studies reported the contribution of NKG2D and its ligands in the solid organ transplantation, and suggest a great potential for therapeutic intervention of this interaction.

NKG2D LIGANDS IN SOLID ORGAN TRANSPLANTATION

The ligands of NKG2D in humans are MHC class I chain-related A and B (MICA, MICB), and ULBP 1–5 (UL-16-binding protein).[11] Mice lack *MIC* genes but express the ULBP homologous proteins, namely RAE-1, H60, and MULT-1. *MICA/B* genes are encoded in the MHC region and share 28–35% homology to human leukocyte antigen class I genes. The *MICA/B* genes are highly polymorphic, with more than 72 *MICA* and 31 *MICB* recognizable alleles (http://www.ebi.ac.uk/imgt/hla).

The main findings have focused on the presence of anti-MICA antibodies in patients with a kidney or heart transplant and their direct involvement in the failure of the allograft. The development of anti-MICA antibodies after kidney transplantation is a significant risk factor in the outcome of the graft, and is independent of the presence of anti-HLA antibodies.[12] Moreover, increased rates of allograft failure in human leukocyte antigen-well-matched patients were observed among those with antibodies against MICA before transplantation.[13] Our group has also described a high correlation between the presence of anti-MICA antibodies and increased risk of acute rejection during the first year after heart transplantation.[14] Because excellent reviews have been published regarding the humoral response against MICA; this article will focus on the cellular immune response.

NKG2D ligands are expressed at low levels in many normal cells. Their expression is increased on stressed cells by infection or malignant transformation, and it alerts the immune system of adverse cellular conditions. Increased MICA/B expression has been observed in several transplanted grafts with histological evidence of rejection and/or cellular injury[14–16] (Table 1). Studies in animal models showed that ischemia/reperfusion injury (IRI), which is caused after transplantation, induces expression of RAE-1 on tubular epithelial cells, permits the infiltration of NK cells inside the graft, and increases the risk of acute allograft rejection by NKG2D-dependent mechanisms.[17,18] Furthermore, the simultaneous expression of Rae-1, H60, and NKG2D during acute heart allograft rejection support the role of this interaction in the immune responses associated with transplantation.[19] Whether an analogous mechanism occurs in humans still needs to be elucidated; however, it is known that IRI leads to the activation of endothelial cells, which increase their expression of adhesion molecules and recruit effector cells into the tissue. Recently, hypoxia-inducible factor-1 was shown to increase the MICA expression on human renal tubular epithelial cells and cardiomyocytes during the hypoxia/reoxygenation process that occurs during IRI.[20,21] These findings provide the first insights on the IRI mechanisms that induce MICA expression, and can spur the development of new strategies to reduce the early renal inflammatory injury after transplantation.

One of the best known mechanisms of immune evasion in tumor cells is the proteolytic release of MICA from the cell surface. The endoplasmic reticulum protein 5 interacts with MICA in the cell membrane and induces a conformational change that is necessary for proteolytic cleavage of the molecule. We identified a soluble form of MICA in the serum samples, from heart transplant patients, obtained during the first year after transplant.[22] Transplant patients with high levels of soluble MICA showed a lower incidence of acute rejection and had higher graft function and survival. *In vitro* studies demonstrated that soluble MICA engages NK cells expressing NKG2D, induces receptor internalization and

Table 1 | Effects of NKG2D and its ligands in solid organ transplantation

Transplant	Animal model/samples	Biological effects	Reference
NKG2D			
Heart	$CD28^{-/-}$ heart allotransplantation model	Increase of NKG2D ligands within the graft amplify the adaptive immune response and increase graft injury Blockage of NKG2D prolongs the cardiac allograft survival	29
Skin	SCID mice bearing MHC class II-deficient skin allograft	Increased expression of NKG2D ligands on indirectly primed CD4+ T cells in the rejecting grafts Activation of NK cells through NKG2D leads to cytotoxicity against graft cells and rejection Blockage of NKG2D prolongs graft survival but does not induce permanent graft acceptance	8
Kidney	Human biopsies and urine	Elevated levels of NKG2D mRNA and NKG2D+ cells in urine and kidney biopsies, respectively, diagnosed with acute and chronic rejection	27
NKG2D ligands			
Kidney	TEC	IRI induces the expression of NKG2D ligand, Rae-1, in TEC NK cell engagement through NKG2D allows NK cell-induced apoptosis	17
Kidney	Renal IRI mice model	IRI increases the expression of Rae-1 and H60 and activates NK and CD8+ T cells	18
Heart	C57BL/6 heart allotransplant model	Simultaneous increase of Rae-1, H60, and NKG2D during acute cardiac allograft rejection	19
Kidney	Human renal proximal tubular epithelial cells (HK2 cell line)	Hypoxia during IRI increases MICA expression through a HIF-1 pathway Enhance cytotoxicity and IFN-γ secretion by NK cells, leading to graft injury	20
Kidney and pancreas	Biopsies	Increase of MICA/B in epithelial cells of kidney and pancreas allografts during acute and chronic rejection	16
Kidney	Biopsies	Upregulation of MICB molecules following renal transplantation associated with evidence of cellular stress	15
Heart	Biopsies	Increase of MICA in endomyocardial biopsies with histological evidence of acute rejection	14

Abbreviations: HIF, hypoxia-inducible factor; IFN, interferon; IRI: ischemia/reperfusion injury; MHC, major histocompatibility complex; MICA, MHC class I chain-related A; MICB, MHC class I chain-related B; NK, natural killer; SCID, severe combined immunodeficiecy disease; TEC, tubular epithelial cells.

degradation, and impairs the NKG2D-mediated allogenic cytolytic responses. This finding supports a new mechanism by which NKG2D ligands could contribute to thwarting allograft rejection, and elucidates its inhibition of effector functions mediated by NK and CD8+ T-cytotoxic cells.

NKG2D MEDIATES ALLOGRAFT INJURY

The NKG2D-activating receptor is expressed in all NK cells, $\gamma\delta$ T, $\alpha\beta$ CD8 T, and NKT cells.[23] NKG2D functions as a primary activating receptor in NK cells, and a co-stimulatory receptor in CD8+ T cells. In these cells, a T-cell receptor-mediated stimulus is necessary for activation, although, under certain circumstances, NKG2D might be able to function as an activating receptor, independent of T-cell receptor stimulation.

Recently, studies in mouse models deficient in NKG2D showed that this receptor is involved in the development, homeostasis, and survival of NK cells. NK cells from NKG2D-deficient mice show a higher proliferation rate of immature NK cells and are more susceptible to apoptosis.[24] NKG2D signaling is also implicated in the effector functions of NKs as these $NKG2D^{-/-}$ NK cells showed a weaker cytolytic response and lower IFN-γ production against target cells expressing NKG2D ligands.

The activation of NK cells through the NKG2D receptor bound to its ligands may regulate multiple immunological pathways involved in the rejection or the outcome of allograft transplantation (Table 1). The best known example of the NKG2D function is reported in bone marrow transplantation of mice.[25] NK cells reject the bone marrow cells expressing the NKG2D ligand, Rae-1, and the blockade with a neutralizing non-depleting NKG2D monoclonal antibody prevents rejection. Similar results were obtained using a mouse model with severe combined immunodeficiency disease recipients bearing MHC class II-deficient skin allografts.[8] Transferred CD4 T cells, which recognize alloantigens only through the indirect pathway, mediate rejection by a NK cell-dependent route. Inflammation induced by indirectly primed CD4+ T cells leads to the upregulation of NKG2D ligands in the allografts. Concurrently, these CD4+ T cells recruit and trigger activation of NK cells through the interactions of the NKG2D-activating receptor with its ligands on donor cells. These results were corroborated so that NKG2D blocking significantly prolonged survival but did not induce a permanent acceptance, probably because of the involvement of other activating NK receptors.

Only some indirect evidence has reported the involvement of NK cells during rejection of kidney transplants. Accumulation of CD56+ NK cells expressing granzyme in kidney biopsies of patients undergoing acute rejection suggests a role of their cytolytic activity in kidney allograft rejection.[26]

Furthermore, IRI likely induces nonspecific recruitment of NK cells via early graft infiltration.[27] A recent study associated the presence of NK cells with the mechanisms of microcirculation injury during antibody-mediated rejection in kidney transplants.[9] They proposed that donor-specific antibodies are able to bind to the endothelium and recruit NK cells that produce IFN-γ and trigger antibody-dependent cellular cytotoxicity. The increased expression of NK-cell transcripts, such as CX3CR1, indicated the recruitment of NK cells to allograft endothelium when donor-specific antibodies are present.

The NK-cell receptors that are directly involved in the immune responses after kidney transplantation have not been well defined, but various studies suggest that NKG2D could have an important role. An elevated NKG2D mRNA expression was correlated to severity of acute rejection, and NKG2D + cells were detected in clusters around tubules in biopsies derived from patients diagnosed with acute and chronic rejection.[28] NKG2D gene expression was also detected in urinary sediments obtained at 2–3 days before the acute rejection episode; these findings raise the possibility that NKG2D may be able to serve as an additional informative biomarker of transplantation outcome.

That is, the NKG2D activating receptor might participate not only in the immune responses that occur initially after transplantation and is promoted by IRI, but it may also contribute to the development of acute rejection. However, further studies are needed to elucidate whether the source of NKG2D receptors is predominantly NK cells or CD8 + T cells.

NKG2D RECEPTOR, A BRIGDE BETWEEN INNATE AND ADAPTIVE IMMUNITY

Maier *et al.*[7] were the first to demonstrate that although NK cells may not be sufficient to directly reject a solid allograft, they can participate in acute rejection by promoting the actions of alloreactive T cells. They demonstrated that $CD28^{-/-}$ mice, which have an impaired T-cell co-stimulation, reject fully MHC-mismatched allogeneic hearts in a manner similar to $CD28^{+/+}$ recipients. However, $CD28^{-/-}$ mice depleted of NK cells show significant prolongation of allograft survival.

The bidirectional crosstalk between NK cells and DCs is involved in the maturation of DCs and the activation of an adaptive immune response. DCs secrete cytokines such as interleukin-12 and interleukin-15, which upregulate both the cytotoxic ability and cytokine production (IFN-γ and tumor necrosis factor-α) of NK cells. Concurrently, these proinflammatory cytokines induce DC maturation and the subsequent activation of T cells, promoting a Th1-like alloresponse. NKG2D has a critical role in the NK–DC interaction, as MICA and MICB expression is upregulated on DCs induced to mature by INF-α.[29] Furthermore, cytokines such as interleukin-2, interleukin-15, or IFN-α increase the expression of NKG2D, and, consequently, the NKG2D-mediated effector functions. In short, the NK–DC crosstalk through interaction of NKG2D and NKG2D ligands contributes to the feedback regulation of T-cell-mediated immune responses.

After transplantation, IRI leads to a rapid upregulation of NKG2D ligands that decreases within a few days. However, a second phase of upregulation of NKG2D ligands was only observed in allogenic transplantation,[30] that is, $Rag^{-/-}$ mice (deficient in T and B cells but intact NK cells) fail to express Rae-1 in the late phase, suggesting that the innate response is insufficient to maintain the expression of these ligands after the initial injury. These data suggest that initial interactions of NKG2D + cells with its ligands amplify the adaptive response, enhance the effector functions of CD8 + T cells and NK cells, and increase graft injury. Blockade of NKG2D significantly prolongs graft survival, prevents CD28-independent rejection of cardiac allografts, and inhibits NK effector functions without depleting NK cells or reducing their migration to the graft. Prolonged treatment with anti-NKG2D is critical in maintaining the blockage, as single doses of monoclonal antibody were ineffective in the same cardiac transplantation model.[6] Activated NK cells after transplantation may be able to directly or indirectly provide T-cell co-stimulatory signals that can promote allograft rejection.

In this way, the increased expression of NKG2D ligands in transplanted allograft might recruit NK cells that directly enhance the NKG2D-mediated cytotoxicity against the graft, function as a bridge between innate and adaptive immunity, and lead to the rejection of the graft.

IMMUNOSUPPRESSION AND NKG2D RECEPTOR

NK cells also have the ability to contribute to allograft tolerance in patients who have undergone organ transplantation, through elimination or inhibition of donor DCs that subsequently reduce the activation of alloreactive T cells or modulate their response by immunosuppressants. Current immunosuppression in solid organ transplantation involves the use of pharmacological and biological agents that mainly prevent the activation of T cells and development of immune responses against the donor. However, the effects of immunosuppressive agents on NK cells have not been clearly defined.

Conflicting results on the influence of calcineurin inhibitors on NK cell phenotype and function have been recently reported. *In vitro* studies demonstrated that cyclosporine and tacrolimus (FK506) produce dose-dependent inhibition of NK cell degranulation and IFN-γ production.[31] Moreover, blood samples from calcineurin inhibitor-treated transplant patients show a reduction of NK cell numbers and impaired effector functions. However, other studies have reported that mycophenolic acid and rapamycin inhibit the acquisition of NKG2A, reduce the expression of NKG2D- and natural cytotoxicity receptor-activating receptors, and lead to the loss of cytotoxicity against target cells,[32,33] but cyclosporine had no effects on the NK receptor repertoire and leaves the cytolytic capacity intact.[32,33] These findings suggest that the distinct mechanisms of action of the

immunosuppressive reagents may contribute to their differential effects. As the effects of cyclosporine are known to be reversible, the distinct experimental designs of these studies may also contribute to the disparate results.

Kidney transplant patients receiving induction therapy with polyclonal rabbit anti-thymocyte globulin showed normal levels of NK cells at 1 month after transplantation, but the repertoire of NK cell receptors was modified.[34] These cells had an increased expression of the inhibitory receptor NKG2A, and reduced expression of killer Ig-like receptors and NKG2D. The global functions of NK cells were not affected and probably were maintained by a compensatory effect of other receptors. The low levels of NKG2D during the first months after transplantation are of special interest because this receptor is involved in the adaptive immune response against cytomegalovirus (CMV) infection, one of the most common viral complications following solid organ transplantation. NK cells have an important role in the initial stages of viral infections, especially when adaptive immunity is not fully active or is damaged by the use of immunosuppressive therapy. NKG2D interacts with its ligand MICA, which is upregulated in CMV-infected cells, and NKG2D functions as a co-stimulatory molecule in the activation of human CD4 + T lymphocytes against CMV.[35] Hadaya *et al.*[36] recently demonstrated an expansion of NKG2D + NK cell population during acute CMV infection, and these NK cells may have a role similar to that of CD4 + T cells. For resolving CMV infections, it is essential to maintain a proper number and function of NK cells during the early time points after transplantation, and decipher how immunosuppression may affect NK activity to minimize the risks.

CONCLUDING REMARKS

In summary, the finding that expression of NKG2D ligands is induced by IRI injury during acute and chronic rejection and the discovery of their interactions with their activating receptor NKG2D open a new route of intervention for improving the outcome of solid organ transplantation. NKG2D + cells were found in kidney biopsies during acute and chronic allograft dysfunction, and blockage of this interaction prolonged the survival in skin and heart-transplanted mice models. Moreover, immunosuppression may influence NK cell functionality and the ability of NK cells to resolve opportunistic viral infections in addition to modulating DC and T-cell responses to the donor. In the future, it is important to determine the *in vivo* effect of these drugs on the repertoire of inhibitory and activating receptors, such as NKG2D, and their modulation of the effector functions of NK sub-populations. In conclusion, blockade of NKG2D with monoclonal antibodies or soluble NKG2D ligands can contribute to prevent the effector functions of NKG2D + cells that participate in the innate and adaptive immune response involved in rejection after transplantation. The interaction of NKG2D–NKG2D ligands would be an interesting target for the development of new therapeutic strategies for transplantation.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This work was supported by Spanish grants from the Red de Investigación Renal (REDinREN) and the FIS PI08/0566 from Instituto 'Carlos III'.

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